STUDIES ON ALKALIPHILES - AN INDUSTRIALLY SIGNIFICANT GROUP OF EXTREMOPHILES

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IN

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BY

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CERTIFICATE

This is to certify that ***Ms. Sunita Borkar*** has worked on the thesis entitled **"Studies on Alkaliphiles - an industrially significant group of Extremophiles"** under my supervision and guidance.

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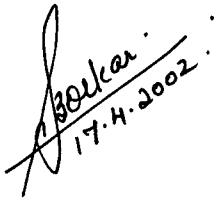
I declare that this thesis has been composed by me and has not previously formed the basis for the award of any degree, diploma or other similar titles.



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Last but not the least, I wish to dedicate this thesis to my late mother who has always been with me as a source of inspiration in my education career.

SUNITA BORKAR.

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LIST OF ABBREVIATIONS

α	Alpha.	ml	Millilitre.
APS	Ammonium persulfate.	mm	Millimeter.
		μ	Micron.
β	Beta.	μ l	Microlitre.
BCA	Bicinchoninic acid method.	μ g	Microgram.
		mM	Millimolar.
BSA	Bovine serum albumin.	M	Molar.
		MM	Mineral medium.
cfu	colony forming units.	nm	Nanometer.
		OD	Optical density.
°C	Degree centigrade.	%	Percent.
CMC	Carboxy methyl cellulose.	PAGE	Poly acrylamide gel electrophoresis.
cp	Centipoise.	PBS	Phosphate buffer saline.
d/w	Distilled water.		
DAP	Diaminopimelic acid.	PPYG	Poly peptone yeast extract glucose agar.
EDTA	Ethylenediamine tetraacetic acid.	rpm	Revolutions per minute.
EP	Exopolymer.	Rf	Resolution factor.
EPS	Exopolysaccharide.	SDS	Sodium dodecyl sulfate.
Fig.	Figure.		
g	Gram (s).	SEM	Scanning electron microscopy.
g/L	Grams per litre.		
GC	Gas Chromatography.	sp	Species.
		TLC	Thin layer chromatography.
h	Hour (s).		
IBIS	International Bio-synthetics.	U	Unit.
		UV	Ultra violet.
kDa	Kilo Dalton.	v/v	Volume / Volume.
L	Litre.	w/v	Weight / Volume.
mg	Milligram.	λ	Wavelength.
min	Minute(s).		

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"The role of the infinitely small is infinitely Large....."

Louis Pasteur

CHAPTER I

"Extremophile" explorer
Koki Horikoshi [260]

INTRODUCTION

1.1 Extreme Environments And Their Inhabitants:

Microorganisms are ubiquitous in nature occupying not only different moderate habitats on earth but are also found to adapt and colonize certain harsh environments, once assumed to be sterile by man. These amazing creatures, thriving in extreme environments are called **Extremophiles** (215,241,361) and are found in econiches with extremes of temperature (**Thermophiles & Psychrophiles**), pressure (**Barophiles**), alkalinity (**Alkaliphiles**), acidity (**Acidophiles**), salinity (**Halophiles**) and low nutrient content (**Oligotrophs**). Some of them can also survive in niches/media containing high levels of organic solvents (**Organic solvent tolerant organisms**), heavy metals (**Metal tolerant organisms**), radiations (**Radio resistant forms**) (246) or extra dry conditions (**Xerophiles**).

Bacterial extremophiles have become the focus of greatly enhanced attention during the past few years. Research in this area was stimulated by the description of *Thermus aquaticus* by Brock in 1969 (45). Since then a surprising number and variety of isolates have been obtained from these heretofore inaccessible environments (Table 1.1).

These microorganisms have developed special adaptations to survive in such extreme habitats, which include new mechanisms of energy transduction, regulating intracellular environment and metabolisim, maintaining the structure and functioning of membranes and enzymes and so on. They thus live in habitats that will support no other form of life and are perhaps oldest on earth with a unique genome believed to have been adapted initially to suit the extreme environmental challenges that the earliest life forms faced on this planet.

Table 1.1. Microorganisms under extreme environments.[295]

EXTREME CONDITION	MICROORGANISM	HABITAT	EXTREME GROWTH CONDITION	METABOLIC CHARACTERISTIC
High temperature	*Pyrococcus furiosus*	Geothermal marine sediments	100°C	Anaerobic heterotroph
Cold temperature	*Bacillus* TA41	Antartic seawater	4°C	Aerobic heterotroph
High pressure	*Methanococcus janaschii*	Deep sea hydrothermal vent	250 atm, 85°C	Growth and methane production stimulated by pressure
High pH	*Clostridium paradoxum*	Sewage sludge	pH 10.1, 56°C	Anaerobic heterotroph
Low pH	*Metallosphaera sedula*	Acid mine drainage	pH 2.0, 75°C	Facultative chemolithotroph
High salt	*Halobacterium halobium*	Hypersaline waters	4-5M NaCl	Aerobic heterotroph
Presence of organic solvents	*Pseudomonas putida IH 2000*	Mud samples of Kyushu island, Japan	Grows in culture media containing more than 50% toluene	Degrades polyaromatic hydrocarbons.
Presence of heavy metals	*Alcaligenes eutrophus*	Environment severely contaminated with heavy metals	Cd^{++}, Co^{++}, Zn^{++}, Ni^{++}, Cu^{++}	Plasmid borne resistance
Ionizing radiations	*Deinococcus radiodurans*	Soil	Ionizing radiation	Tolerates massive DNA damage.

Table 1.2. Industrial applications of Extremophiles.[3,70,361]

EXTREMOPHILIC GROUP	ENZYMES, ENDOGENOUS COMPOUNDS	APPLICATIONS, PRODUCTS
Thermophiles 50-110°C	Amylases Xylanases Proteases Taq polymerase Guar gum fluidising enzyme	Glucose, fructose for sweetners Paper bleaching Amino acid production from keratins, food processing, baking, brewing, detergents, biosensors, bioremediation. Genetic engineering, PCR, sequencing Oil and gas companies
Psychrophiles 5-20°C	Neutral proteases Proteases Amylases Lipases Polyunsaturated fatty acids Ice-protein, ω- 3 Fatty acids	Cheese maturation, dairy production Detergent for cold water washes Detergent for cold water washes Detergent for cold water washes, perfumes Pharmaceuticals Artificial snow, Production of Dietary supplements
Barophiles Optimal growth > 1atm	Proteases	Proteolysis in leather treatment Food processing Oil-well stimulation
Alkaliphiles pH > 9.0	Proteases Amylases Lipases Cellulase 103 Xylanases Cyclodextrins Antibiotics	Detergents Detergents Detergents Detergents Paper Stabilization of volatile substances Pharmaceuticals.
Acidophiles pH < 2.0	Sulfur oxidation Acid tolerant enzymes	Desulfurisation of coal Animal feed addtives
Halophiles 3-20% salt	Antibiotics Carotene Glycerol Compatible solutes Membranes Enzymes Bacteriorhodopsin	Pharmaceuticals Food coloring Pharmaceuticals Pharmaceuticals Surfactants for Pharmaceuticals Waste treatment, peptide synthesis, enhanced oil recovery from wells Light sensors, computer biochips
Organic solvent tolerant organisms	Dioxygenases	Crude oil degradation

Table 1.3. Selected companies with extremophile organism or molecular Program.[287]

COMPANY	PROGRAM
Altus Biologics (Cambridge, MA)	Artificial extremozymes; cross-linked enzyme crystals
Amersham Pharmacia Biotech (Cleveland, OH)	Thermostable alkaline phosphatase
Archaenzyme (Jerusalem, Israel)	Extremozymes from Archaea
Diversa (San Diego, CA)	Extremophile expression libraries
DuPont Merck (Nutley, NJ)	Protease stabilization in oxidizing, alkaline and hot environments
Genencor International (Rochester, NY)	Protease stabilization in oxidizing, alkaline and hot environments
Hoffman La Roche (Basel, Switzerland)	Thermostable Taq polymerase for PCR
Maxygen (Santa Clara, CA)	DNA shuffling of extremozyme genes
Novo Nordisk Biotech (Davis, CA)	Extremozyme cloning and expression
Pfizer Central Research (Groton, CT)	Extremophile screening

Studies on Extremophiles have intensified recently, as scientists have recognized their potential use in an array of industrial applications. The enzymes from such organisms, which function at unusual parameters, are referred to as **"Extremozymes"** (270,286). Such enzymes have already been commercialized example, Taq Polymerase, Cellulase 103 etc. (Tables 1.2 & 1.3).

1.2 Aim and scope of work:

This investigation was undertaken to study one of the extremophilic groups "ALKALIPHILES" on the following aspects:

1. **Survey the biodiversity of alkaliphiles in natural and manmade alkaline and non-alkaline environments and screen their enzyme profiles.**
2. **Characterisation of the selected pre-dominant isolates growing at extreme pH and determination of their buffering capacities.**
3. **Amylase production, purification and characterisation from alkaliphilic *Bacillus* sps SB-D and SB-W.**
4. **Adhesive viscous exopolymer production by an alkaliphilic bacterium and characterisation of the same by chemical and chromatographic analysis.**

Goa has a diverse alkaline ecosystem both natural and manmade; not much explored for isolation and characterisation of alkaliphiles. This study would hence be of significance:

1. To advance the scientific knowledge on Alkaliphiles.

2. For industrial applications with respect to the utility of their extra cellular enzymes active under alkaline conditions, specifically in detergent industries.

3. To utilise alkaliphiles for the synthesis of novel products such as exopolymers with varied applications.

The studies would thus be beneficial for industrial applications with respect to enzymes and exopolymers of alkaliphiles stable and active at high pH, and give an insight on the bio-diversity of alkaliphiles in this state of India.

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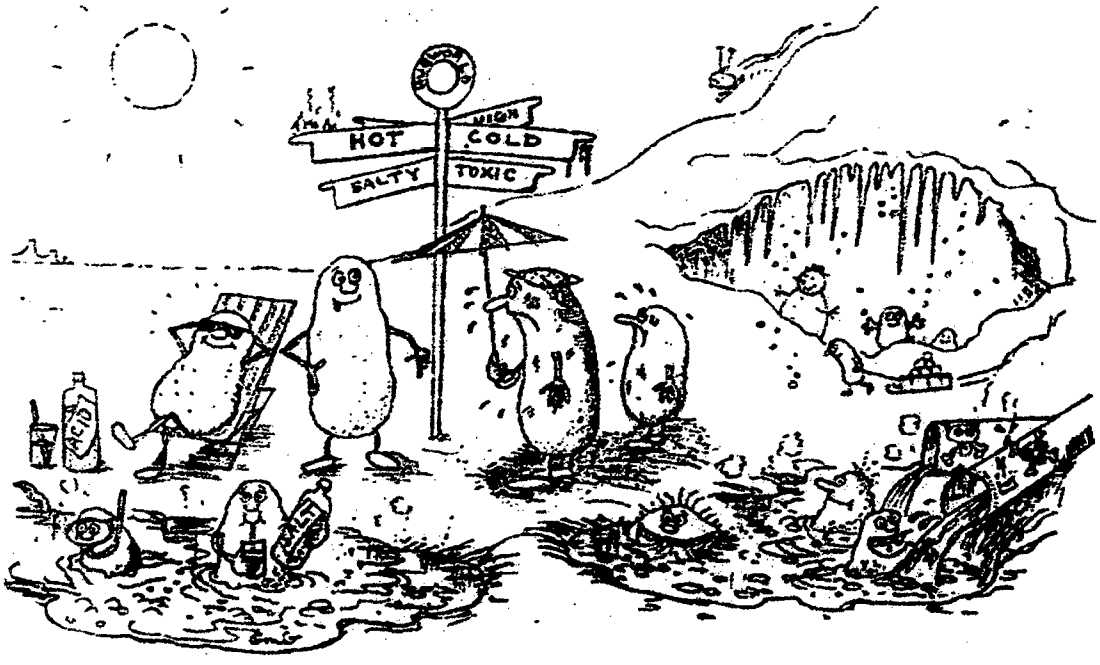
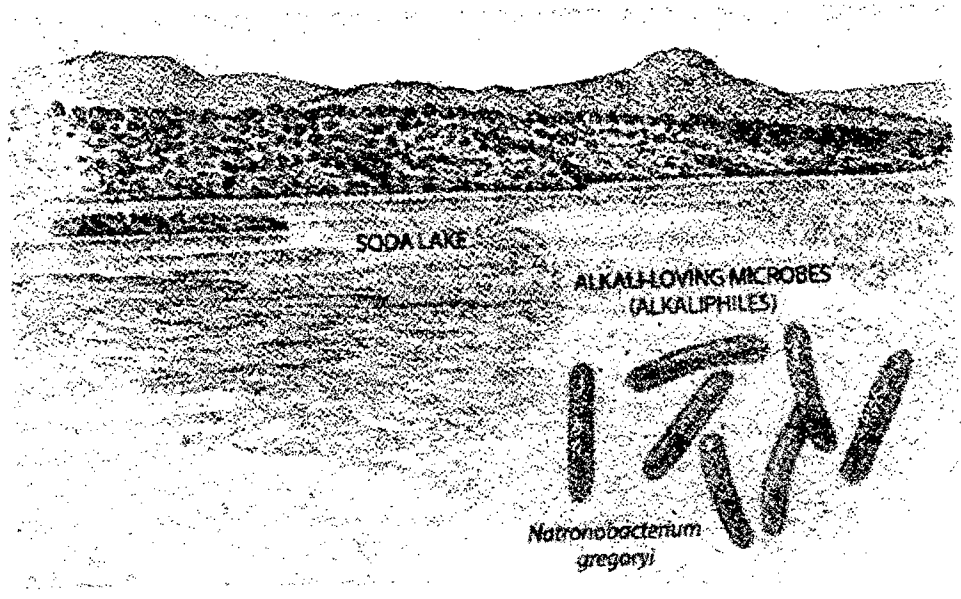


"What do you mean 'extreme'? We love it here!"[19]

CHAPTER II

REVIEW OF LITERATURE

SECTION A



ALKALIPHILES

2.1. Definition and Classification of Alkaliphiles:

Alkaliphiles are a diverse group of organisms that thrive in highly alkaline environments with pH optima for growth being 9.0 or above. Based on pH preference such alkaliphiles are grouped into two broad categories: **Alkalitolerant** organisms that show optimal growth in the pH range of 7.0 - 9.0 but cannot grow above pH 9.5 and **Alkaliphilic** organisms that show optimal growth between pH 10.0 and 12.0. The extreme alkaliphiles are further subdivided into **Facultative alkaliphiles**, which show optimal growth at pH 10.0 or above but can grow well in neutral pH range and **Obligate alkaliphiles** which show optimal growth above pH 10.0 but do not grow below pH 9.0 (221).

2.2 Ecological niches of prevalence of alkaliphiles:

Most natural environments on the earth are essentially neutral having pH values between 5.0 and 9.0 harboring neutrophiles. However, there are specified ecosystems that show unnaturally high pH ranging between 9.0 and 12.0 (Fig. 2.1.A_1). Interestingly, during the past 20 years, a number of studies have shown that microorganisms can indeed survive and grow at high pH of above 10.0 (155,157) as seen in figure 2.1.A_2.

Alkaliphilic microorganisms, particularly prokaryotes are widely distributed and can be found not only in alkaline environments but also in non-alkaline, such as neutral and acidic soils, although counts of alkaliphiles are higher in alkaline environments (121,157,221). In addition, alkaliphiles can be isolated from man made alkaline

pH	(H^+) (Molarity)		Common examples
0	10^{-0} (1.0)	Increasing Acidity	Concentrated nitric acid
1	10^{-1}		Gastric contents, acid thermal springs
2	10^{-2}		Lemon Juice Acid mine drainage
3	10^{-3}		Vinegar, ginger ale Pineapple
4	10^{-4}		Tomatoes, orange juice Very acid soil
5	10^{-5}		Cheese, cabbage
			Bread
6	10^{-6}		Beef, chicken Rain water Milk Saliva
7	10^{-7}	Neutrality	Pure water Blood
8	10^{-8}		Seawater
9	10^{-9}		Strongly alkaline soil Alkaline lakes
10	10^{-10}		Soap
11	10^{-11}		Household ammonia
12	10^{-12}		Saturated calcium hydroxide solution
13	10^{-13}		Bleach Drain opener
14	10^{-14}		
		Increasing Alkalinity	

Fig. 2.1.A_1 pH Scale.[248]

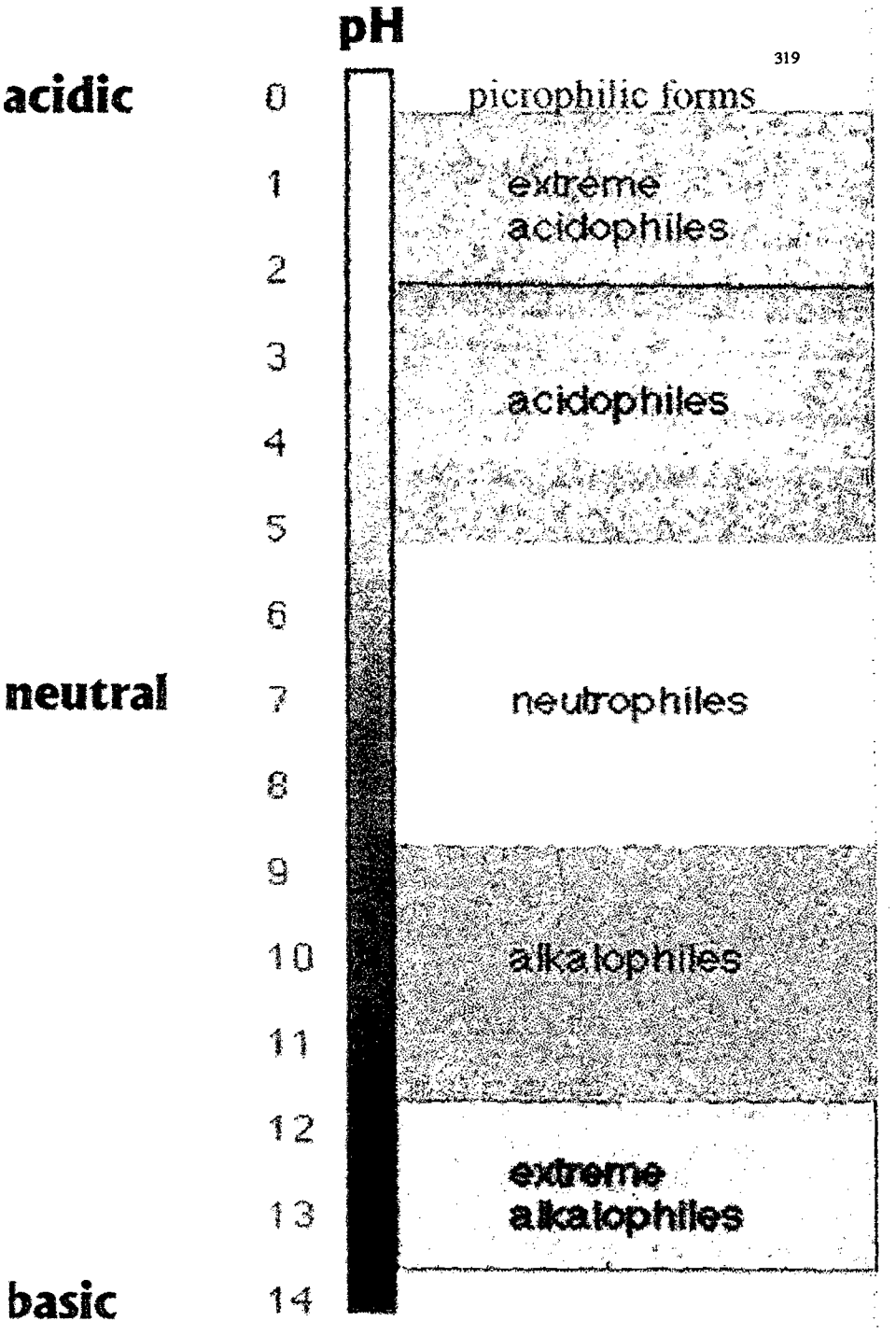


Fig. 2.1.A_2 Extremophilic groups at various pH ranges.

environments such as Indigo dye balls and alkaline effluents of various industries, which support the growth of alkaliphiles (Table 2.1.A)

2.3 Genesis of natural alkaline environments:

The most stable naturally occurring alkaline environments such as eutrophic soda (Na_2CO_3) lakes, soda deserts and alkaline groundwaters are caused by a combination of geological, geographical and climatic condition (121). Lake Magadi in Kenya, Lake Wadi Natrum in Egypt of the East African rift valley and the Western soda lakes of United States are probably the most stable and highly alkaline environments on the earth with a consistent pH of 10.5 to 12.0. These environments are characterized by the presence of large amounts of sodium carbonate and sodium chloride, formed by the evaporative concentration, giving rise to environments that are both alkaline and saline. In India, such saline and alkaline lakes are Lake of Lonar situated in Maharashtra having an average pH 10.5 (175) and Deodani Kyars salt lake in Rajasthan (372).

The genesis of alkaline lakes is due to vulcanism, contributing to a source of sodium carbonate to the surface environment or biological activity and weathering, which produce CO_3^{-2}, charged surface waters, thus forming a bicarbonate-carbonate solution that leaches surrounding minerals. In most environments, such ground waters rapidly become saturated with Ca^{+2} and Mg^{+2} resulting in the precipitation of calcite ($CaCO_3$), magnesite ($MgCO_3$) and dolomite ($MgCa(CO_3)_2$). Carbonate is removed from the solution and the genesis of alkaline brine is inhibited, as it is the case for most ground waters. However, when the CO_3^{-2} concentration exceeds that of Ca^{+2} and Mg^{+2},

Table 2.1.A Ecological niches showing presence of Alkaliphiles.

Source	Ref No.	Source	Ref No.
Natural environments		**High Organic content ecosystems**	
Alkaline springs		Biogas plants	157
Aqua de Ney, Siskiyou country, California	338	Chicken manure digestor	238
Black bird valley, Stanislaus country, California	338	Compost	157
Ground water calcium springs in Oman.	121	Alkaline soil	132
Hyperthermal springs in Bulgaria	80	Japanese forest soil	264
Yellow stone National Park	234,381	Decomposed manure	157
Hveragerdi Iceland	138	Dung	117
Hot water spring in Bombay, Thane, India.	54	Feces of man & animals	157
Soda lakes		Horn meal	117
Alkaline Soda Lake, Ethiopia	109	Indigo dye ball	157,353
Big Soda Lake, Nevada	121	Lime treated garden soils	129
Hungarian Soda Lakes	363	Sewage	40,295
Lake Ashanti, Bosumatwi, Ghana, Africa	119	Termite infested mound soil	301
Lake Bogoria, Kenya Lake Hannington, Kenya	119 119	Bioteriorated casein containing building material	179
Lake Magadi, Kenya	93,122,187, 205,307,363	Neutral Garden soil	47,156,162 204,398
Lake Canyan Diable, Arizona, USA	157	**Industrial Effluents**	
Lake Nakuru (Pine Lake)	121	Battery cell manufacturing units	157
Lake Quebec, Labrador, Canada, North America	157	Cement manufacturers & Casting	121
Lake Wadi Natron, Kenya	121,157	Detergent factories	157
Lonar Lake, Maharashtra, India	175	Electro plating	121
Owenn's Lake	157	Lye treatment of animal hides	121
Soda Lake of Kulunda steppe	119	Paper processing factories	121
Soda Lake in Taiwan	274	Potato / food processing plants	106,121
Tibetan soda Lake	394	Textile mills	157,162
Soda Lakes of Tuva	108,188	**Other environments**	
Alkaline Salterns		Air contaminants	117
Dead Sea	281	Acidic soil	157
Great Salt Lake, Utah	238	Deep sea water	95,117
Sambhar Salt Lake, Rajasthan, India	372	Water	382

alkalinity develops, usually with Na^+ as the dominant cation. (Fig.2.2.A).

Bacterial sulfate reduction in surrounding swamps has also been proposed to contribute towards the alkalinity of Wadi Natrum depression and Hungarian soda lakes (121). Transient localised alkaline conditions can also be generated due to animal excreta and biological activities such as proteolysis, ammonification, sulphate reduction or photosynthesis (157).

2.4 Biodiversity and Groups of Alkaliphiles:

2.4.1. Biodiversity:

The earliest reports of microorganisms in alkaline environments appeared in 1922 when Meek and Lipman (253) isolated alkaline tolerant nitrifying bacteria *Nitrosomonas* and *Nitrobacter* sp. followed by isolation of alkaliphilic *Streptococcus faecalis* by Downie and Cruikshank in 1928 (83). Later, a strain of *Bacillus pasteurii*, which grew well at pH 11.0 and required NH_3, was discovered by Gibson (110). Vedder isolated *Bacillus alkalophilus* growing at pH 8.6 - 11.0 in 1934 (377). Twenty-five years later Kushner and Lisson (1959) (229) reported the discovery of many alkali tolerant strains such as *Bacillus cereus* and *Bacillus circulans*.

The use of such alkaliphilic microbes has a long history in Japan, since from ancient times, indigo has been naturally reduced under alkaline conditions in presence of sodium carbonate in a traditional process called " Indigo Fermentation". Takahara and co-workers in 1960 (353) isolated the indigo reducing bacterium *Bacillus* sp. No. S-8 from an indigo ball and improved the indigo fermentation by adding this strain

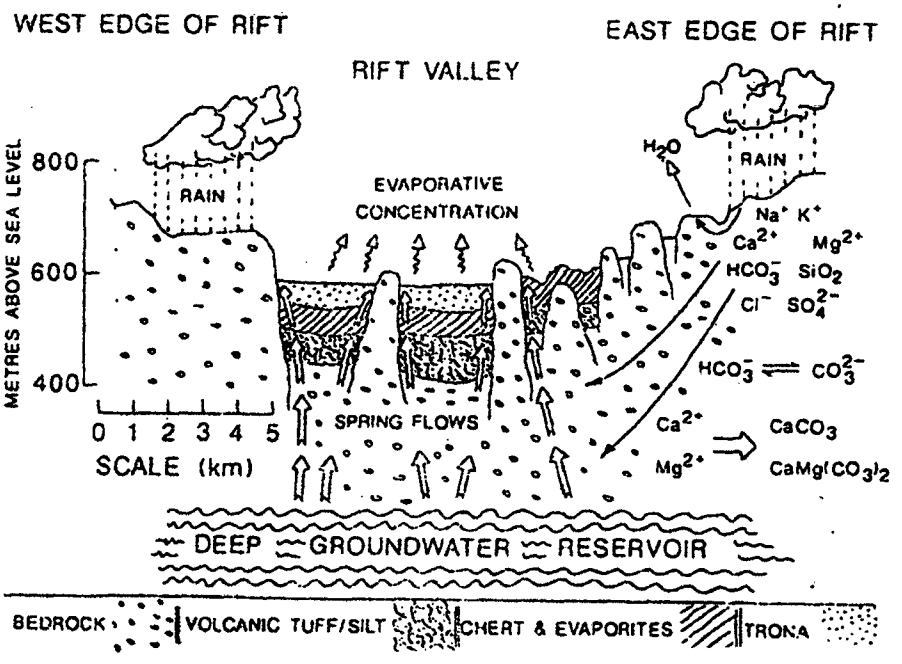


Fig. 2.2.A. Schematic representation of the possible mechanisms involved in the function of alkaline saline lakes (L. Magadi)[121].

during fermentation under alkaline conditions.

Koki Horikoshi, "The explorer of extremophiles", conducted further microbiological studies who isolated a large number of alkaliphiles since 1969 and purified many alkaline enzymes (151-159). The alkaliphilic microorganisms are now known as a diverse group, often adapted to multiple environmental challenges such as high/low temperature, high pressure or high sodium concentrations in addition to high pH. The diversity of alkaliphilic bacteria (Table 2.2.A) comprise a heterogeneous collection of eubacteria belonging to diverse genera (4,7,23,34,40,41,86,102,119,132, 157,175,179,182,187,204,216,234,327,334-336,367-369,401,406-408,) including *Cyanobacteria* (47,71,93,108,294,329,380) to archaebacteria such as *Natronobacterium, Natronococcus, Natronorubrum, Thermophilus, Methanosarcina* (122,183,188,247,340,344,363,364,371,394,400) with reports also on eukaryotes growing at high pH (33,157,282).

2.4.2 Groups of Alkaliphiles:

Alkaliphiles consist of two main physiological groups;

A. **Alkaliphiles:**

These organisms require an alkaline pH of 9.0 or more for growth with optimal growth pH of 10.0. They can be isolated on alkaline media with alkalinity generated using sodium carbonate, sodium bicarbonate, sodium hydroxide, trisodium phosphate or sometimes sodium borate. These salts are added in concentration of about 0.5-2.0% depending on the microorganism used giving a pH of 8.5 to 11.0 (106,121,157).

Table 2.2.A Diversity of alkalophilic bacteria.[221]

GROUP	pH RANGE FOR GROWTH	NATURAL HABITAT	COMMENTS
Eubacteria - Gram-negative photoautotrophs			
Spiruluna sp.	8.0-11.0	Alkaline soda lakes, Rift valley, pH 10.5	Halotolerant cyanobacterium
Synechococcus sp.	6.5-10.0	Yellowstone, 55°C, pH 5.5	Alkalitolerant, Halotolerant cyanobacterium
Ectothiorhodospira sp.	8.0-10.0	Mud from alkaline salt lakes, pH 11.0	Anoxygenic purple sulfur bacterium
Eubacteria - Gram-negative nonendosporeformers			
Aeromonas sp.	7.0-12.0	Soil	Facultative anaerobe
Flavobacterium sp.	7.0-11.2	Soil	Facultative anaerobe
Vibrio alginolyticus	6.0-9.2	Seawater	Halotolerant,alkalitolerant
Eubacteria - Gram-positive nonendosporeformers			
Exiguobacterium sp.	7.0-11.5	Potato processing effluent	Facultative anaerobe
Actinomyces sp.	8.0-11.5	Soil	Heterotrophic facultative anaerobe
Streptococcus sp.	5.0-11.0	Alkaline potato processing effluent	Fermentative facultative anaerobe
Micrococcus sp.	7.0-10		Psychroalkaliphile
Coryneform bacteria	6.6-9.5	Seawater	Halotolerant facultative anaerobe
Eubacteria - Gram-positive endosporeformers			
Clostridium paradoxum	8.0-11.3	Alkaline springs	Strict anaerobe, alkali thermophile
Clostridium proteolyticum	8.0-12	Chicken manure digestor	Strict anaerobe, alkali thermophile
Bacillus alcalophilus	8.5-11.5	Soil (acid-alkaline)	Aerobic heterotroph
Bacillus strain A007	9.0-11.0	Soil	Aerobic heterotroph
Bacillus strain WN13	8.0-11.5	Soil	Aerobic, heterotrophic halophile
Archaebacteria - Aerobes			
Natronobacterium gregoryi *Natronobacterium. magadi* *Natronobacterium. pharaonis* *Natronobacterium. vacuolatum* *Natronococcus occultus.*	9.0-10.0	Alkaline saline lakes -- Wadi Natrum, Egypt and Lake Magadi, Kenya	Halophilic (3M NaCl)
Archaebacteria - Anaerobes			
Methanobacterium thermoalcaliphilum	6.5-10.0	Biogas plant	Thermophilic autotroph

Although most of the alkaliphiles are aerobic or facultatively anaerobic, there are reports of few alkaline tolerant strictly anaerobic strains e.g. *Clostridium* and *Methanobacterium* (179,182,234,238,272,406,408). The first facultative anaerobic xylan using alkalophile lacking cytochrome, quinone and catalase was reported by Niimura *et al* (272). Subsequently, many anaerobic sporulating alkaliphiles were isolated by conventional methods. *Clostridium proteolyticum* was isolated from a chicken manure digester and *Clostridium collagenovorans* from a sewage sludge digester (238). *C. bifermentans* and *C. sporogenes* have been isolated from biodeteriorated casein containing building materials having maximal pH tolerance of 12.2 and 11.7 respectively (179). Kevbrin *et al* (187) isolated alkalophilic obligately anaerobic *Tindallia magadi* from deposits of lake Magadi Kenya.

There are alkaliphiles reported to grow at high temperature under aerobic (200) and anaerobic (86) conditions. Enzymes from these thermophilic alkaliphiles are both alkalostable and thermostable, used for protein engineering and production of thermostable enzymes from aerobes, e.g. Thermostable xylanase (80,279), protease (96,97,354,367), lipase (381) and chitinase (34,35).

Anaerobic alkalithermophiles have been isolated from alkaline hot spring in Yellowstone National park in North America (234,291,389). Stetter's group isolated *Thermophilus alcaliphilus* sp. nov, a new hyperthermophilic archaeon growing on polysulfide at alkaline pH and at temperatures between 56 and 90°C (183).

Interestingly Kimura and Horikoshi have isolated several strains of bacteria which can grow at high pH and temperatures as low as 0°C (191,193). Alkaliphilic and

barophilic bacteria have also been reported from deep-sea sediments collected from depths up to 10,898 meters of Mariana trench (107,159,181,356).

B. **Haloalkaliphiles:**

These organisms isolated from alkaline and highly saline environments such as alkaline soda lakes of Wadi Natrum in Egypt, lake Magadi in Kenya (307,363,364) and Sambhar lake, India (372) also require high salinity achieved by addition of 20% NaCl to the isolation medium. *Natronobacterium* and *Natronococcus* are two well-recognized genera of haloalkaliphilic bacteria growing only at pH values above 8.0-9.0 and extremely high salt concentration (363). A novel haloalkalophilic archaeon isolated from Lake Magadi contains large gas vacuoles in stationary phase of growth forming bright pink colonies and is designated as *Natronobacterium vacuolata.* Xu *et al* (394) isolated two haloalkalophilic archaea from soda lake in Tibet belonging to a new genus *Natronorubrum.* A number of anaerobic halophilic eubacteria have been isolated from hypersaline alkaline environments such as Big Soda Lake, Aqua de Ney spring (272). A novel osmolyte, 2 sulfotrehalose was discovered in several *Natranobacterium* species of haloalkalophilic archaea (77). The ecology, physiology and taxonomy of haloalkaliphiles has been reviewed by Duckworth 1996 (84) and Jones *et al,* 1998 (174).

2.5. Molecular and Biochemical adaptations in Alkaliphiles:

Organisms that grow at extreme pH values are faced with a central problem of pH homeostasis. The mechanisms by which the organisms solve and develop survival

strategies have been extensively studied and investigated during the past decades. Yet, the understanding of any of the specific adaptation in all extreme alkaliphiles needs more attention. A number of adaptation and regulatory mechanisms have been elucidated in aerobic, akaliphilic *Bacillus* sp. which can be characterized as passive and active mechanisms.

A. Passive mechanisms:

Alkaliphilic bacteria are known to change external pH values to a pH suitable for growth, creating their own environment. e.g. *Bacillus* sp. No. 221 grows slowly at neutral pH changing the pH of culture broth to 9.0, further showing rapid growth and producing large amount of alkaline protease (157). This phenomenon from the ecological point of view explains how alkaliphiles can create a microcosm and live in non-alkaline and acidic soils.

Alkaliphilic bacteria also show significantly higher external (Bo) and internal cytoplasmic (Bi) buffering capacities under alkaline conditions than any of the non-alkaliphiles (217,218). It is postulated that this property is conferred by high levels of basic proteins (113,213) or polyamines in alkaliphilic cells. The high polyamine content of alkaliphilic cell envelope has been confirmed by Yonezawa and Horikoshi (1978) (397). Hamana *et al* (1989) (135) further detected the presence of 3 polyamines-putrescine, spermidine and spermine, spermidine being the major polyamine in alkaliphilic *Bacilli.*

The cell wall components of alkaliphiles also play a key role in protecting the cell from alkaline environments as the protoplasts of alkaliphilic *Bacillus* strains lose their stability in alkaline environments (13,233). Components of the cell walls of several alkaliphilic *Bacillus* sp. have been investigated in comparison with those of neutrophilic *Bacillus subtilis* (7-10,12,15,18). In addition to the peptidoglycan, alkaliphilic *Bacillus* sp. grown at pH 10.0 contain acidic polymers such as galacturonic acid, glucuronic acid, glutamic acid, aspartic acid, phosphoric acid and teichuronopeptides which are more negatively charged than in cells grown at pH 8.0. The negative charges on the acidic non-peptidoglycan components adsorb sodium and hydronium ions and repulse hydroxide ions assisting cells to grow in alkaline conditions/environments/econiches.

The cytoplasmic membrane of alkaliphiles has higher concentration of membrane lipid: membrane protein ratio with high cardiolipin content and fatty acid composition consistent with a very fluid membrane (218). *Bis* (monoacyl-glycero) phosphate (BMP), a novel lipid, which does not occur in neutrophiles, is found in alkaliphiles (157,273). Like other *Bacilllus* sps, the alkaliphilc Bacilli possess a diverse group of branched chain fatty acids in their membrane lipids and also contain variable amounts of saturated and unsaturated straight chain fatty acids. Further, all alkaliphilic bacilli have squalene and dehydro or tetrahydro squalene with substantial quantities of C_{40} and smaller quantities of C_{50} isoprenoids in neutral lipid fraction of cell membrane along with phosphatidyl ethanolamine and phosphatidyl glycerol (220,224).

Facultative alkaliphiles however, are found to contain much lower content of unsaturated fatty acids and branched chain fatty acids than obligate alkaliphiles. Studies (Clejan *et al*, 1986 (61); Dunkley *et al*, 1991(85)) indicate that fatty acid composition of obligate alkaliphile membrane is a factor precluding growth at low pH values. Specifically, the combination of high concentration of branched chain fatty acids and substantial fraction of unsaturated fatty acids may make the membrane leaky at near neutral pH, although it functions effectively at high pH (140). Facultative alkaliphiles lack desaturase activity and loose their ability to grow at near neutral pH when supplemented with an unsaturated fatty acid (85). Alkaliphilic bacteria have more acidic amino acid composition of proteins and reduced basic amino acid content that are secreted into the external environment (218,220,222). Further, one of the salient features of alkaliphiles is that their cytoplasmic membranes contain a high concentration of respiratory chain components such as cytochromes including a -, b- and c-type and are red in color (220,299), believed to be crucial to life at high pH. This high concentration of respiratory chain components may provide the mechanism by which the alkaliphiles maximise productive proton tranferring collisions between respiratory chain components and the ATPase (16,69,112,126,131,199,217-225,233, 278,402).

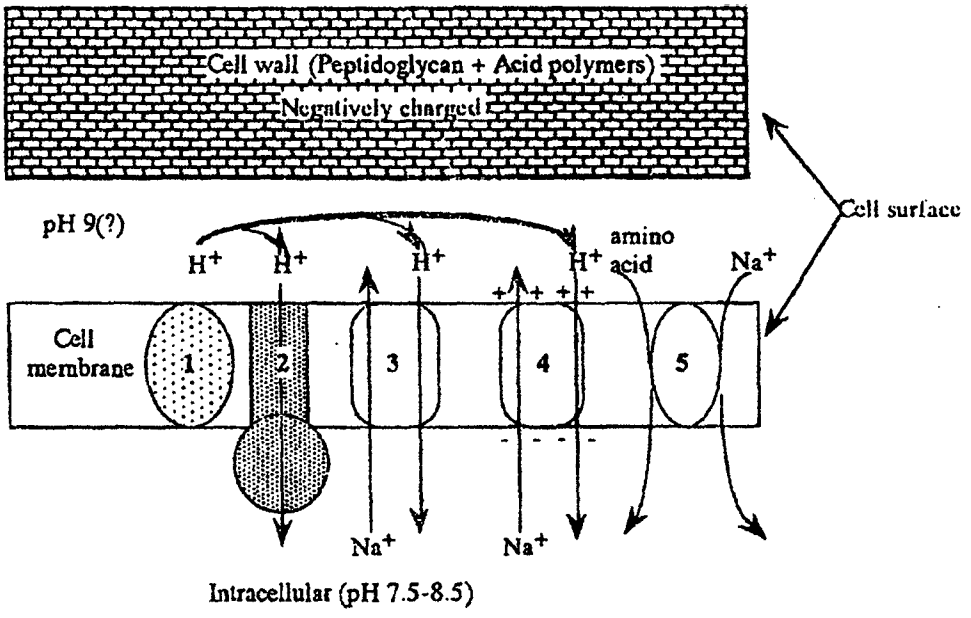
B. Active mechanisms:

A typical alkaliphile, which grows at an alkaline pH range of 9.0 to 11.0, maintains an internal pH 1 to 2 units lower than the external pH (159, 222, 223) (Table 2.3.A) (Fig.2.3.A). This mechanism is found to be present in obligate as well as facultative alkaliphiles but is absent in alkali tolerant bacteria which cannot grow at pH values above 9.0. (126). As a result of this, the pH gradient is reversed (acid in) with respect to a chemiosmotic driving force. Yet, the cells of alkaliphiles like other aerobic bacteria and mitochondria, extrude protons during respiration acidifying a narrow region near the membrane. In alkaliphiles, however, primary proton extrusion is followed by a Na^+ dependent proton accumulation resulting in a proton gradient (pH) H^+ in > H^+ out and a Na^+ gradient Na^+ in < Na^+ out. This function is perfomed by Na^+/H^+ antiporter, which exchanges internal Na^+ for external H^+, both the ions, transported against their concentration gradients at high pH (101,120,126,127,134,166, 201-203,218,220,222,341). Most alkaliphilic bacteria therefore require the presence of Na^+ not only for growth (125,196-198,345) but also for motility, (128,145) and sporulation (211,226). When alkaliphilic bacilli are either transferred to Na^+ free buffers or subjected to a shift in external pH from near neutral to highly alkaline conditions, in the absence of Na^+, a complete failure of pH homeostasis is observed when the cytoplasmic pH rapidly equilibrates to the alkaline external pH. This alkalinisation of the cytoplasm is immediate inspite of the observation that the

TABLE 2.3.A Intracellular pH values in Alkaliphiles at different external pH values.[159]

MICROORGANISM	OPTIMUM pH FOR GROWTH	INTERNAL pH	EXTERNAL pH
Bacillus alcalophilus	10.5	8 7.6 8.6 9.2	8 9 10 11
Bacillus firmus	10.5	7.7 8 8.3 8.9 9.6	7 9 10.8 11.2 11.4
Bacillus strain YN-2000	10.5	8.5 7.9 8.1 8.4	7.5 8.5 9.5 10.2
Bacillus halodurans	10.5	7.3 7.4 7.6 7.8 7.9 8.1 8.2 8.4	7 7.5 8 8.5 9 9.5 10 10.5
Protoplasts		7.5 7.9 8.2 8.4 8.6	7 8 8.5 9 9.3

Extracellular (pH 10.5)



1: Respiratory chain; 2: FoF1-ATPase

3: ΔpH dependent Na^+ / H^+ antiporter

4: Δψ dependent Na^+ / H^+ antiporter

5: Amino acids/Na^+ symporter

Fig. 2.3.A. Schematic representation of cytoplasmic pH Regulation [159].

cytoplasmic buffering capacity of the bacilli is relatively high in alkaline range (127, 217,219,224). Further, all alkaliphilic bacilli growing at alkaline pH possess F_1 F_0 ATPase localised within the cytoplasmic membrane also helping in the translocation of protons (144,225).

A diagrammatic summary of properties of alkaliphilic bacilli relating to their bioenergetics is as represented in Fig. 2.4.A. (220).

2.6. Industrial applications:

Alkaliphilic microorganisms have made a large impact with their application and manufacture of mass-market consumer products in industries. The so-called "Biological Detergents" contain enzymes obtained from alkaliphilic and alkali tolerant bacteria. The traditional craft industry of leather tanning uses a series of highly alkaline and unpleasant processes where the application of alkaline enzymes has brought significant process improvements. Paper and textile processing industries also require enzymes for enzymatic pulping process under alkaline conditions, which help the plant tissues to swell and facilitate the degradation of pectic substances. Alkaliphiles have also been used for the production of cyclodextrins from starch by the action of cyclomaltodextrin gluconotransferases. These cyclodextrins are used as emulsifying, foaming, stabilizing agents and as molecular capsules to wrap up fragrances or drugs for slow release and hence find wide applications in food, fine chemicals and pharmaceutical industries. Alkaline enzymes have significantly been used in waste treatment and appear as useful

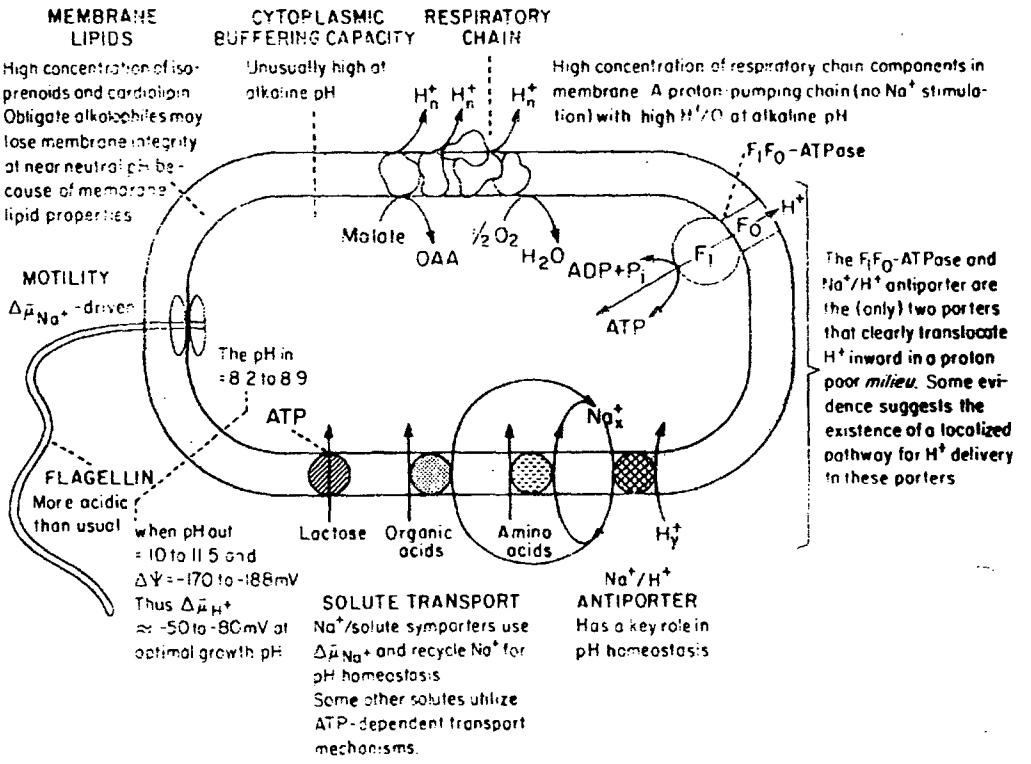


Fig. 2.4.A. A diagrammatic summary of properties of Alkalophilic bacilli relating to their bioenergetics [220].

target for bioremediation of sites contaminated with toxic chlorinated compounds, since increase in pH can increase the bioavailability of these compounds with the resultant decrease in the toxicity. Indigo fermentation is the first industrial application of alkaliphilic bacteria in the world where reduction of indigo from indigo leaves is brought about by alkaliphilic *Bacillus* sp. (120, 121, 157,158, 159,216, 221).

The immense potential of alkaliphiles has resulted in their exploitation for use in industries, particularly with their enzymes active at high pH. Such Alkaline Enzymes are reviewed in the following section.

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SECTION B

ALKALINE ENZYMES

2.1.B. Introduction:

Enzymes are commercially exploited biocatalysts performing a multitude of chemical reactions. They are highly selective catalysts operating under mild reaction conditions viz. ambient temperature, pressure and neutral solutions. However, over the past two decades, the recognition and discovery of the Extremozymes from Extremophiles, catalysing reactions under extreme conditions of temperature, pH, Salt concentration, non-aqueous milieu, very high heavy metal concentration, etc. has opened new avenues for applications of these enzymes in various industries (Tables 1.2 & 1.3) (Chapter I).

2.2.B Alkaliphiles-A source of novel enzymes:

Enzymes stable and active at high pH are called alkaline enzymes that belong to the group of extremozymes. The search of these enzymes catalysing at an alkaline pH range was initiated during the major interest in the use of such enzymes in detergent, although paper, textile, leather and waste treatment industries also require enzymes that function at high pH. Hence there is a considerable commercial demand to develop such alkaliphilic enzymes as biocatalysts in modern biotechnology (121,157). Studies of alkaliphiles have led to the discovery of several enzymes stable at high pH with numerous applications (Table 2.1.B). Some of these enzymes have been produced on an industrial scale.

2.3.B. Alkaline enzymes as detergent additives:

The most commercially important field of application for alkaline enzymes is their addition to household and industrial laundry detergents and in household dishwashers.

TABLE 2.1.B Enzymes produced by Alkaliphiles.[158]

ENZYMES	OPTIMUM pH	STABILITY pH	MW X 10^4	ENZYMES	OPTIMUM pH	STABILITY pH	MW X 10^4
Alkaline protease				**β- 1,3-glucanase**			
No. 221	11.5-12.0	4.0-11.0	3	No. K-12.5	5.5-9.0	6.0-8.0	4
No. 8-1	10.5-11.0	6.0-9.0	3	No. 221	8.5	5.0-9.0	3.6
No. D-6	10.5-11.0	4.0-12.0	2-3				
Alkaline amylase				**Xylanase**			
No. A-40-2	10.5	7.0-9.0	7	No. C-59-2	5.5-9.0	5.0-9.0	3.58
No. H167	10-11	6.0-11.0	6	No. 212	5.7	4.0-9.0	2.3
			7.3		6.8		3.7
			8		7.8		1.45
N0. 17-1	4.5-10.0	6.0-10.0	5-6	No. C-125	6.0-7.0	5.0-11.0	1.6
N0. 38-2	4.5	6.0-10.0	8.8		6.0-10.0	4.0-12.0	4.3
	7	6.0-9.0	8.5				
	8.0-9.0	------	8.5	**α-Galactosidase**			
No. 313	8	6.0-8.0	6.4	No. 31-2	7.7	7.5-8.0	-
Alkaline pectinase				**β-Galactosidase**			
No. P-4-N	10	5.0-9.0	6-7	No. 31-2	6.5	5.5-9.0	18.5
Alkaline Lipase				**Pencillinase**			
No. 26.1-B	9.5	5-11	-	No. 170	6.0-7.0	7.0-10.0	2-3
Alkaline pullulanase				**Maltose dehydrogenase**			
No. 202-1	9	6.0-10.0	9.2	No. 93-1	10.2	6.0-10.0	3.9
					9.8	7.0-8.0	4.8
Alkaline cellulase				**Glucose dehydrogenase**			
No. N-4	6.0-11.0	5.0-11.0	4-8	No. 93-1	9.8	6.0-8.0	5.1
No. 212	6.0-8.0	5.0-11.0	5				
No. 1139	9	5.0-11.0	9.2				
Alkaline alginase				**Uricase**			
No. M-2	9	8.0-10.0	4	No. H-3	9	10	10
Alkaline catalase				**Polyamine oxidase**			
No. Ku-1	10	7.0-9.0	12.58	No. PO-1	4	3.0-6.0	6.4 X 2
					6	-	
Alkaline RNase				**β- Mannanase**			
No. 243	9	6.0-10.0	1.2	No. AM001	9	8.0-9.0	5.85
					9	8.0-9.0	5.95
Alkaline DNase					8.5	8.0-9.0	4.02
No. M-29	9	6.0-10.0	4				
Restriction enzyme				**β- Mannosidase**			
No.170	7.5	------	------	No. AM001	6	6.0-5.0	9.4

The use of microbial enzymes in laundry processes is not a new idea e.g. Biotex® a pre-wash laundry detergent containing an alkaline protease called Alcalase® was launched in the early 1960s (121). However, during the 1980s there has been a dramatic revival for detergent enzymes due to remarkable technical innovations and improvements including the non-use of sodium tripolyphosphates (STPP), realising the environmental concern of phosphate pollution.

For an enzyme to be useful as a detergent additive it must:

1) Be active in solutions at alkaline pH because of the alkalinity of detergent composition with a working pH range between 8-11.
2) Be stable in the presence of detergent additives such as anionic and non-ionic surfactants, chelating agents, bleach agents, bleach activators, perfumes, optical brightners, solvents and proteinases under washing conditions.
3) Be highly stable over a wide temperature range due to variations in washing temperatures.
4) Exhibit long term stability in the detergent product.
5) Be active in the absence of calcium ions due to presence of sequestering agents in detergents.
6) Be active in the presence of organic solvents such as ethanol (5-10%), added to thixotrophic (liquid) detergents (121).

Since detergent enzymes account for 30% of the total worldwide enzyme production, the continuous screening of improved alkaline enzymes from alkaliphilic

microorganisms represents a good example of a successful commercial exploitation of biology by industry.

Companies with a significant interest and commercial stake in detergent enzymes include International Bio-synthetics (IBIS), The Netherlands, a joint venture of shell and Gist-Brocades, Novo industries, A/S, Denmark and Miles Laboratories, U.S.A, a division of Bayer. Presently, the products from these industries are used globally by the two largest detergent manufacturers, Procter & Gamble and Unilever (121).

The most commonly used enzymes in detergent formulations are alkaline amylases and proteinases with the recent introduction of cellulases and lipases, as discussed below:

1. **Amylases**: Amylases are one of the most widely exploited enzymes to date in the laboratory and in industry which hydrolyse starch (Figs. 2.1.B-2.3.B), including raw starch (1,186,390) with tremendous potential in different industries like food, detergent, textile, paper, adhesives, fine chemicals, pharmaceutical and fermentation. Although amylases produced by members of the plant and animal kingdom have been traditionally used for various commercial purposes (Table 2.2.B), microbial intracellular (317,385) or extracellular amylases are preferred for commercial exploitation due to their multifold properties, easy extraction procedure, lower production cost and potential of unlimited supply (302).

Many of the industrial processes require amylases, which function at high pH/temperature (379). These enzymes are known to be advantageous for industrial

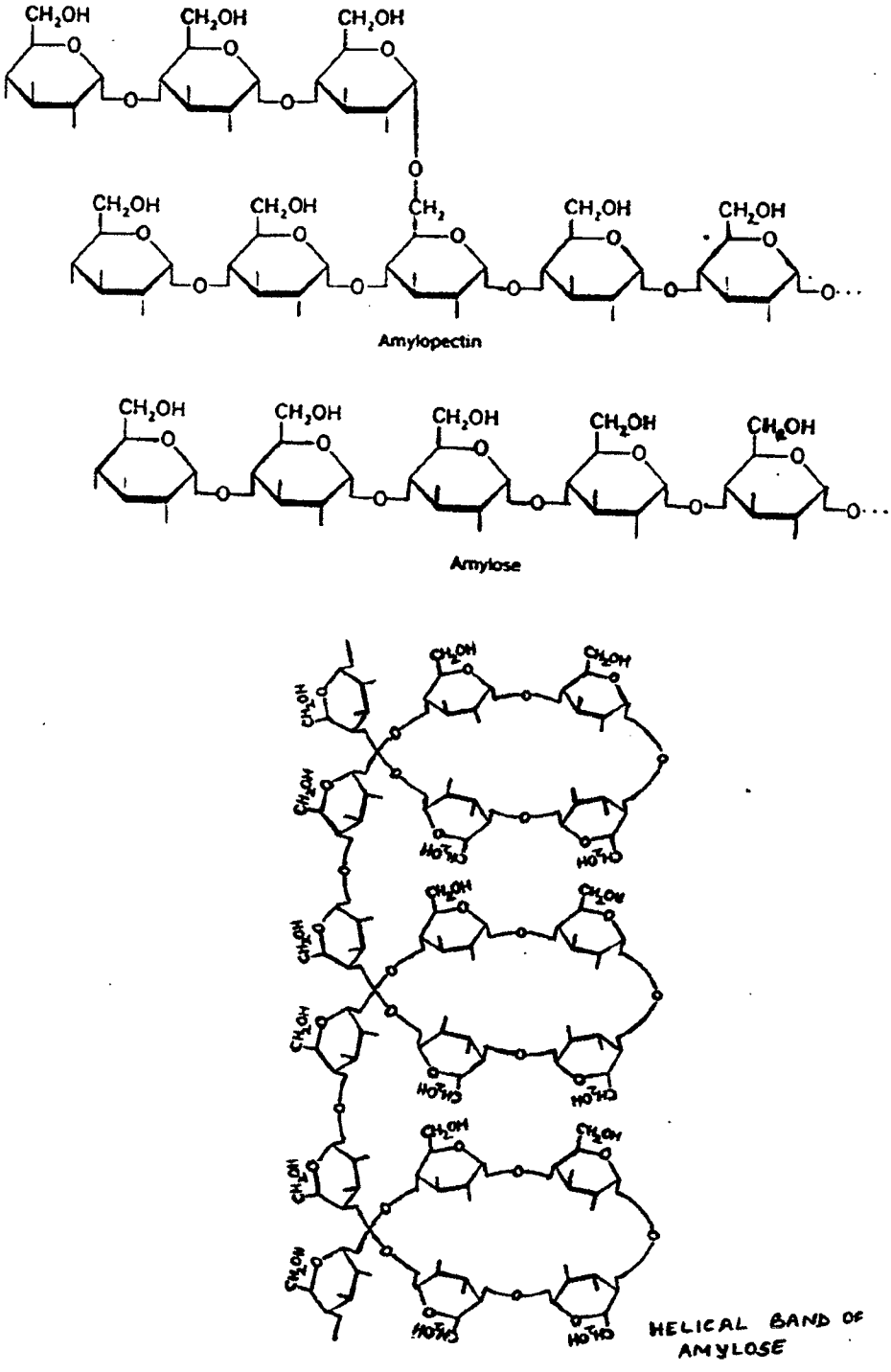

Fig. 2.1.B. Chemistry of starch.

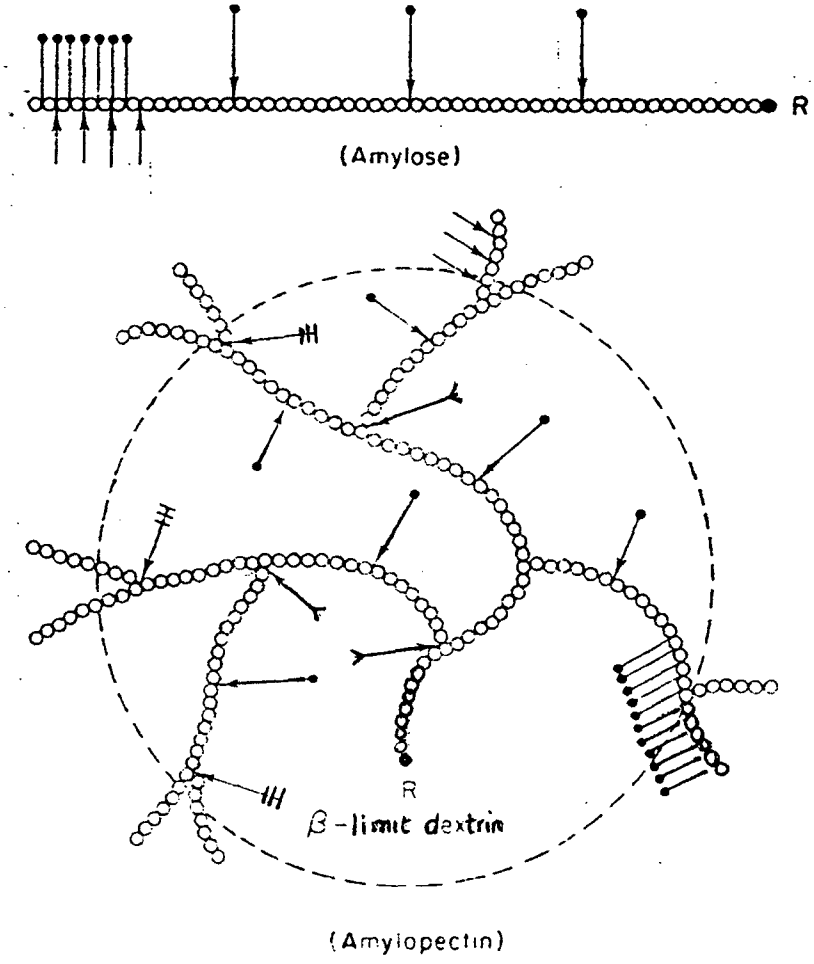

Fig.2.2. B Reaction mechanisms of various amylases. Key to figure.[392]

- ●⟶ amylo(1→4)dextrinase, α-amylase
- ⟶ amylo(1→4)maltosidase, β-amylase
- ●— amylo(1→4, 1→6)glucosidase, amyloglucosidase
- ⟩⟶ amylo(1→6)dextrinase, isoamylase
- ⫴⟶ amylo(1→6)glucosidase ?

R = Reducing terminal

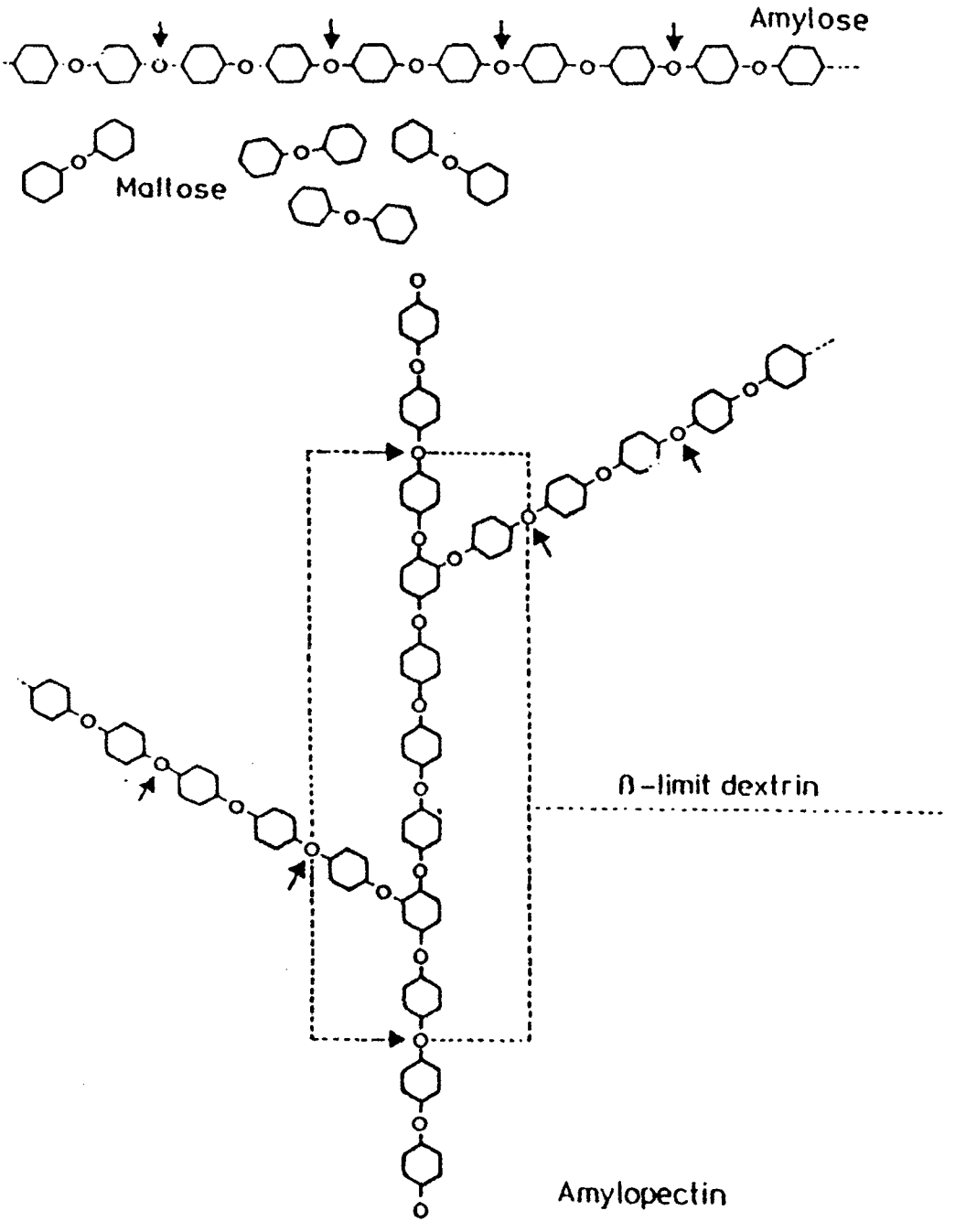

Fig. 2.3.B. Enzymatic action of β-amylase on amylose and amylopectin [3(

Table 2.2.B Sources of Amylases.[392]

Origin	%	
	Type of enzymes	Main products from starch
Plant		
Malt	α, β,	M
Sweet potato	β	M
Soy beans	β	M
Animal		
Saliva	α	D,M
Pancreas	α	D,M
Microorganisms		
Bacteria		
B. subtilis	α	G,M
B. stearothermophilus	α	D,M
Clostridium	AG	G
Mold		
Rhizopus	AG, α	G
A. niger	AG, α	G
A. oryzae	AG, α	G
Others		
Endomycopsis	AG, α	G
Oospora	α	D

Key to abbreviations: AG-Amyloglucosidase; M-Maltose; G-glucose; D-dextrin.

Table 2.3.B Characteristics of Bacterial Amylases Under Extreme Conditions.[163]

Strain	pH optimum	Temperature optimum (°C)
Thermostable α – amylases		
Thermomonospora curvata	5.5-6.0	65
Thermoactinonyces vulgaris	5.9-7.0	60°C optimum activity, unstable above 70°C
Thermophile V-2	6.0-7.2	Optimum of 70°C but stable at 80°C for 2hours
B. stearothermophilus	5.0-6.0	70°C optimum activity, varies according to growth temperature and enzyme isolation.
B. licheniformis	5.0-8.0	76°C optimum activity; 50% of maximal activity at 90°C
Alkaline α-amylases		
Bacillus alkaliphilus	9.2	51
Bacillus No. A-40-2	10.5	50-55
Pseudomonas stutzeri	8	Inactivated above 70°C
Acidic α-amylases		
B. acidocaldarius 104-1A	4.5	60-63
B. acidocaldarius Agnano 101	3.5	75
Clostridium acetobutylicum	4	50

applications as they reduce bacterial contamination, increase substrate/starch solubility, enhance enzymatic reactions and are stable to denaturing action of solvents, detergents, proteolytic enzymes etc. Such thermostable amylases have been reported from several microorganisms including bacilli (176,370,371, 396), archaebacteria (46,88,115,169,208,209,277,373) and fungi (258). These enzymes have been purified and some of their properties have been studied (66) (Table 2.3.B).

a) Biosynthetic aspects of amylase production:

Amylase production in microorganisms is greatly influenced by the growth phase, nutritional factors such as the carbon and nitrogen source and presence of inorganic elements.

Amylase is generally produced during the idiophase with variations occurring in different organisms. Some organisms produce amylase during the stationary phase while others form during the logarithmic growth phase in parallel with cell mass. In the industrial processes, where crude media are used, the maximum amylase production takes place in the stationary phase. Slow metabolic rate is known to favor amylase production (163).

Effectiveness of the carbon source for amylase production is governed by the metabolic rate of the organism. The greater the velocity of carbon metabolism, the more strongly is amylase formation inhibited (392). Hence glucose, fructose and sucrose which are most effective in promoting respiration are ineffective in enzyme formation whereas lactose, galactose, maltose and starch are known to support good

growth and amylase production (243, 392). Undefined carbon sources such as barley corn flour and oil seed cakes are known to induce a high level of amylase production in many bacterial strains (243).

Further, the effect of proteins as nitrogen sources depends largely upon the amino acid constitution. Casein, soyameal and gelatin have been found to be excellent nitrogen sources for amylase production whereas, bacto liver, yeast extract, bacto beef have no correlation with regard to amylase excretion (392). However, a high level of amylase activity using proteose peptone and tryptone as nitrogen sources has been observed by Mamo and Gessesse (243). Further, amines such as putrescine, cadaverine and spermine have also been found to stimulate amylase formation.

Most of the α-amylases of *Bacillus sps* have been found to be dependent on calcium ions for stability and activity. The presence of calcium is required for enzymic activity and for the protection of the enzyme from protease. Therefore α-amylases are regarded as calcium metallozymes. However, α-amylase of *B.licheniformis, B. coagulans* and *B. brevis* have been reported to be calcium independent and stable to EDTA action (21).

b) Amylases as detergent additives:

Amylases as detergent additives at high pH remove starch-based stains often working synergistically with proteinases to dissolve protein-starch combinations in food stains. Products include Termanyl® (Novo) and Maxamyl® (IBIS), which are effective up to 100°C and exhibit a good performance up to pH 10.0. Rapidase® is used in desizing

and softening of denim (159). The first alkaline amylase was produced in Horikoshi II medium by cultivating alkaliphilic *Bacillus* sp. A-40-2 (152). Boyer and Ingle reported an alkaline amylase in strain NRRL B-3881 that was the second report of an alkaline amylase (40,41). Many alkaliphilic strains have now been reported to produce alkaline amylases (141,142,161,186,190,192,193,194,205,206,252,390,395).

2. **Proteases**: These are the most commonly used enzymes in detergent formulations and have the largest market segment obtained from alkaliphilic microorganisms (120,121,151,158,159,216,221). Serine proteinases produced from alkaliphilic *Bacillus* sps. are endopeptidases with a reactive serine molecule at the active site. Commercial products include Alcalase® and Esperase® (Novo industries) and Maxatase® and Maxacal® (International Biosynthetics- IBIS). Maxatase® is an alkaline protease active between pH 7.0 and 11.0 with an optimum activity at pH 9.5 to 10.0, the pH of many detergents. In their encapsulated form, they are added as 0.4-0.6 % to detergents for hydrolysis of proteins, and removal of proteinaceous stains as blood, egg, grass and those from body secretions. Besides lifting proteinaceous soil, proteinases ensure that coagulated protein is not redeposited on the fabric during the wash, which gives a Grey unclear appearance to whites. (157).

Alkaline proteases have also been used in the hide dehairing process, where dehairing is carried out at pH values between 8.0 and 10.0 (159). In the traditional dehairing process, the hides were placed in a bath containing calcium hydroxide and sodium sulfide at pH around 12.0. This process had the disadvantages of being

detrimental to the hairs (which sometimes have commercial value), causing swelling of the skins resulting in difficulties in processing and causing unpleasant effluent problems. The application of bacterial enzymes in the manufacture of leather has now resulted in a quicker and more reliable process avoiding the use of sulfide and giving a higher quality product. Salt cured hides are soaked in alkaline liquor to swell the skins. The uptake of water and cleaning of the hides is improved by the addition of detergent proteinases such as Alcalase® (Novo) and Milezyme® (Miles Laboratories) and Reverdase® (IBIS) (120,121). The dehairing process relies on dissolving the proteinaceous material binding the hair (keratin) in the hair follicle without damaging the fibers (collagen) of the skin and in some cases without damaging the hair (wool), which is also a valuable product.

The bating process, which is usually performed at pH 7.0-9.5, uses proteases such as Batinese® (IBIS) used to modify the matrix proteins, elastin and keratin making the leather supple or rigid depending on the desired character of the finished product.

An interesting application of alkaline protease was developed by Fujiwara and coworkers (96,97) for the removal of the gelatinous coating of X-ray films for recovery of silver. Alkaline proteinases from alkaliphilic *Bacillus* sps, *Thermoactinomyces, Streptomyces* and haloalkaliphiles have been reported (55,207,274,355,366,367,401, 404).

3. **Cellulases**: alkaline cellulases with an optimum pH of 10.0 for activity were first reported by Horikoshi and coworkers who found two *Bacillus* sp. strains N4 and 1139

producing extra cellular alkaline CMCases (156). *Bacillus* sp. strain N-4 (ATCC 21833) was found to produce multiple CMCases active over a broad pH range of 5.0 to 10.0. This discovery led to an industrial application of cellulases as laundry detergent additives. Cellulase 103, introduced by Genecor International (Rochester, NV) and launched as "Attack®" detergent with added cellulases, isolated from alkaliphilic bacterium is the first large-scale commercial application of an extremomolecule.

Cellulases exhibit fabric softening and color brightening properties, besides removing soil (157) by removing or opening up the microfibrils that appear on the surface of the cotton fabrics due to wear and repeated washing, thereby restoring the original appearance and smooth fiber structure. Cellulases can also be used in waste treatment, since the undigested material is mainly cellulose (326). Ito *et al* (164, 165) have extensively studied the use of alkaline cellulases in detergents while Park et al (285) have reported alkaline cellulases from *Streptomyces.*

4. Lipases: These enzymes degrade fats into more hydrophilic fatty acids and find great applications as detergent additives, as hydrophobic stains caused by cosmetics, body fats and oil based foods are more difficult to remove. Introduction of alkaline lipases as detergent additives is only recent, although their market value is as good as proteases. Extensive screening for alkaline lipase producing microorganisms from soil and water samples has been conducted by Watanabe et al (382). Wang and Shen (381) reported purification and characterization of thermophilc and alkaphilic tributyrin esterase from *Bacillus* A-30-1, isolated from a hot spring area of Yellow Stone

National Park. Bhushan (33) found a lipase produced from an alkaliphilic *Candida* sp. by solid state fermentation. A Japanese company has produced a laundry detergent, containing a non-alkaliphilic fungus lipase, produced by recombinant DNA technology. Many alkaline lipases tested with detergents are inhibited in the presence of either alkylbenzene sulfate or dodecyl benzene sulfonate (157,158).

In addition to these enzymes, there are reports on alkaline xylanases (80,109,132, 148,149,154,256,264,272,279), Pectinases (50,153,358,359), Phosphatases (275), Catalases (228), Chitinases (35,369) and β-lactamases (346), Cyclomaltoglucono transferases (107,263,313), Pullulanase (262), Glucosidase (185), Succinate dehydrogenase (112), 1,4 β glucanases (399), Xylose Isomerase (54) and Urease (329).

Alkaliphiles also exhibit remarkable versatility regarding the production of their secondary metabolites such as 2-phenylamine (136), Carotenes (11), Siderophores (103, 104), Cholic acid derivatives (196), Organic acids (283), Antibiotics (368) and enzyme inhibitors(23). **Exopolymer (Reviewed in section C)** production has been reported in alkaliphlic *Cyanobacteria* (71,380).

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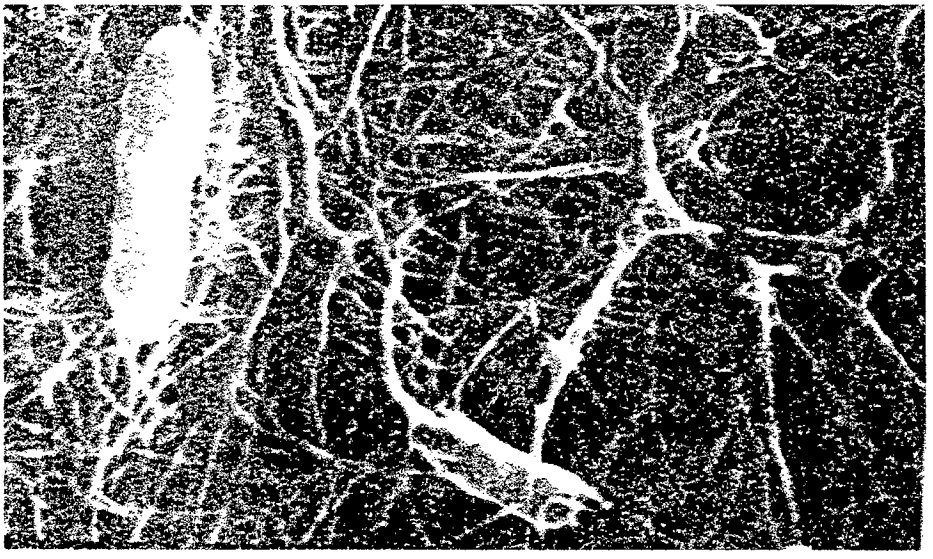
SECTION C



Bacterial cellulose

EXOPOLYMERS

2.1.C. Introduction:

Microbial exopolymers are high molecular weight secretions of bacteria belonging to wide genera (73,91,123,236,237,240,289,292,297,303,308,309,318,322,325,342,357, 360,374), yeasts (298,328) and microalgae (71,72,143,380), ranging from capsules which closely surround the cells to the slime matrix released into the environment forming highly viscous aqueous solutions (74). Chemically, these polymers are composed of repeating single unit monosaccharides joined by glycosidic linkages. Additional structural complexity is achieved by the introduction of branches into the polymer, thereby representing a rich source of structurally diverse molecules with unique physical and chemical properties (293).

History of mircobial exopolymers can be traced back on to the primitive earth in the Archaeon era, more than 3.5 billion years ago as suggested by microfossil records of *Cyanobacteria* producing copious amounts of EP in the form of sheaths, capsules and slimes (143). However, the interest in microbial exopolymers as possible industrial fermentation products received major impetus in 1955 through the commercialisation of the polysaccharide B-1459, "Xanthan Gum" produced by a mutant of *Xanthomonas campestris* NRRL-B-1459 (52), now marketed under the trademark "Kelzan®" and "Keltrol®" (52). Similarly, "Gellan®", from *Sphingomonas paucimobilis* strains is marketed under the trademark "Gelrite®" or "Kelcogel®". Dextran derived "Sephadex®", bacterial cellulose as "Biofill®" (350) have also proved to be valuable despite the fact that they are available from non-bacterial sources (Table 2.1.C). Microbial EP are preferred for commercial exploitation due to their diversity,

Table 2.1.C : Commercially important Ploysaccharides.[316]

Type of Polysaccharide	Examples
Starch and derivatives	Raw starches, pregelatinized starches, modified starches
Cellulose derivatives	Microcryatalline cellulose, carboxymethly cellulose, methylcellulose
Sea weed extracts	Alginates, carrageenans, agar, furcellaran
Plant extracts	Gum arabic, gum karaya, gum tragacanth
Seed gums	Locust bean gum, guar gum
Plant extracts	Pectins
Microbial gums	Xanthan gum, gellan gum

multifold properties such as biodegradability (311), ecofriendly nature, low toxicity, specific activity, lower production cost and potential of unlimited supply.

EP producing bacteria have been identified in a variety of ecological niches both hostile and extreme (269). Extremophilic microorganisms (Table 2.2.C) offer a number of novel exopolymers active at extreme temperature, pH and salinity, which are exploited for a number of applications ranging from emulsifiers to adhesives. Extremophilic biopolymers are a significant and unexplored biological resource of industrial interest.

Although extracellular polymer production is frequently encountered in different organisms, only a fraction of these organisms produce substantial amount to be of economic interest (250). Cellulose, starch, water soluble gums and biosurfactants are the polymers for which effective markets have been obtained in different industries used as thickening, emulsifying, stabilizing, gelling and viscocifying agents. Studies of the chemical structure and identification of these polymers along with their chemical and physical properties are essential for understanding the possible applications in industries. Further, although the type and amount of the polymer produced depends on the producer organism, factors like growth phase, rate of aeration, temperature and pH, carbon/nitrogen nutrients, trace elements, also affect their production.

Table 2.2.C : Exopolymer producing Extremophiles.

Extremophile	Category	Source of isolation	Ref.
BACTERIA			
Sulfolobus solfataricus	Acidophile, archaea	Hot acidic spring of Agnano naples	269
Bacillus thermoantarcticus	Thermophile	Mount Melbourne Antarctica	244
Methanosarcina thermophila	Thermophile	-	340
Streptococcus thermophilus	Thermophile	Yoghurt	87,231, 343.
Haloferax mediterranei	Halophile,& archaea	-	6
Bacillus sp. Strain SP018	Halothermophile,	-	289
Alteromonas infernus sp. nov	Barophile	Deep sea thermal vent	300
Clostriduim perfringens	Anaerobe	-	305
CYANOBACTERIA			
Anabaenopsis circularis	Barophile	Benthic region	26
Cyanospira capsulata	Alkalophile	Alkaline soda lake Magadi-kenya	380
Microcystis flos aquae C340	Alkalophile	Alkaline soda lake Magadi-kenya	72
Cyanothece sp	Alkalophile	Somaliland saltpan	71
Spirulina	Alkalophile	Soda lake	71
Synechococcus, Mastigocladus	Thermophile	Thermal springs	71
Trichodesmin	Oligotroph	Nutrient poor open oceans	72
Chroococcidiopsis	Psychrophile	Polar deserts	72
Phormidium	Barophile	Benthic	26

2.2.C Physiology and regulation of Exopolymer production:

Prokaryotic exopolymers are released as water soluble material into the surrounding medium and cause a progressive increase of its viscosity. The amount of exopolymer produced depends upon the microbial strain and culture conditions. A slight change in growth condition such as growth phase, culture medium composition and environmental factor may significantly increase or decrease the yield of the desired product (124,330,378). Various process parameters, which influence EP production and secretion, have been studied (288,391). The factors playing significant role are described below.

a) **Growth phases in batch culture**: Growth phase is an important parameter in EP synthesis and varies in different cultures. In some microorganisms, EP is secreted continuously during growth (30,59,303,352), whereas in others, EP production is a feature of the late log phase and stationary phase (288,347,391). Further, some bacteria secrete EP capsules early in growth and loose slime later on; while other bacteria produce only slime (39,59). The tight capsules change in their consistency and other properties, resulting in slow dissolution of the capsule from the log to stationary phase, thus getting loosely associated and eventually released extracellularly (74,347-349). Under laboratory conditions, most bacterial strains release large quantities of EP during the stationary phase of growth (349).

Emulsan is accumulated on the cell surface in precursor form during logarithmic growth and subsequently released in the stationary phase or after inhibition of protein synthesis (310). Bacterial alginate synthesis by *A. vinelandi* starts in the exponential phase of growth but the rate of production increases after cessation of growth (150).

EP chemistry also varies with cellular mode of growth (28). It is reported that EP produced by the cells in log phase is different in composition from the EP produced by the same strain of cells in stationary phase (59,300,349). Further, morphological changes can also affect polymer synthesis e.g. Pullulan synthesis by *Aureobasidium pullulans* starts at the same time as hyphal budding (52).

b. Environmental factors:

Environmental factors independently affect the fermentation kinetics and greatly influence the production of EP. These factors can lead to optimum fermentation yields and also affect chemical composition, macromolecular structure and physical properties of these polymers.

1. **Aeration and Agitation:** Oxygen transfer is one of the key parameters for the process optimisation of EP production with variations existing in different cultures. Generally, EP is produced under well-aerated conditions in liquid media, containing a carbon and nitrogen source, with potassium phosphate buffer (pH 7.0), $MgSO_4$ and trace minerals, although release of EP by *Pseudomonas* sp. has been observed in static conditions (393). Agitation plays a role in maintaining oxygen tension and improving mass transfer of nutrients between the medium and the cells.

Xanthan production is reported to depend on the overall volume and agitation power, requiring above 20% air saturation (49,261). In alginate production by *Azotobacter vinelandii* during low impeller speed, 40% of the substrate was converted into the alginate but at higher rates, much of the sucrose was wasted as CO_2 and only 8% yielded polysaccharide (347). However, in *Acetobacter xylinum*, less bacterial cellulose was formed in shaken flask than in static batch cultures (348). During pullulan synthesis by *Aureobasidium pullulans,* static conditions produced more polymer than did cultures incubated in shaker flasks (52).

2. pH and Temperature: Usually, the optimum pH for the growth of the organism is optimum for polymer production. e.g. optimum pH and temperature values for growth and xanthan production are 7.0 and 28°C respectively (49,250). However, the pH of the medium plays an important role in molecular weight of Pullulan by *Aureobasidium pullulans.* Initial pH values of 5.0 - 6.5 led to the production of high molecular weight polysaccharide and a low molecular weight product at initial pH of 7.0 - 8.0 (52).

Enhanced production of EPS by many Gram negative strains is favored by growth at lower temperatures (15-18°C) in medium deficient in N_2 and high in glucose (210,309). It has been observed that EP production is frequently favored by incubation at sub-optimal temperature, although there are reports of no change in the amount of EP by temperature changes (39).

c. Influence of the composition of the culture medium:

1. **Carbon and Nitrogen source**: EP production has been observed by different organisms on various substrates; the substrates ranging from simple sugars (51,53,100), phenolic acids (257), amino acids to hydrocarbons (79,236,237). Organisms with inherent property of EP production form the polymer under conditions of high carbon and low nitrogen irrespective of the source of carbon available (49,71,75,78,254,276, 328). While a nitrogen source is necessary for cell growth, an excess of nitrogen generally reduces conversion of the carbohydrate substrate to the polymer (250).

It is interesting, however, that while excess of carbon in the medium increases EP production, it is not a necessity for EPS production. Many bacterial species can synthesize EP in the absence of the utilisable carbohydrate in the medium via utilisation of amino acids as a carbon source (347-350). EP formation under low carbon conditions has been found in *Azotobacter* (171,172). Some organisms are reported to release EP during complete energy and nutrient starvation conditions (393).

A number of industrial waste products such as cheese whey which are complex in carbohydrate and amino acid composition have been tested and utilised for large scale polymer production such as xanthan (24).

d. Effect of Ions: Various ions are known to affect polysaccharide synthesis required either for substrate uptake or as cofactors (5,31,32,42,74,89). Gel like consistency of EP is governed by the tertiary interactions of adjacent polysaccharide chains requiring cations such as Ca^{+2} and / or Mg^{+2} which act as ionic bridges forming cross links

between adjacent sugars on different chains. Ca^{+2}/Mg^{+2} ions are also necessary for the adhesion purpose and are used both intracellulary in the transport and secretion of EP and extracellularly as crosslinking agents (74). It has been observed that a rapid disruption of the gel polymer results if the bacteria are transferred to cation deficient medium, restored by the addition of Ca^{+2}/Mg^{+2} ions. The requirement of these ions in the gelling of EP is more pronounced at extreme pH (74).

The ability of bacteria to produce exopolysaccharide under varying conditions is therefore thought to reflect the important functions of these secretions under fluctuating nutrient and environmental conditions and / or by-products of metabolic pathways under the same conditions.

2.3.C. Composition, Biosynthesis and recovery of EP:

Bacterial exopolymers range from simple α-1,4 linked unbranched glucose polymers such as dextran (Fig 2.1.C) homopolysaccharide to highly complex branched and substituted heteropolymers made up of repeating oligosaccharide subunits such as xanthan (Fig 2.2.C), further substituted with pyruvate, acetate, formate, sulphate, phosphate and other groups (Table 2.3.C). This produces a wide heterogenecity among EP, thus representing a rich source of structurally diverse molecules with unique physical and chemical properties. Uronic acid (carboxylated form of sugars) residues confer a net negative charge while pyruvate residues contribute to the water binding properties of the EP (74).

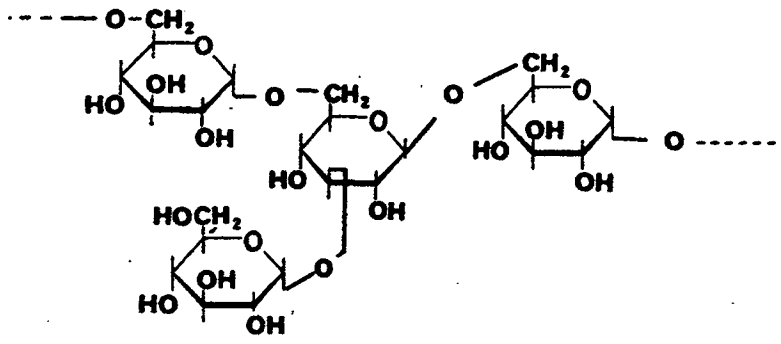


Fig. 2.1.C. Part structure of dextran [348].

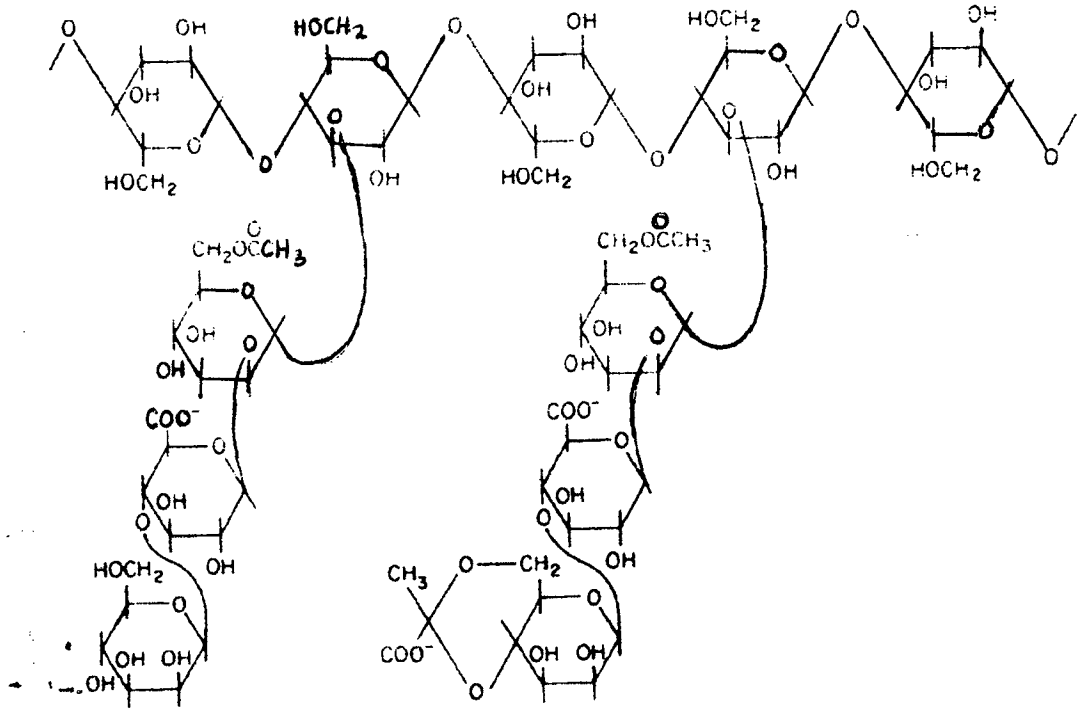


Fig. 2.2.C. Structure of extracellular polysaccharide of *Xanthomonas campestris*, according to Jansson *et al.* (1975) [170]

Table 2.3.C : Composition of Bacterial Exopolymers.

Component	Types
CARBOHYDRATE	
Simple hexoses	*D*-Glucose *D*-Galactose *D*-mannose
Neutrat sugars	6-deoxy L-mannose [Rhamnose] 6-deoxy L-galactose [Fucose]
Polyols	Glycitols, Glycerol, Ribitol
2 deoxy 2 amino sugars as N-acetyl derivatives	*D*-glucosamine *D*-galactosamine, D-mannosamine
Uronic acids	*D*-glucuronic acid *D*-galacturonic acid *D*-mannuronic acid *D*-guluronic acid
NON-CARBOHYDRATE ORGANIC	
Acyl substituents	*O*-acetyl, succinyl, formyl
Ketal substituents	Pyruvic acid
INORGANIC	Phosphates, Sulphates

Exopolymer synthesis occurs at the cytoplasmic membrane utilizing activated precursors glycosyl donors i.e. nucleotide diphosphate sugars or more rarely nucleotide monophosphate sugars and isoprenoid lipid molecules (C55 alcohol bactoprenol), both synthesized intracellularly with their enzymes present in the cytoplasm or loosely associated with the cell membrane.

The EPS synthesis therefore requires enzymes for the uptake of the substrate, production of each nucleotide sugar precursors, separate transferases for each monosaccharide in the subunit, one or more polymerases and proteins involved in the export of the polysaccharide. General pathway for the biosynthesis of EP is as shown in (Fig 2.3.C.).

After formation and partial assembly, the EPS chains are extruded into the extracellular environment where further elongation may occur in Gram positive organisms (Fig.2.4.C), requiring a significant amount of energy expenditure by the bacteria (386).

EP, depending on the nature as sheaths, capsules and slimes can be separated (Scheme 1 pg.58) and recovered from the broth by various methods (Table 2.4.C). The genetic control of the synthesis of these polymers is either due to chromosomally located genes (343) coding for the enzymes used in various stages of synthesis and assembly or associated with transposons, insertion elements and plasmids (375).

2.4.C. Role of EP in:

1. **Producer cells:** EP in producer cells have several key functions including

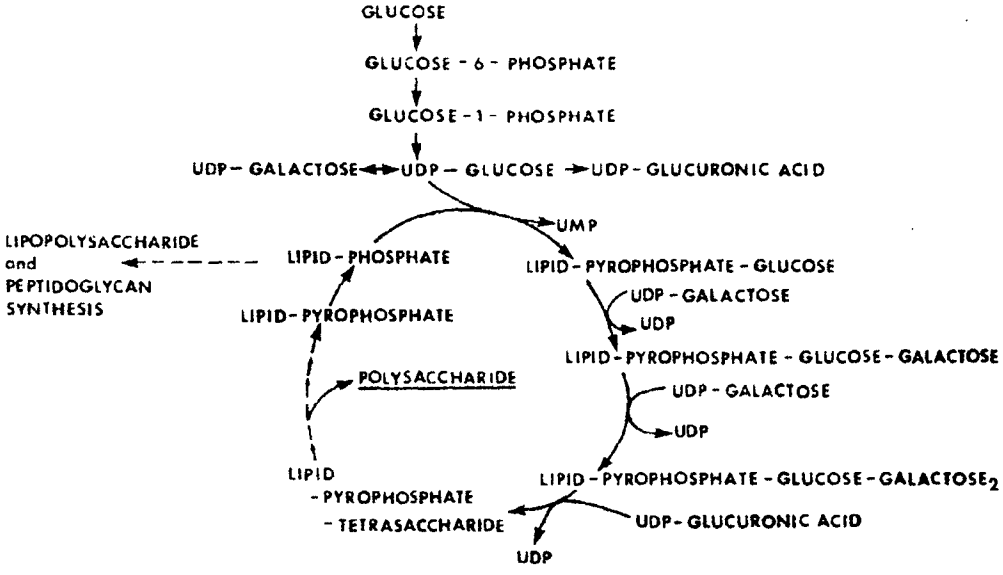


Fig. 2.3.C. Pathway for the biosynthesis of an exopolysaccharide [347]

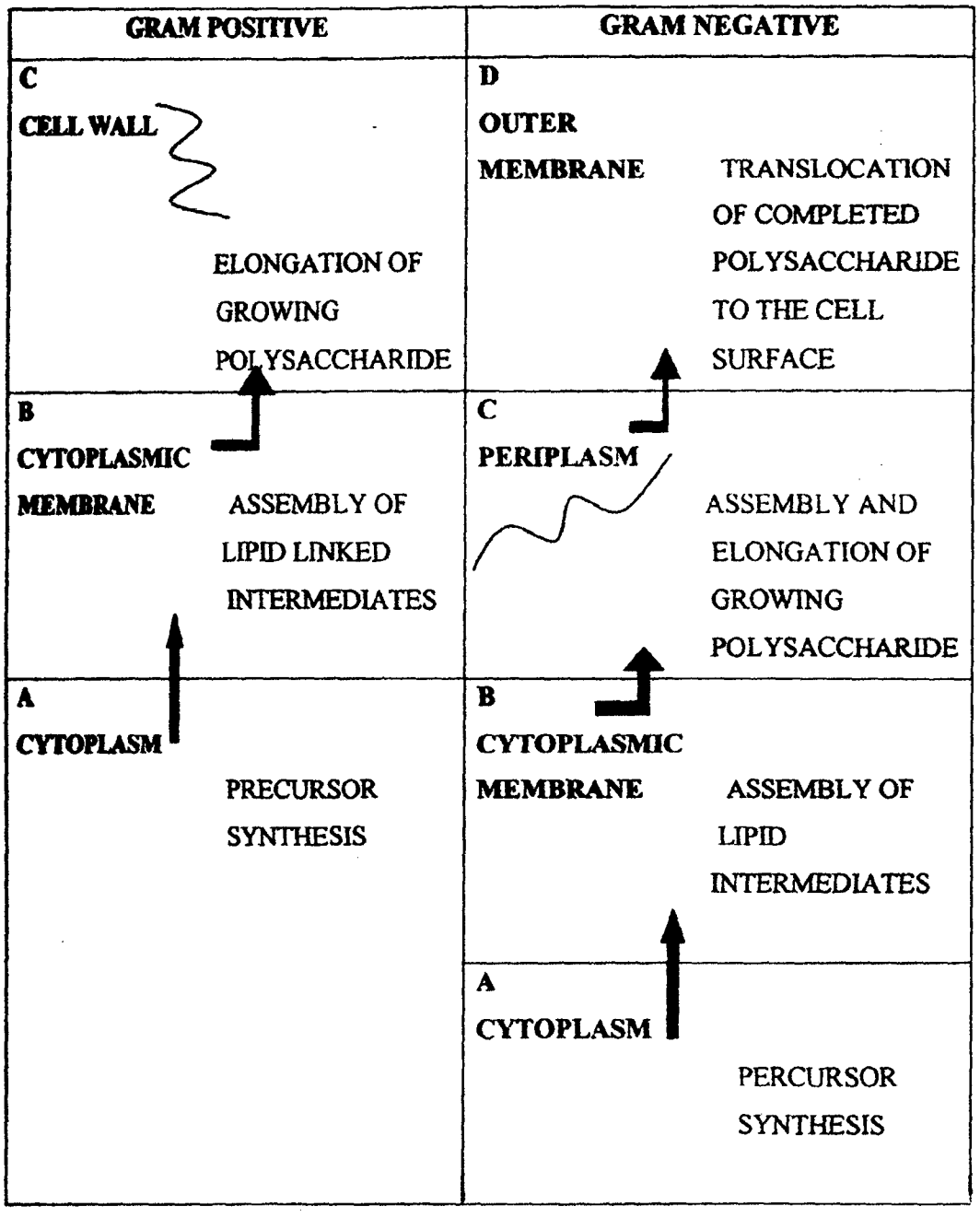
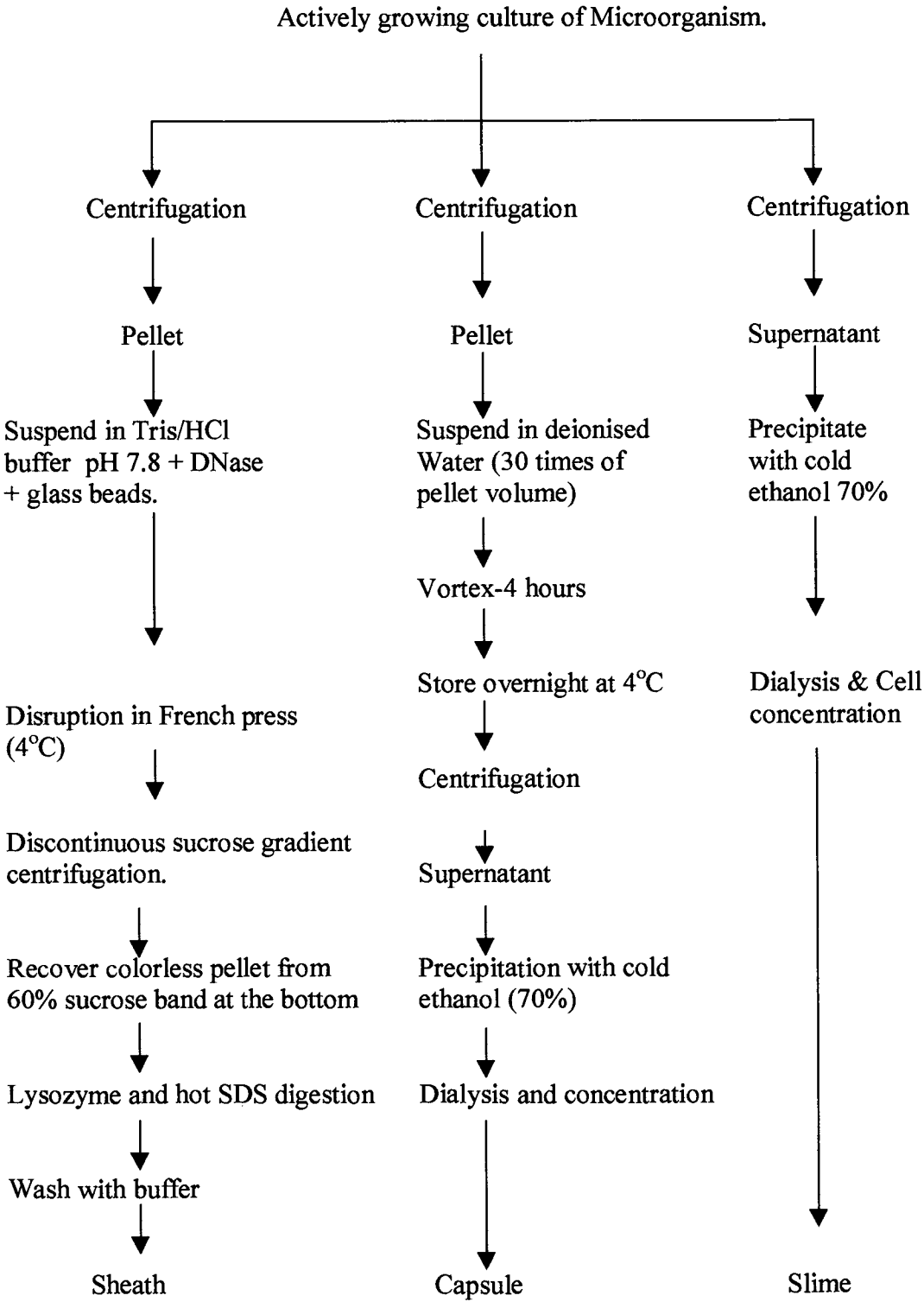


Fig. 2.4.C. Possible mechanism for the synthesis and assembly of bacterial EPS .[386]

Table 2.4.C : Downstream processes for recovery of important Exopolymers & Biosurfactants.[76,324]

No.	Processes
A.	**Batch mode**
I	***Ammonium sulphate precipitation***
a	Emulsan
b	Biodispersan
c	Bioemulsifier
d	Capsular polysaccharides
II	***Sodium Chloride***
III	***Quarternary ammonium salts***
a	Cetavlon
IV	***Organic solvents***
I	Acetone precipitation
a	Bioemulsifier
b	Ploysaccharides
ii	Ethanol precipitation
iii	Isopropanol precipitation
	Trehalolipids
	Sophorolipids
	Liposan
V	***Acid precipitation***
	Surfactin
VI	***Crystallisation***
	Cellobiolipids
	Glycolipids
B	**Continuous mode**
I	***Centrifugation***
	Glycolipids
II	***Adsorption***
a	Rhamno lipids
b	Lipopeptidesan
c	Glycolipids
III	***Foam separation and precipitation***
	Surfactin
IV	***Tangential flow filration***
	Mixed biosurfactant
V	***Diafiltration and precipitation***
	Glycolipids
VI	***Ultrafiltration***
	Glycolipids
VII	***Adsorption***

Scheme 1. Schematic representation for isolation of sheaths, capsules and slimes.[2]



storage (78) which enhance the survival and competitive success of microbial cells under varying natural conditions. While they have been studied in other disciplines, the investigation of exopolymers in extremophiles has been largely overlooked and represents a relatively novel area. Accumulating evidence however suggests that the ability to produce EP is a direct and logical response to selective pressures in natural environment. However, EP should not be considered "essential" to the cell per se, because a cell can remain viable, metabolize and reproduce without an EP coating (74).

The presence of EP buffers cells against quick ionic and environmental changes such as pH, salinity or nutrient regimes (42), thus creating a microenvironment around the microbial cell which allows it to operate, metabolize and reproduce more efficiently.

Exopolymers sequester and concentrate nutrients, important in oligotrophic (65) and highly alkaline environments in which the nutrients are insoluble (71,72). They also localize and maintain the activity of enzymes (232). The remarkable water holding capacity of EP enable the producers to maintain moisture in their immediate environment thereby providing protection against desiccation (143,280,304), predation by phagotrophic protozoans and antibacterial agents such as antiobiotics. EP enhances pathogenecity and survival strategies in pathogenic bacteria by protecting from evasion of phagocytosis (65,305) and in adhering to the gastric epithelium (160).

2. **Biofilm formation:** EP are known to enhance adhesion of microorganisms to surfaces as "surface adhesins" due to the presence of abundant carboxyl groups

resulting in accretion of biofilms. Biofilms colonising artificial surfaces are often a nuisance in a wide variety of technical applications e.g. marine fouling, contamination of pipes in food processing plants and artificial blood vessels (27,29,56,65, 67,297,362,388). Corrosion may be caused by the metabolic products of the cells such as organic/inorganic acids or direct metal chelating activity of the polymer (30-32). Slimy microbial polymers can cause severe processing and quality problems, especially in cane and beet industries during sugar beet storage and processing resulting in sucrose loss (357).

3. **Control of environmental pollution:** As biosurfactants, EP emulsify hydrocarbon-water mixtures resulting in hydrocarbon degradation, including recalcitrant forms of pesticides. Therefore, they are potentially useful for oil spill management and remediation of hydrocarbon and crude oil contaminated soils (76). Further, The ability of EP to bind a wide variety of metal cations such as lead, zinc, cadmium, cobalt, copper, iron, magnesium, strontium, silver and nickel have immense potential in biosorption of these ions from aqueous solutions (90,105,177,249,255,323). These metal ions emerge from various electro plating, tanning, canning, pesticides, film processing industries and nuclear reactors. In recent years, wide spread concern is shown on the removal of these heavy metals from waste water since they have detrimental effects on the entire biota.

EP being high molecular weight compounds with abundant carboxyl and hydroxyl groups can bioconcentrate cations more than 10,000 times (384). Further, reactions

between metallic ions and EP are strongly influenced by pH level, being higher at elevated pH due to increase in the number of ionised acidic groups (74,90). This suggests that mineral development on microbial surfaces and exopolymers may be more widespread at high pH, with microbial metal immobilisation property to be more attributed to alkaliphilic bacteria. Thus, biosorption of heavy metals from industrial effluents using metal resistant alkaliphiles/exopolymer producing alkaliphiles is an important field of research in toxic metal bioremediation. Pollution control through bioremediation is the most economical and ecofriendly approach.

4. Industries (Table 2.5.C): Microbial exopolymers such as Xanthan, dextran, gellan, alginate etc. are widely accepted commercial products of biotechnology with wide range of potential applications as food additive and in non food industries such as pharmacology, cosmetics, oil industry used as thickening, emulsifying and viscosifying agents (324,350,383,384).

Exopolymers are key ingredients in many processed foods such as gelatins, puddings and ice creams (316). Xanthan is incorporated into foods to alter the rheological properties of the water present, thereby modifying the texture of food. Alginates are ingredients of many different types of processed foods with their unique properties of emulsification, retaining water, forming gels and controlling ice crystallisation. Pullulan is used as a probiotic to selectively promote the growth of *Bifidobacterium* sps in the human intestine following its incorporation into specialised dietary foods (350).

Table 2.5.C : Established applications of microbial Exopolysaccharides.[350]

	Use	Polymer
Biological properties :	Antitumour agents	β-D-Glucans
	Eye and joint surgery	Hyaluronic acid (*Streptococcus* EPS)
	Heparin analogues	*Escherichia coil* K5 EPS
	Wound dressings	Bacterial cellulose
Chemical properties:	Enzyme substrates	*Escherichia coil* K4 and K5 EPS
	Oligosaccharide preparation	Curdlan, pullulan, scleroglucan
Physical properties:		
Emulsion stabilization	Foods, thixotropic paints	Xanthan
Fibre strength	Acoustic membranes	Bacterial cellulose
Film formation	Food coatings	Pullulan
Flocculant	Water clarification, ore extraction	Various
Foam stabilization	Beer, fire-fighting fluids	Xanthan
Gelling agents	Cell and enzyme technology	Gellan
	Foods	Curdlan, Gellan
	Oil recovery (blockage of permeable zones)	Curdlan, Xanthan
Hydrating agent	Cosmetics, pharmaceuticals	Hyaluronic acid
Inhibitor of crystal formation	Frozen foods, pastilles and sugar syrups	Xanthan
Shear thinning and viscosity control	Oil-drilling 'muds'	Xanthan
Suspending agent	Food	Xanthan
	Paper coatings	Various
	Agrochemical pesticides and sprays	Xanthan
Viscosity control	Jet printing	Xanthan

Exopolymers are used as textile print paste thickeners and in pigment dye dispersions, hence finding use in textile, printing and dyeing industries (250). Xanthan gum is an excellent suspending agent in ceramic glazes and many hard to suspend mixtures, finding great utility in manufacture of paints, pigments and ceramics.

Exopolymers are also very attractive in health care and cosmetic industries Xanthan gum has excellent properties as a bodying agent for toothpaste and ointments helping the formulations to spread with more ease (250). Deodorant gel products contain Gellan (350).

Bacterial cellulose, a highly pure polymer, free from lignin and other noncellulosic material as the product "Biofill®" is manufactured in the form of wound dressings for patients with burns, chronic skin ulcers or other excessive loss of tissue (350).

In the oil industry, EP is useful for in situ microbially enhanced oil recovery and desludging of crude oil storage tanks (325). The adhesive (bonding of dissimilar molecules) properties of some EP make them promising cements (383,384).

5. Laboratories: Microbiological and biochemical experiments also use exopolymers. Gellan as "Gelrite®" forming thermoreversible gels is a good replacement for agar into microbiological and cell culture media yielding high clarity gels; mostly used for the culture of thermophilic microbial species. Cross-linked Dextran "Sephadex®" finds applications in gel filtration and as molecules used for rapid molecular weight determination. Alginate based gels are used for the

immobilisation of cells and enzymes. Pullulan is used to prepare accurate molecular mass standards of low polydispersity for calibrating HPLC columns used for the size exclusion chromatography of water soluble polymers (350).

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CHAPTER III

Biodiversity of Alkaliphilic Bacteria in varied econiches and their enzyme profiles.

Biodiversity is an assemblage of variability within all living organisms, leading to a rich bioresource that needs to be properly tapped. Such diverse microbial resources are considered to be potentially rich for exploitation.

Over the past decades, microorganisms from different ecosystems have been extensively studied. The focus of investigations is now on extreme environments where a high degree of microbial diversity is found to exist. Like the other extremophiles, "ALKALIPHILES" have also attracted attention because of the extracellular enzymes produced by them active at high pH range, finding applications in a variety of industrial settings.

This chapter describes the prevalence of alkaliphiles in various natural and man made alkaline and non-alkaline environments and a study of their enzymes profiles.

Materials and Methods:

3.1 Collection and analysis of samples:

Four composite alkaline samples were collected from the spent water treatment tank of an Agrochemical factory in Goa. Samples representing neutral, acidic and alkaline econiches were included in the study. In addition, different samples were collected from Deodani Kyars Sambhar Salt Lake, Jaipur, Rajasthan representing a standard saline and alkaline ecosystem with high sodium chloride concentration and high pH (between 8.5-11). The pH of the various samples was tested using pH paper on site and using LABINDIA μp controlled pH analyser (PHAN) in the laboratory.

3.2 Enumeration of Alkaliphiles:

3.2.1 Diluent: Samples were serially diluted in (a) physiological saline (pH 7.0) and (b) carbonate-bicarbonate buffer (pH 10.0) (Appendix B.2) and spread plated on the alkaline media.

3.2.2. Media: Three different media were used for scoring and isolating alkaliphiles (a) Polypeptone yeast extract glucose agar (PPYG), (b) Horikoshi I and (c) Horikoshi II (Appendix A), while haloalkaliphiles were scored on medium with high pH and salt concentration (Appendix A).

Solutions of 10% glucose, starch and sodium carbonate were autoclaved separately and mixed into the basal medium before pouring into plates/slants. The final pH of the freshly prepared medium was maintained at pH 10.5. The plates were incubated for 24-48 hours at room temperature. Plates with fewer than 300 colonies were counted to estimate cfu/ml or g.

3.3 Purification and maintenance of the cultures:

Cultures were subcultured successively on PPYG medium (pH 10.5) to obtain pure cultures and maintained on PPYG agar slants, sealed with parafilm and preserved at 4°C. The isolates were periodically checked for purity. Stock cultures were subcultured every three months and stored at 4°C. Working cultures, subcultured from stock cultures were subcultured every month or when required.

3.4 Selection and characterisation of bacterial strains:

Predominant isolates (126+12) growing at high pH were selected and characterised for colony characteristics, Gram character, shape, mode of cell arrangement, pigmentation, spore formation, oxidase and catalase reactions.

3.5 Distribution of alkaliphilic and temperature tolerant bacteria:

Isolates (126+12) from PPYG (pH 10.5) were replica plated on two sets of PPYG agar plates with pH 7.0, 9.0 and 10.5. One set of plates was incubated for 48 hours at room temperature for growth of mesophiles and the other set at 55°C for thermophiles. Colony size, consistency and growth of the colonies were recorded.

3.6 Studies on the enzyme profiles of the isolates:

The enzyme activities of the isolates (126+12) from PPYG agar (pH10.5) were determined qualitatively by spot inoculating on media with specific substrate: example, Horikoshi II agar for amylase, skimmed milk agar for protease, carboxymethylcellulose (CMC) agar for cellulase and tributyrin/tween 80 agar for lipase, all at pH 10.5. (Appendix A). The plates were incubated for 48 hours at room temperature and checked for enzyme activity. Starch and cellulose hydrolysis was determined by flooding the plates with 1% iodine solution and 0.03% (w/v) congo red solution respectively. (Appendix A). Proteolytic and lipolytic activities were directly observed as colonies sorrounded by a white halo on specific medium.

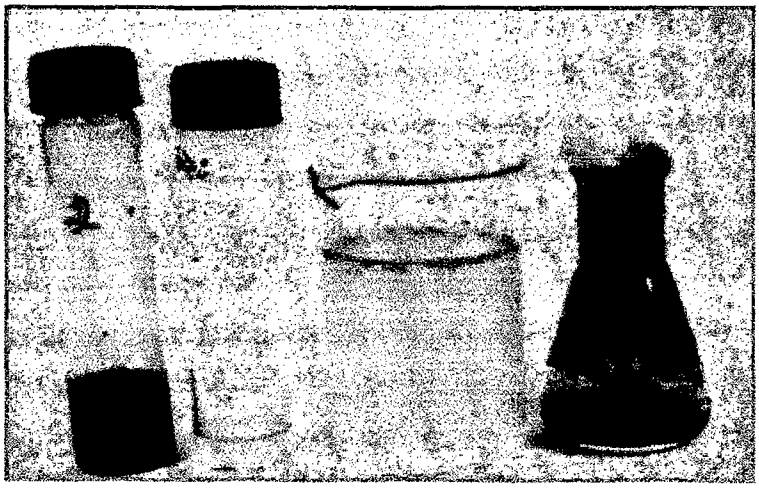
3.7 Plate assay for primary screening of lipase activity (212, 314):

In addition to the screening of lipase producers on agar plates using tributyrin / Tween 80 as the substrate, lipase production was confirmed by streaking the lipase producing strains on agar plates containing Tween 80 and Rhodamine B at pH10.5 (Appendix A). Lipase production was monitored by exposing the plates to UV light at 254 nm after incubation for 24 hours.

Results & Discussions:

Samples were collected from diverse alkaline and non-alkaline econiches, both natural and manmade and plated on media with pH 10.5. The medium used for isolation of alkaliphiles has a pH of 10.5 and above (106,157), which in the present study has been maintained using sterile sodium carbonate. It has been reported that the pH of the medium can be increased using higher concentrations of sodium carbonate (120,204). However, our studies indicate that the pH of the medium does not rise above pH 10.3 inspite of increasing the concentration of sodium carbonate. (Appendix A.8). It has also been reported that sodium ions are essential for maintenance of cytoplasmic pH 1-2 units below the external pH. Hence the sodium salts are favored as the compounds for pH change for growth of alkaliphiles (157).

Amongst the 22 samples collected, water samples of the Agro chemical factory showed the highest alkaline conditions (Plate 3.1 & Table 3.1) This Agro chemical factory, situated at Sancoale, Goa is involved in the production of fertilizers such as



1 2 3 4

Plate 3.1. Samples from Zuari Industries Ltd.-Goa
(Description as per Table no. 3.1)

Table 3.1 Description of Alkaline samples from treatment tank of an Agrochemical factory, Goa.

Sample No.	pH	Temperature	Description
1	9.50	60 °C	Black Sediment
2	8.50	40 °C	Oily Brown liquid from the side of tank
3	10.00	60 °C	Turbid spent water from tank
4	9.00	50 °C	Scrapings from the side of the tank

Table 3.2 Effect of diluent on *viable count of samples from Agrochemical factory.

Sample No.	c.f.u./ml or g	
	Diluent-Na_2C0_3-HCO_3 Buffer (pH 10.0)	Diluent -saline (pH 7.0)
1	1.04×10^9	1.9×10^2
2	3.22×10^9	1.4×10^2
3	5.3×10^9	2.2×10^2
4	1.5×10^{11}	4.0×10^3

*** Counts taken on PPYG agar (pH 10.5)**

UREA and NPK. Four composite samples were collected and the counts determined using different diluents. Interestingly, the samples diluted in carbonate-bicarbonate buffer (pH 10) showed considerably higher counts ranging from 10^9-10^{11} cfu/ml or g as compared to those which were diluted in physiological saline at pH 7 where the counts were found to be 10^2 cfu/ml or g (Table 3.2). The further analysis of all the samples was therefore carried out using the diluent carbonate bicarbonate buffer (pH 10.0).

All the samples were found to yield the presence of alkaliphilic bacteria and gave higher counts on PPYG agar as compared to Horikoshi I and Horikoshi II media (Tables 3.3 A, B & C). However, the natural alkaline samples such as droppings of birds, piggery waste, coconut rhizosphere, manmade alkaline samples like paper pulp, raw and treated sewage and non alkaline samples e.g. garden soil, droppings of goat, termite mound soil did not show significant differences within the counts on these three media. All the samples, with pH ranging from 8-10, showed highest counts of alkaliphiles in the order of 10^9-10^{11} cfu/ml or g. Interestingly, samples with acidic pH (3.19) also showed the existence of alkaliphiles with higher counts of 2.20×10^4 cfu/ml or g on PPYG, 1.0×10^2 cfu/ml on Horikoshi I and 4.0×10^2 cfu/ml or g on Horikoshi II media. Although the samples rich in agricultural and plant material yielded higher counts on Horikoshi II medium, however, mangrove water samples did not show significant counts on this medium, giving least counts of 2.5×10^2 cfu/ml as compared to all the other samples.

Table 3.3 A * Total viable cell counts of Alkaliphiles from natural alkaline environments.

Samples Analysed	pH	c f.u./ml or g		
		PPYG	Horikoshi I	Horikoshi II
Salt Pans	8.00	2.57×10^6	1.55×10^4	1.2×10^4
Mangrove Sediments	8.00	4.50×10^3	3.25×10^2	2.1×10^2
Mangrove water	7.80	1.00×10^2	2.50×10^1	1.0×10^1
Compost	8.70	2.50×10^{10}	5.20×10^8	3.0×10^{11}
Cowdung	8.50	4.56×10^{11}	2.4×10^8	2.8×10^8
Droppings of Birds	8.70	1.55×10^8	1.8×10^8	2.0×10^8
Piggery waste	8.00	4.2×10^5	1.8×10^5	1.3×10^5
Coconut rhizosphere	7.80	4.2×10^5	1.5×10^6	4.0×10^4
Alkaline soil	8.50	2.50×10^4	1.2×10^2	-

*** Serial dilutions were prepared in Carbonate - Bicarbonate buffer (pH10).**

Table 3.3 B * Total viable cell counts of Alkaliphiles from man made alkaline environments.

Samples Analysed	pH	c.f.u./ml or g		
		PPYG	Horikoshi I	Horikoshi II
Agro Chemical Factory	10.00	5.30×10^9	1.50×10^5	1.3×10^4
Mining effluent	8.00	1.60×10^8	1.8×10^5	2.0×10^4
Dairy effluent	8.50	6.20×10^6	8.0×10^4	5.6×10^3
Biogas digester sample	8.67	3.60×10^8	2.4×10^4	4.0×10^5
Paper pulp	8.50	2.0×10^{10}	1.6×10^8	$4..8 \times 10^7$
Polyester dyeing sample	8.01	2.0×10^8	1.6×10^3	1.2×10^3
Raw sewage	8.00	2.8×10^7	5.3×10^7	1.74×10^8
Treated Sewage	7.50	3.7×10^3	4.5×10^3	8.0×10^4

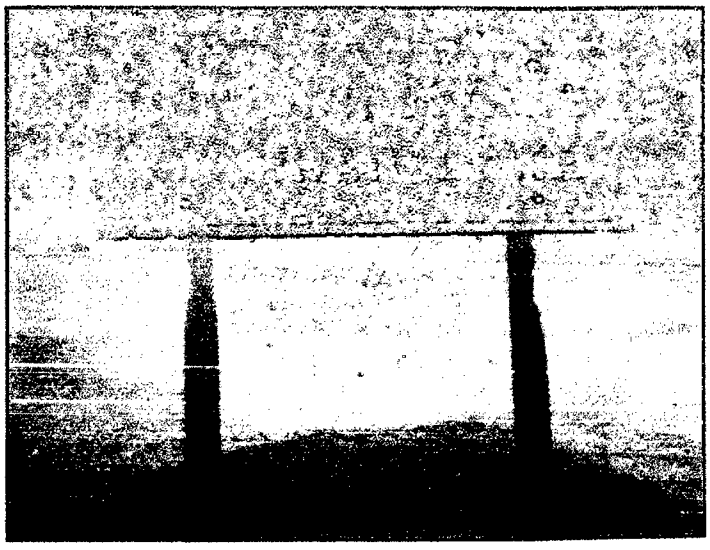
Table 3.3 C * Total viable cell counts of Alkaliphiles from non alkaline environments.

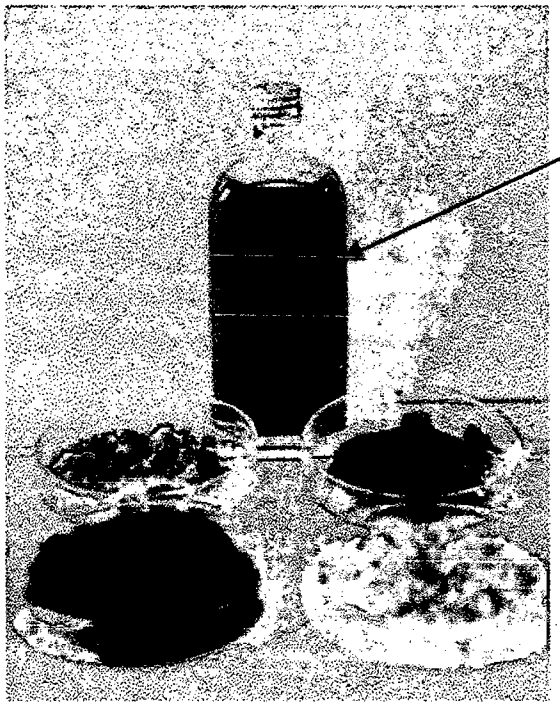
Samples Analysed	pH	cfu/ml or g		
		PPYG	Horikoshi I	Horikoshi II
Marine Water	7.354	1.20×10^7	4.0×10^6	2.0×10^7
Acidic Soil				
a) White	5.80	3.3×10^3	2.0×10^1	-
b) Green	6.00	2.06×10^5	1.2×10^3	1.5×10^1
c) Red	5.90	2.9×10^3	1.50×10^1	-
d) Yellow	5.80	2.0×10^3	1.70×10^1	-
Garden soil	7.00	7.00×10^8	6.20×10^8	6.50×10^8
Droppings of goat	7.50	7.50×10^8	5.00×10^8	5.50×10^8
Gluten Water	3.19	2.20×10^4	1.0×10^2	4.00×10^2
Termite mound soil	7.00	2.0×10^4	1.1×10^4	1.56×10^5

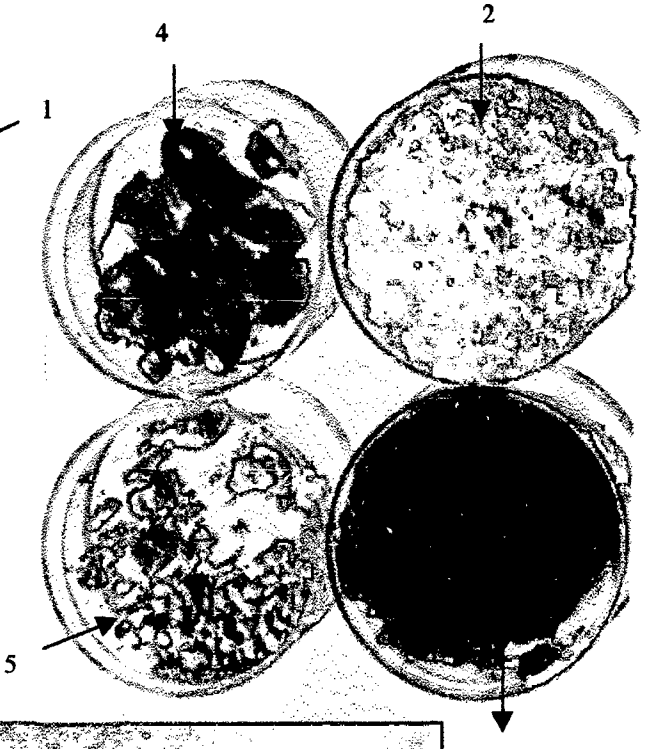
Alkaliphiles have been isolated from various sources including acidic soils (Table 2.1.A). This reflects on the interesting property of alkaliphiles, of changing the pH of the environment to suit their growth, a phenomenon very interesting from the ecological point of view. Alkaliphilic bacteria when inoculated at pH 12 and incubated, grow slowly by changing the pH of the medium, finally attaining a pH value of about 10. In acidic conditions, the bacteria can increase the pH value to about 9 at which point they show almost the same rate of growth as that at pH 10.5 (157,184).

True alkaliphiles live in soda lakes and soda deserts such as those found in Egypt, the Rift Valley of Africa and the Western U.S. and Alkaline salterns. (Table 2.1.A). Microbes in these saline and alkaline environments are hence adapted to both high alkalinity and high salinity.

A typical Salt lake of Rajasthan named as "Deodani Kyars Sambhar Salt Lake" was selected as an ideal ecosystem for isolation of typical alkaliphilic and haloalkaliphilic bacteria. The water and salt samples collected from different sites (Plate 3.2 & Table 3.4) were red in colour with a pH in the range of 8-10 while the sediment samples were found to be black in colour. These samples showed no variations in count when plated on three different media with a count ranging from 10^3-10^5 cfu/ml of alkaliphiles. Further, the samples were also plated on PPYG with 25% NaCl for enumerating the haloalkaliphiles which showed a count of 10^2-10^3 cfu/ml or g (Table 3.5). These colonies were found to be red in colour, which may be attributing the colour to the Sambhar Lake. A similar bright red coloration due to blooms of haloalkaliphilic archaea has also been reported in Soda and Salt Lakes of Lake Magadi,







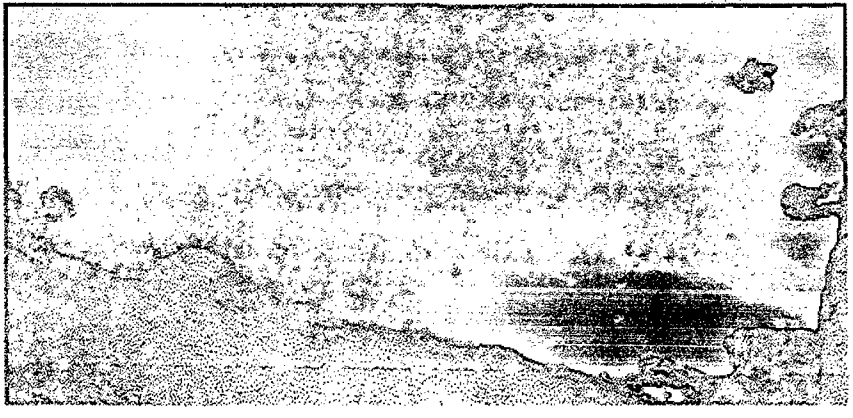


Plate 3.2. Samples from DEODANI KYARS Sambhar lake, Rajasthan.

Table 3.4 Description of Samples from Deodani Kyars Sambhar Salt Lake, Rajasthan.

Sample No.	pH	Temp.	Description
1	10.00	50 °C	Red coloured water
2	9.00	40 °C	Red Salt
3	9.00	45 °C	Red Salt + black sediment
4	9.00	45 °C	Black sediment
5	8.00	40 °C	Sand

Table 3.5 * Total viable cell counts of Alkaliphiles and Haloalkaliphiles from Sambhar Salt Lake samples.

Sample No.	cfu/ml or g			
	Alkaliphiles			Haloalkaliphiles
	PPYG	Horikoshi I	Horikoshi II	PPYG + 25 %NaCl
1	4.0×10^3	5.5×10^3	2.0×10^3	1.0×10^3
2	8.6×10^3	9.0×10^3	7.0×10^3	1.5×10^3
3	7.8×10^4	6.5×10^4	4.0×10^4	6.0×10^2
4	9.3×10^4	6.8×10^4	1.1×10^5	1.0×10^2
5	3.0×10^4	4.2×10^4	3.1×10^4	-

Lake Wadi Natrun, Yellowstone National Park and the Dead Sea (121,281,296,307, 364). These red pigmented microbes reach number of 10^2-10^5 cfu/ml (221). The isolates from these environments consist of rods and cocci belonging to *Natronobacterium* and *Natronococcus* genera and have carotenoid pigments, the bacterioruberins (121,122,281).

All the samples, when plated on different media showed precipitation of salt (plate 3.3). This phenomenon was found to be a physical change due to loss of moisture, which was also observed in the control plates when incubated at low temperature.

Predominant isolates from all the samples were purified and characterised. From the Salt Lake samples, 11 alkaliphiles and 1 haloalkaliphile were selected, while the remaining samples yielded 126 predominant alkaliphilic bacterial strains. The isolates had varied cultural and morphological features (Appendix C.3, Plates 3.4.1-3.4.12) varying from sporulating type to rods, cocci, and pleomorphic forms. A large number of organisms were found to be filamentous with varying degrees of branching. All organisms were found to be Gram positive except isolate number 23 (Appendix C.3). The isolates further varied in their tolerance to high pH and temperature (Table 3.6 & 3.7). Equal distribution of obligate alkaliphiles (61) and facultative alkaliphiles (65) was observed. It was interesting to note that 39.3% of obligate alkaliphiles and 29.23% of facultative alkaliphiles were also tolerant to high temperature (55°C). A wide variety of organisms were pigmented with the colour ranging from buff-yellow-orange-pink-red-green as has also been reported (11,106,339,382). Interestingly, all the cultures

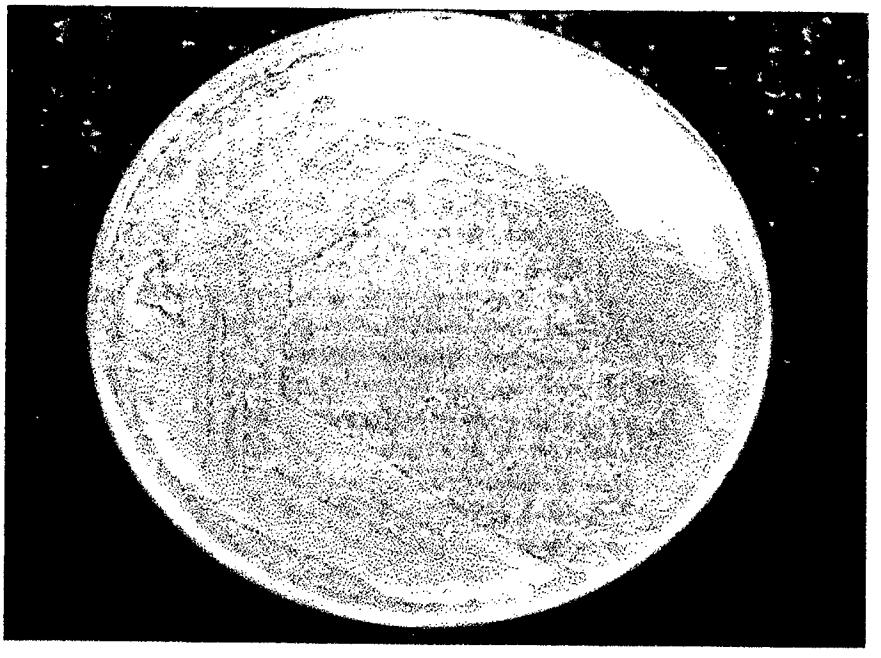


Plate 3.3. Characteristic salt precipitation at pH (10.5)

Plate 3.4.1.

Plate 3.4.2.

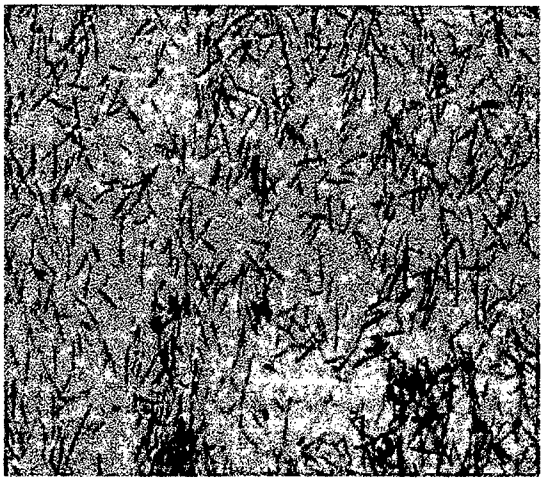


Plate 3.4.3.

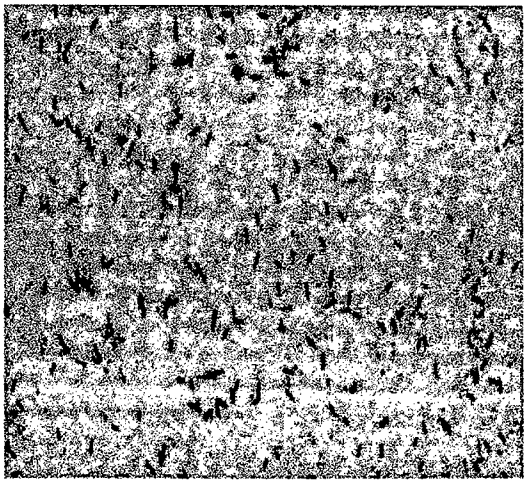


Plate 3.4.4.

Morphological characteristics of Alkaliphilic isolates

Plate 3.4.5

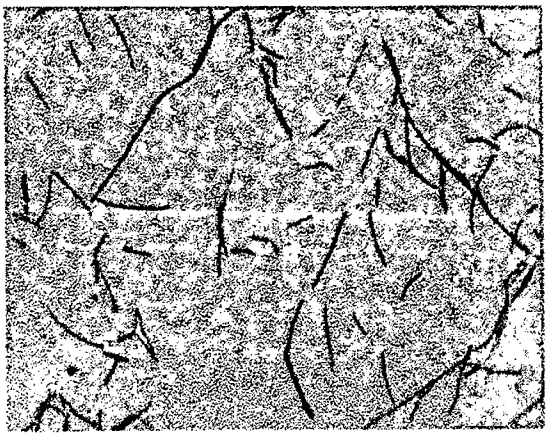


Plate 3.4.6

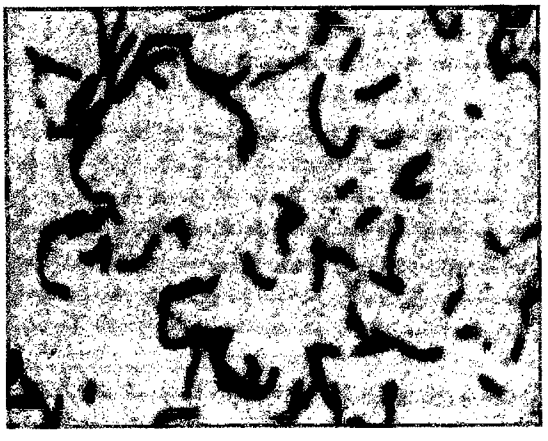


Plate 3.4.7

Plate 3.4.8

Morphological characteristics of Alkaliphilic isolates

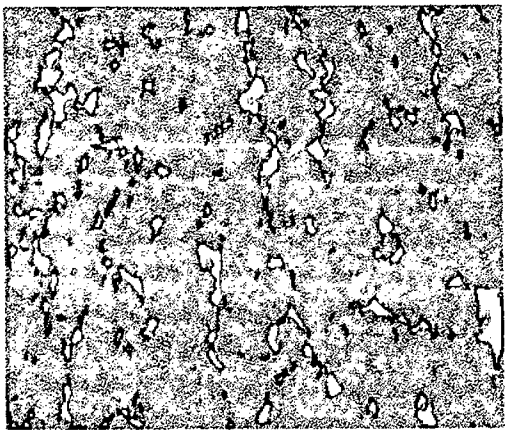


Plate 3.4.9

Plate 3.4.10

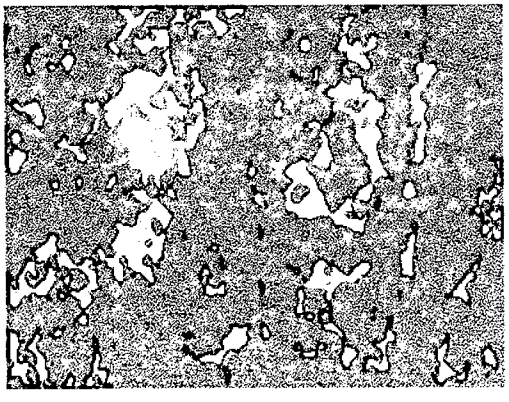


Plate 3.4.11

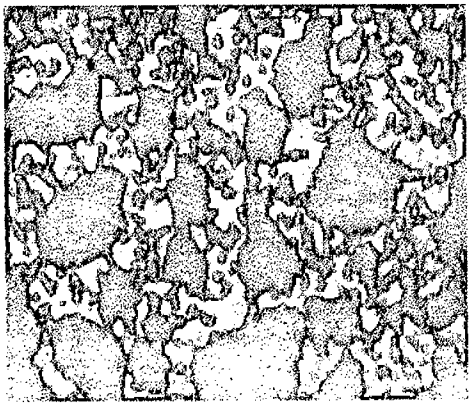


Plate 3.4.12

Morphological characteristics of Alkaliphilic isolates

Table 3.6 - A Comprehensive presentation of the results on prevalence of Alkaliphiles in diverse econiches.

Category	Strain Numbers
Obligate Alkaliphiles (OA)	3,4,5,6,7,9,10,12,13,18,19,21,22,24,25,27,28,29,36,37,40,41,42,43,44,45,46,47,48,49, 50,51,54,56,60,69,73,76,78,79,83,86,87,93,94,102,104,105,106,107,108,109,110,111, 112,120,121,122,123,125,126,**127,129,130,131,133,134,136,137.**
Facultative Alkaliphiles (FA)	1,2,8,11,14,15,16,17,20,23,26,30,31,32,33,34,35,38,39,52,53,55,57,58,59,61,62,63, 64,65,66,67,68,70,71,72,74,75,77,80,81,82,84,85,88,89,90,91,92,95,96,97,98,99, 100,101,103,113,114, 115,116,117,118,119,124,**128,132,135.**
Thermophilic (OA)	5,6,7,12,18,19,22,24,27,28,37,40,41,42,44,46,49,60,83,87,93,102,108,121, **127,131,136,*138.**
Thermophilic (FA)	15,20,52,57,59,61,67,68,70,72,75,82,88,95,99,114,115,116,117,**128,132,135.**
Mesophilic (OA)	3,4,9,10,13,21,25,29,36,43,45,47,48,50,51,54,56,69,73,76,78,79,86,94,104,105, 106,107,109,110,111,112,120,122,123,125,126,**129,130,133,134,137.**
Mesophilic (FA)	1,2,8,11,14,16,17,23,26,30,31,32,33,34,35,38,39,53,55,58,62,63,64,65,66,71,74,77,80, 81,84,85,89,90,91,92,96,97,98,100,101,103,113,118,119,124.
Endospore formers(OA)	22,25,29,36,45,46,48,50,54,56,60,69,78,83,87,94,102,104,108,109,110,112,125, **129,133,134,137.**
Endospore formers (FA)	11,15,17,32,34,57,58,59,64,65,67,68,72,74,77, 80,84,85,88,89,92,96,97,99,113,115.
Pigmented (OA)	3,4,6,7,9,10,12,13,21,24,25,26,27,28,29,36,37,40,43,44,45,48,49,50,56,60,76,78,79,83, 94,104,107,108,110,111,112,125,**130,131,133,134,136.**
Pigmented (FA)	2,14,15,17,20,31,32,33,38,53,55,61,62,63,64,65,67,72,74,81,84,92,97,98,99,100,101,
	103,124,**135.**
Rods (OA)	19,22,25,29,45,46,48,50,54,69,83,87,93,102,109,110,112,**133,137.**
Rods (FA)	11,15,17,20,32,34,58,59,65,67,77,80,84,85,89,96,97,99,115.
Pallisade Rods (OA)	4,9,12,13,26,36,44,51,56,78,86,104,108,125,**130,134.**
Pallisade Rods (FA)	16,57,63,64,74,75,90,91,**135.**
Long Rods (OA)	18,28,94,**131.**
Long Rods (FA)	68,82,88,116.
Thick Rods (OA)	60,**129.**
Thick Rods (FA)	72,92,113.
Cocci (OA)	3,21,37,43,73,79,105,106,111,120,122,123,126.
Cocci (FA)	1,2,14,30,31,33,35,38,39,53,55,61,62,66,71,81,98,103,118,119.
Filamentous Forms (OA)	6,7,24,40,41,42,49,69,121,**127,136.**
Filamentous Forms (FA)	27,52,70,95,117,**128,132.**
Pleomorphic Forms (OA)	5,47,76,107.
Pleomorphic Forms (FA)	101,114.
L-Forms (OA)	10.
L-Forms (FA)	8,100,124.
Coco bacilli (FA)	23.

NOTE: Numbers in bold are isolates obtained from Sambhar salt lake.

(OA)- Obligate alkaliphile, (FA)- Facultative alkaliphile, * - Obligate Haloalkaliphile.

were found to show catalase and oxidase activity (Table 3.7). Alkaliphilic property is maintained in the cultures by various molecular and bio chemical addaption mechanisms such as increased content of cytochromes resulting in the oxidase activity (16,69,112,199,233).

In the present study, the diverse variations of morphological forms indicate the presence of alkaliphilic nature amongst wide range of organisms. Extreme alkaliphilic eubacteria are known to be members of Gram positive group belonging to genera such as *Bacillus* (12,34,40,80,107,112,233), *Micrococcus* (4,195), *Staphylococcus* (132), *Arthrobacter* (157, 327), *Corynebacterium* (23,157,204), *Streptomyces* (102,285,401), *Actinomyces* (367-369), *Clostridium* (63) and *Exiguobacterium* (62). However, few reports are also available on gram negative alkaliphiles belonging to the genera like *Pseudomonas* (157,175, 382) and *Flavobacterium.* (157,338)

The distribution of obligate and facultative alkaliphilic organisms in the varied samples tested is indicative of their versatile metabolic activities in relation to the source of the samples. The isolates were therefore scored for the enzyme activity related to degradation of natural organic matter. The studies on enzyme production showed the presence of extracellular enzymes by obligate and facultative alkaliphiles at high pH (Table 3.8). Presence of cellulase was found in 79.35% of the total isolates with amylase being shown by only 58.72% and protease by 47.61%. It was interesting to note that amylase and cellulase were equally distributed amongst the obligate alkaliphiles while amylase and protease were shown by facultative alkaliphiles. However, lipase activity was shown by only 29.35% of the total isolates (Fig. 3.1).

Table 3.7 Distribution and characterisation of Alkaliphiles.

Distribution	Obligate Alkaliphiles		Facultative Alkaliphiles		Total	
	Total No.	%	Total No.	%	Total No.	%
	61	48.41	65	51.58	126	
Thermophilic	24	39.34	19	29.23	43	34.13
Mesophilic	37	60.65	46	70.76	83	65.87
Category						
Gram Positive	61	100	64	98.46	125	99.20
Pigmented	38	62.29	29	44.61	67	53.17
Sporulating rods	23	37.7	26	40	49	38.88
Rods inChains	17	27.86	19	29.23	36	28.57
Pallisade rods	14	22.95	8	12.3	28	22.22
Long Rods	3	4.918	4	6.15	7	5.55
Thick Rods	1	1.639	3	4.61	4	3.17
Cocci	13	21.31	20	30.76	33	26.19
Filamentous forms	8	13.11	5	7.69	13	10.31
Pleomorphic forms	4	6.55	2	3.07	6	4.76
L - forms	1	1.639	3	4.61	4	3.17
Cocobacilli	0	0	1	1.538	1	0.79
Catalase Positive	61	100	65	100	126	100
Oxidase Positive	61	100	65	100	126	100

The rhodamine plate assay method for lipolytic cultures showed an orange fluorescence on irradiatiang the plates with U.V. while non-lipolytic cultures accumulated rhodamine B and formed pink coloured colonies. It has been reported that the rhodamine plate method for lipase activity is insensitive to pH changes and allows re-isolation of organisms without any change of physiological properties (212,314).

In the present study, it was interesting to note, that many of the isolates showed the production of more than one enzyme being active at alkaline pH (Table 3.9) (Figs. 3.2 & 3.3). It is of particular interest, that amongst the isolates 16% showed the ability to produce all the varied enzymes, cellulase, protease, amylase and lipase.

Wide range of unique types of enzymes has been isolated from alkaliphiles, which have industrial applications (Table 2.1.B) such as Alkaline cellulase 103, isolated from the alkaliphilic *Bacillus* strains by Ito and co-workers, used as laundry detergent additive (164,165) and also reported by others (68,98,99,133,156,315,326). The production of alkaline amylase by *Bacillus* sps. has been reported by Horikoshi. (152), Boyer and Ingle (40,41) and others (139,141,142,161,185,186,190,192-194,205,206, 251,252,395,403). Alkaline serine proteases from alkaliphilic *Bacillus* sps. strain 221 with optimum pH of 11.5 with 75% of the activity maintained at pH 13 (151) and other proteases have also been reported. (38,55,111,207,274,344,355,365-367,401,404) Fujiwara *et al* purified thermostable alkaline proteases from thermophilic alkalophilic *Bacillus* sps. strain B-18 (96, 97) while Takami *et al* isolated protease from *Bacillus* sps strain A H-101 (354). Alkaline lipase production has been reported by Watanabe *et al* (382) by Gram negative *Pseudomonas nitroreducens* and *Pseudomonas*

Table 3.8 A Comprehensive Analysis of Enzyme Activity of Alkaliphiles .

Enzyme	Strain Numbers.		
	Obligate Alkaliphiles	**Facultative Alkaliphiles**	**Total**
Cellulase	4,5,6,7,9,10,12,13,18,19,24,25,26,29,36,40, 41,44,45,46,47,48,49,50,51,54,56,60,69,76, 87,93,94,102,104,105,106,107,108,109,110, 111,112,121,123,125,**127,129,130,131,134, 136,137.**	1,2,8,14,16,17,23,27,31,32,33,34,39,52, 53,57,58,59,61,62,63,64,65,66,67,68,70, 71,72,74,75,77,80,81,82,85,88,89,90, 95,96,97,98,99,100,101,103,114,115, 116,117,118,119,124,**128,132.**	109
Amylase	3,4,7,9,10,12,13,18,19,24,25,26,28,29,36,41, 42,44,45,50,51,54,56,60,69,76,78,79,83,86, 87,93,94,104,105,106,107,108,109,110,111, 112,120,121,122,123,**127,129,130,133,134, 136,137.**	8,11,14,15,16,17,20,23,27,52,57,58,59, 61,62,63,64,72,77,82,84,85,88,89,90,95, 96,113,**128,132.**	83
Protease	6,9,19,24,28,29,36,40,41,42,44,45,50,54,56 ,60,69,78,79,86,93,94,102,104,105,107,108 109,110,111,112,121,**127,129,134,136.**	1,2,16,23,27,52,57,58,59,61,62,63,64,65, 66,67,72,77,80,81,84,90,95,96,101,103, 113,124,**128,132,135.**	68
Lipase	5,6,7,9,22,29,42,49,50,56,60,93,94,106,109, 110,111,112,**129.**	17,20,27,57,58,59,61,63,64,65,66,67,90, 95,96,97,98,99,100.	38

NOTE: Numbers in bold are isolates obtained from Sambhar salt lake.

Table 3.9 Multiple enzyme production by Alkaliphiles.

Enzyme	Strain Numbers		Total
	Obligate Alkaliphiles	Facultative Alkaliphiles	
Cellulase (C)	46,47,48,125,**131.**	31,32,33,34,39,53,68,70,71,74,	21
		75,114,115,116,117,118,119.	
Amylase (A)	3,83,120,122,**133.**	11,15.	6
Protease (P)	-	**135.**	0
Lipase (L)	22.	-	1
C + A	4,10,12,13,18,25,26,51,76,87,	8,14,82,85,88,89.	17
	123,**130,137.**		
C+P	40,102.	1,2,80,81,101,103,124.	9
C+L	5,49.	97,98,99,100.	6
A + P	28,78,79,86.	84,113.	6
A + L	-	20.	1
P + L	-	-	0
C + A+P	19,24,36,41,44,45,54,69,104,	16,23,52,62,72,77,**128,132.**	19
	105,107,108,121,**127,134,136.**		
C+A+L	7,106.	17.	3
C+P+L	6.	65,66,67.	4
A+P+L	42.	-	1
C+A+P+L	9,27,29,50,56,60,93,94,109,	57,58,59,61,63,64,90,95,96.	21
	110,111,112,**129.**		
C,A,P,L,Not detected	21,37,43,73,126.	30,35,38,55,91,92.	11

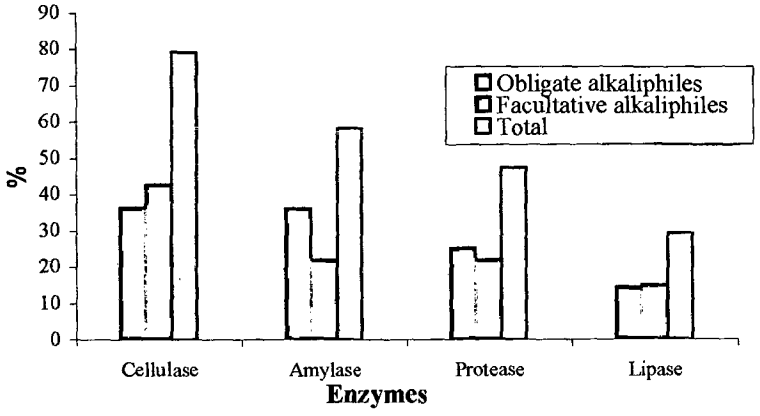


Fig.3.1 % Distribution of enzyme activity in alkaliphiles.

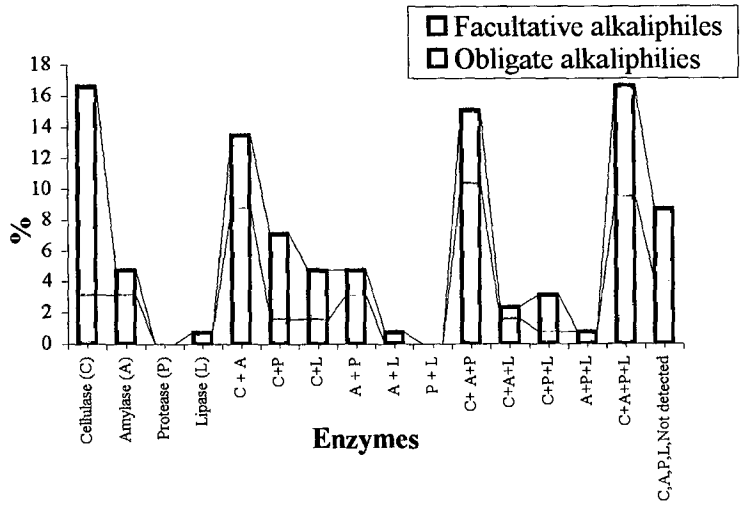


Fig 3.2 % Distribution of multiple enzyme production by Alkaliphiles.

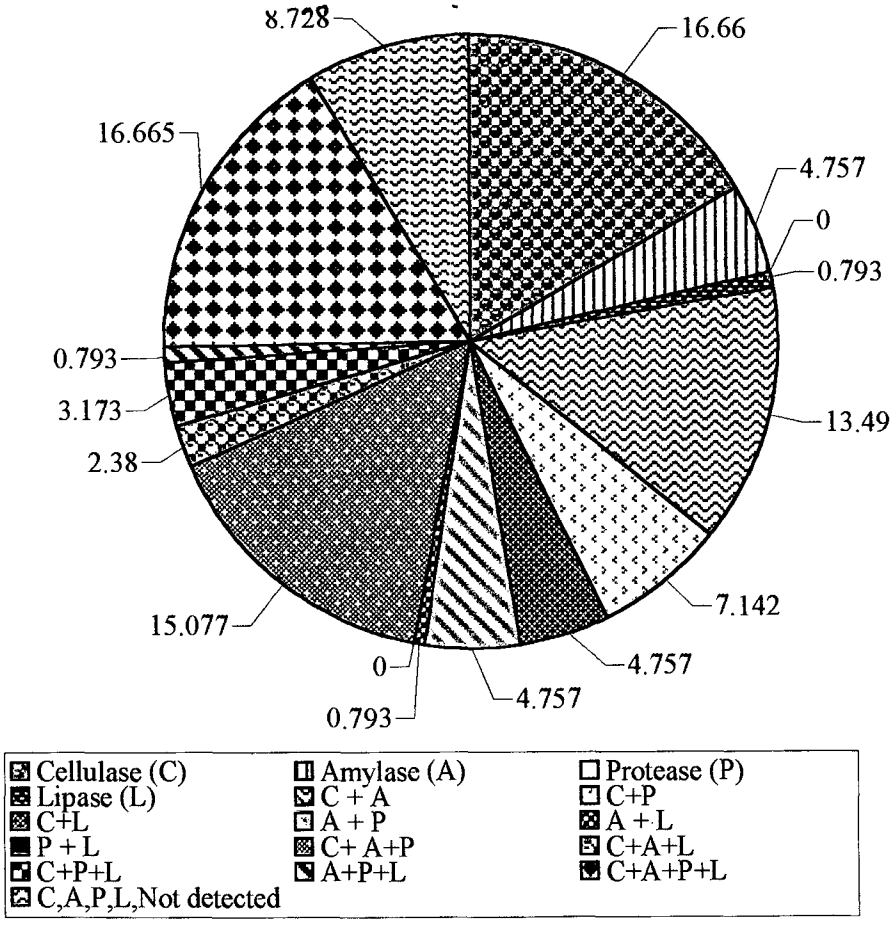


Fig 3.3 Total multiple enzyme production by alkaliphiles.

fragi, and others (33,232,381). Multiple enzyme production has been reported by Nihalani *et al* (271).

The present investigation has thus highlighted:

1) The existence of alkaliphiles in varied environments both alkaline and non-alkaline.

2) The production of enzymes active at high pH with relation to the substrate available in the samples.

To quote, "It has been stated that once researchers have extremophiles in their petri dishes, the next step is to see just what these organisms can do for applications in industries (286). Hence for further studies potential cultures were selected.

The following chapter describes the characterisation of two selected isolates:

1) **Strain no. 56** - an obligate mesophilic alkaliphile designated as **SB-D** and

2) **Strain no. 57**- a facultative thermophilic alkaliphile, designated as **SB-W** in the study, both isolated from Agro chemical factory, showing amylase production.

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CHAPTER IV

Characterisation and studies on Buffering Capacities of the isolates SB-D and SB-W.

Bacteria that grow at extreme pH values encounter a variety of biological and, specifically, bioenergetic challenges that derive from a central pH homeostasis. The pH homeostatic mechanism in alkaliphiles is due to the cytoplasmic buffering capacity which can be determined using whole cells and cells treated with 10% Triton X-100. All alkaliphilic strains studied so far have shown the requirement of sodium ions for growth and are known to change the pH value of the medium to the optimum pH.

During the course of investigation, one hundred and thirty seven isolates growing at extreme pH 10.5 were isolated from diverse econiches, purified, characterised and their enzyme profiles were studied. Two isolates, strain number 56, a mesophilic obligate alkaliphile (SB-D) and strain number 57, a thermophilic, facultative alkaliphile (SB-W) were selected for further studies. The two isolates were found to be best amylase producers at alkaline pH. Besides, SB-D produced needle like crystals and a metabolite increasing the viscosity of the culture broth (Chapter VI).

This chapter discusses the:

(1) Growth patterns of the two isolates in different media, under varying environmental parameters.

(2) Buffering capacities, scanning electron micrographs and protein profiles of whole and triton treated cells.

(3) Biochemical, chemotaxonomic characteristics of the isolates SB-D and SB-W and their identification.

Materials and Methods:

4.1 Growth Profiles:

50 ml each of mineral medium, with and without 0.5% yeast extract, Horikoshi I and Horikoshi II media at pH 10.3 (Appendix A) were inoculated with 1% of 18 hour old culture and incubated under shaker conditions at 200 rpm at room temperature. The flasks were removed after every 4 hours of incubation and growth was monitored at 540 nm for mineral medium and 600 nm for Horikoshi I and II media. pH variation was monitored using Labindia pH analyser. SB-W isolate was also grown at 55°C and pH 7.4 in Horikoshi I medium to monitor the variations in growth. Effect of different concentrations of glucose and starch on growth was studied. Further, concentration of glucose in Horikoshi I medium and starch in Horikoshi II medium during growth were determined using glucose oxidase method and Iodine blue value respectively (Chapter V).

4.2 Determination of Intracellular pH (339):

Bacteria grown in Horikoshi I medium were harvested in the exponential phase of growth by centrifugation at 10,000 g for 15 minutes at 4°C, washed twice in 150 ml water adjusted to pH 10.5 with 10% Na_2CO_3 and finally resuspended in 10 ml water at pH 7. Cells were ruptured by sonication in B. Barun Biotech International-Labsonic- U Sonicator The pH of the lysate was recorded.

4.3 Effect of sodium ions:

The bacterial strains SB-D and SB-W were grown in Horikoshi I medium with or without 50 mM of sodium ions. pH of the medium was adjusted to 10.3 with 3 N KOH. Growth was monitored as absorbance at 600 nm after every 4 hours of incubation against uninoculated medium as blank.

4.4 Studies on buffering capacities (130, 217, 409):

4.4.1 Harvesting of Cells: Cells were harvested by centrifugation at 10,000 x g for 15 minutes in their logarithmic phase of growth. They were then washed twice with and resuspended in 200 mM KCl solution. (Appendix C.4)

4.4.2 Protein Estimation: 1 ml of cell suspension in 200 mM KCl solution containing 1 ml of 0.1 NaOH was pre digested at 80°C for 30 minutes, cooled, mixed well and centrifuged. 0.5 ml of the supernatant was diluted with equal volume of distilled water and used for protein estimation by Bicinchoninic acid method (Appendix D) using bovine serum albumin as the standard.

4.4.3 Titration: Appropriate volume of cell suspension corresponding to 5 mg of cell protein was taken for titration against 0.05 M KOH (Appendix C.4). Experiments were conducted in 50-ml glass beaker using 10 microlitre aliquots of 0.05 M KOH and the pH change was noted using a calibrated pH analyser-Labindia. The whole cell buffering capacity (Bo) was measured as nanomoles of hydroxyl ions consumed to change one pH unit per mg of protein (Appendix C.4).

4.4.4 Permeabilisation of cells: Volume of intact cell suspension in 200 mM KCl corresponding to 5 mg protein was treated with 10 ml of 10% Triton X-100, prepared in 200 mM KCl (Appendix C.4). The content was mixed gently, and allowed to stand for 5 minutes and centrifuged. The supernatant (extract) was stored in screw-cap tubes and used for chemical analysis of cell components. The pellet obtained was washed and resuspended in 200 mM KCl and titrated against 0.05 M KOH till the pH changed by one unit. Buffering capacity of these treated cells (Bt) was measured and the internal or cytoplasmic buffering capacity (Bi) was determined from the formula Bi = Bt - Bo (Appendix C.4)

4.4.5 Chemical analysis of extracts of Triton X-100: The permeabilisation extracts obtained after Triton X-100 treatment were analysed for 1) Proteins by B.C.A. method 2) Sugars by phenol sulphuric acid method and 3) Lipids by Thin Layer Chromatography in Chloroform: Methanol: water solvent system visualising with α-naphthol sulphuric acid reagent (Appendix E.1).

4.4.6 Effect of Incubation period and medium composition on Buffering capacity: The cells were harvested after 12, 16 and 20 hours of growth in Horikoshi I medium and the Bo, Bt and Bi determined as explained above. Buffering capacities of the cells grown in mineral medium with and without yeast extract, Horikoshi I and II media were also analysed.

4.5 Scanning Electron Micrographs of cultures (387):

The cultures SB-D and SB-W grown in Horikoshi I medium before and after triton treatment were smeared on the stub, prepared for scanning electron micrographs and observed under JEOL - 5800 LV Scanning Electron Microscope (Appendix E.4).

4.6 SDS-PAGE profiles (60, 320):

Whole cell protein profiles of SB-D and SB-W were determined using Sodium dodecyl sulphate-polyacrylamide gel electrophoresis. (Appendix E.3).

4.7 Cultural, Morphological, Biochemical and Chemotaxonomic characteristics of two alkaliphilic isolates:

4.7.1 Cultural and Morphological studies: Cultural characteristics of the isolates grown on Horikoshi I agar for 4 days were observed. Cultures were stained for Gram character, presence of spores and capsules using standard methods (Appendix B.1). Motility was observed in 18 hour cultures by hanging drop technique.

4.7.2: Biochemical Tests (116,333): The two isolates SB-D and SB-W were identified based on the tests described in Bergey's Manual of Systematic Bacteriology (333). All media used for identification were supplemented with 1% Na_2CO_3 to raise the pH to 9.5.Fermentation of carbohydrates was checked using *O*-cresol red as the indicator (106). Standard methods were used for Nitrate reduction, Indole production, Citrate utilisation, Catalase and Oxidase, M.R.V.P. Test, Gelatin, Starch and Casein hydrolysis, growth in Sodium Chloride, resistance to Lysozyme, egg yolk lecithinase reaction, oxidative/fermentative metabolism of glucose. (Appendix C.1).

4.7.3: Chemotaxonomic Studies: Chemotaxonomic analysis for cell wall amino acids and sugars; cellular quinones and lipids were undertaken as described in Chemical methods in bacterial systematics by Goodfellow and Minnikin. (118) (Appendix C.2).

Results & Discussions: Large number of organisms adapted to living in the extreme pH of 10.5 have been isolated and characterised. This character is found to be stable and is not lost on further transfers. Also, the alkaliphilic property is found to be more prevalent in Gram positive organisms as compared to gram negative.

The isolates, an obligate alkaliphile SB-D and a facultative alkaliphile SB-W selected for further studies showed luxuriant growth in Horikoshi medium with glucose and starch as the carbon source. However, it was noted that the cultures SB-D and SB-W could not grow in mineral medium unless subcultured several times and no significant increase was observed in mineral medium supplemented with yeast extract (Figs. 4.1 & 4.2). It has been reported that the alkaliphiles do not grow in synthetic mineral salts medium, perhaps due to precipitation of insoluble salts of magnesium and calcium. Most of the media devised to date for the isolation and growth of alkaliphiles are complex containing yeast extract and peptone as carbon and nitrogen sources (120).

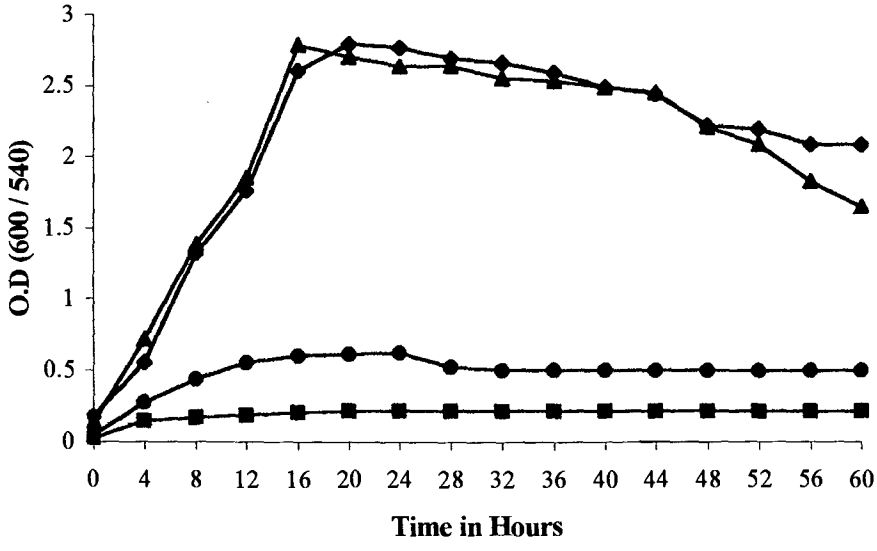


Fig. 4.1 Growth profile of SB-D in different media at pH 10.3 & 25 ° C.

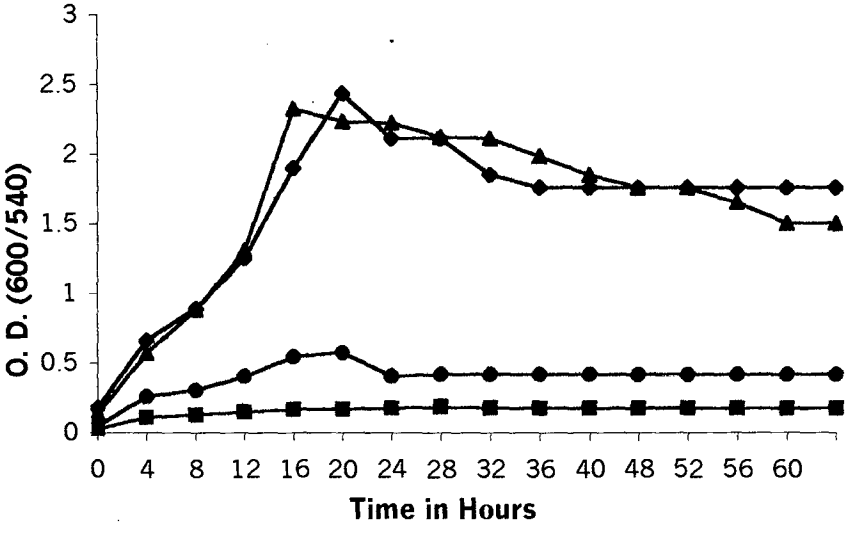
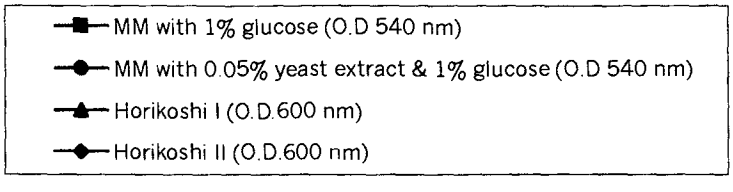


Fig 4.2 Growth profile of SB-W in different media at pH 10.3 & 25 ° C.



Growth profiles of the isolates monitored in Horikoshi medium showed no lag phase while the exponential phase continued upto 16 hours in Horikoshi I medium and 20 hours in Horikoshi II medium for both the cultures at 25°C (Figs. 4.1 & 4.2).

Since SB-W could also grow at 55°C, the growth monitored at this temperature showed no lag phase and the exponential phase was completed within 8 hours (Fig. 4.3). Further, temperature variations had significant effects on growth of SB-W, a facultative alkaliphile as compared to pH variations (Fig. 4.4).

As growth was maximum when glucose and starch were used as the carbon source, the effect of different concentrations of glucose and starch (0%-7% w/v) on growth was studied. It was noted (Fig. 4.5) that glucose and starch at a concentration of 1 % supported maximum growth of both the isolates at 25°C. Higher concentrations of glucose beyond 3% were inhibitory to the growth of the organisms while this inhibitory effect was observed with starch at a concentration of above 1%. Further, the inhibition was more pronounced with glucose than starch.

Uptake of glucose and starch at 25°C revealed a rapid utilisation of glucose by both the isolates depleting within 16 hours with the utilisation of 50% glucose within 4 hours by SB-W culture. However, a lag of 8 hours was observed in the uptake of starch by SB-D isolate being utilised in 24 hours while SB-W showed no lag, utilising starch in 20 hours (Fig. 4.6). Guffanti and Hicks (131) have also demonstrated that both facultative and obligate strains of *Bacillus* use their substrates as efficiently as aerobes at neutral pH values.

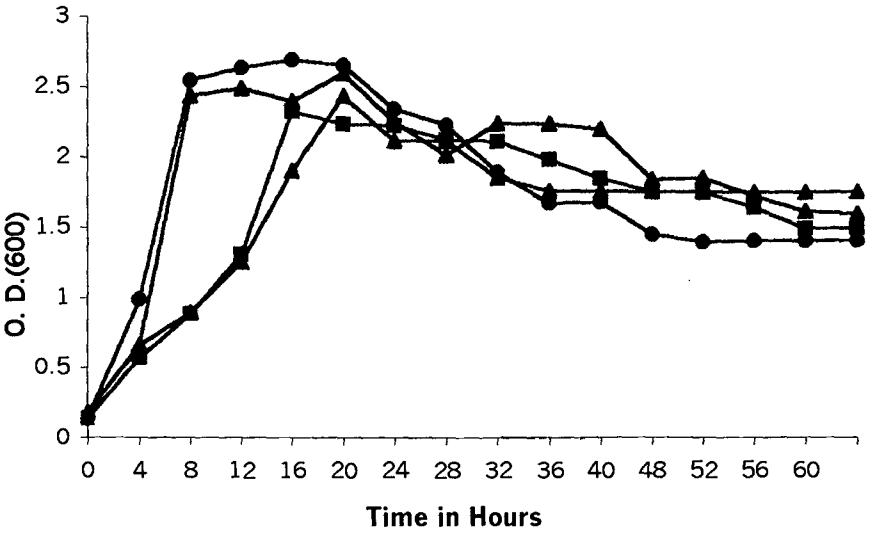


Fig 4.3 Growth profile of SB-W at 25°C & 55°C.

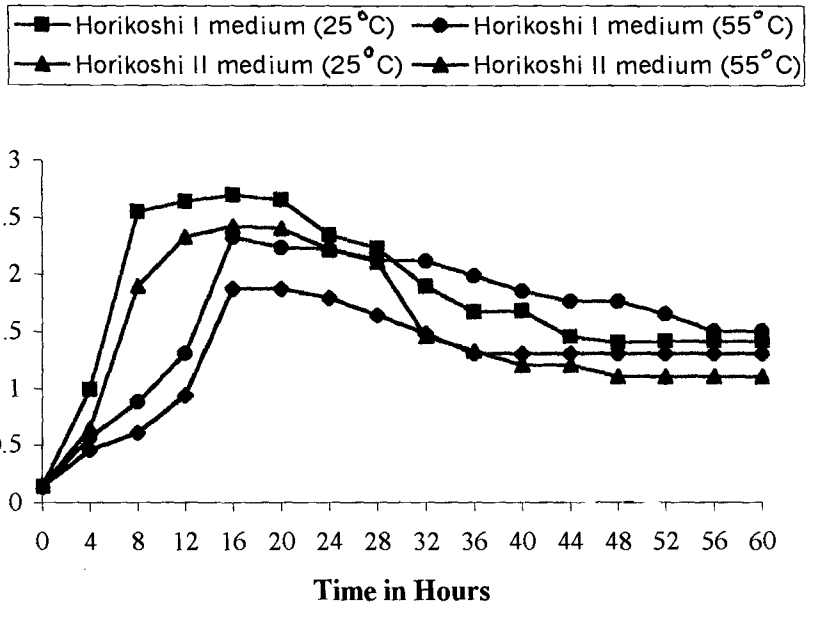


Fig 4.4 Growth profile of SB-W in Horikoshi I medium at pH 7.4 & 10.3 at 25°C & 55°C.

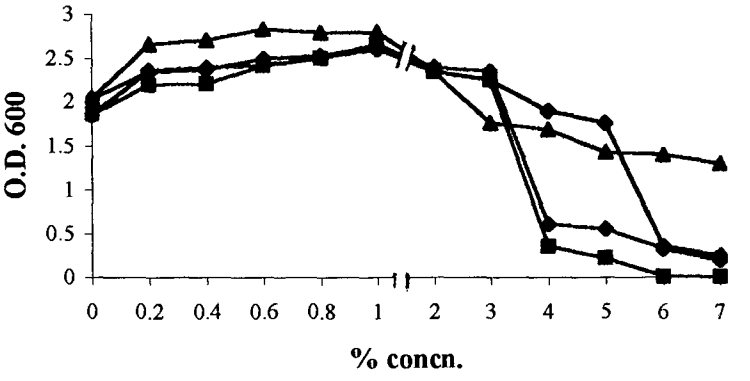
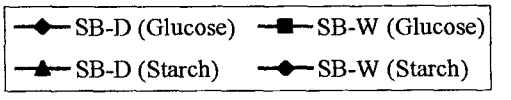


Fig.4.5 Effect of glucose & starch concn. on growth of SB-D & SB-W at 25°C.



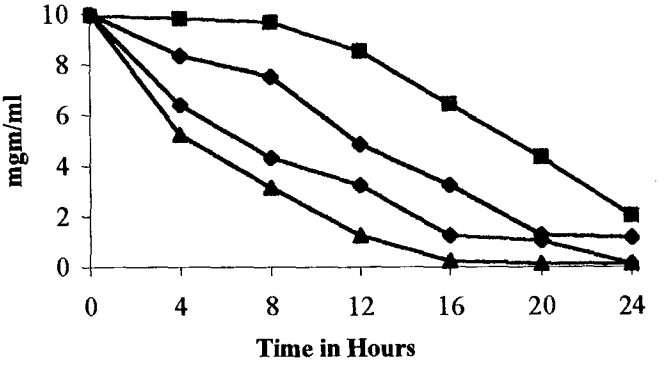
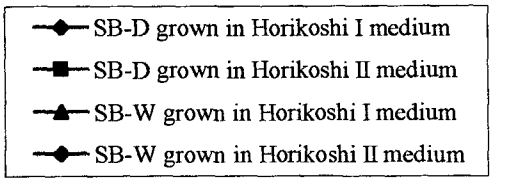


Fig 4.6 Concn. of glucose & starch in Horikoshi medium during growth of SB-D & SB-W at 25°C.



Note : Methods of estimation used :
Glucose - Glucose oxidase method.
Starch - Iodine blue value.

The effect of sodium ions on growth of both the cultures was further studied. As seen in Fig. 4.7 growth of SB-D was observed only in the presence of sodium ions at pH 10.3. Interestingly, the presence of sodium was also needed for the growth of the facultative alkaliphile at not only pH 10.3 but also at pH 7.4.(Fig. 4.8). Absence of sodium failed to support the growth of both the isolates at alkaline pH.

As seen with strains SB-D and SB-W, it has been reported that alkaliphilic *Bacillus* strains cannot grow in the absence of sodium ions in the range of pH 7-10 (157). Enhancement of growth of facultative alkaliphile at pH 7.5 and 10.5 by addition of sodium to the medium has also been observed by Guffanti *et al* (131). Requirement of sodium by obligate and facultative alkaliphiles in the growth medium is for maintaining a cytoplasmic pH that is much lower than that of the external milieu by catalysing the exchange of extra cellular protons for intracellular sodium (157,197,198, 201-203,214). pH measurement of broken cell lysate is a simple technique of determining intracellular pH (157), although more complex techniques such as distribution of weak bases across the cell membrane and use of pH sensitive fluorescent probes (17,43,48,58,63) can be used.

The variations in pH in Horikoshi medium during growth and the intracellular pH of the isolates were monitored. It was interesting to note that there was no significant difference in the extracellular pH during growth of both the isolates remaining alkaline (Fig. 4.9). However, the intracellular pH of SB-W was found to be only 7.7 as compared to the obligate alkaliphile SB-D which exhibited a pH of 8.02. (Table 4.1). Reports have indicated several active and passive protective mechanisms, which enable

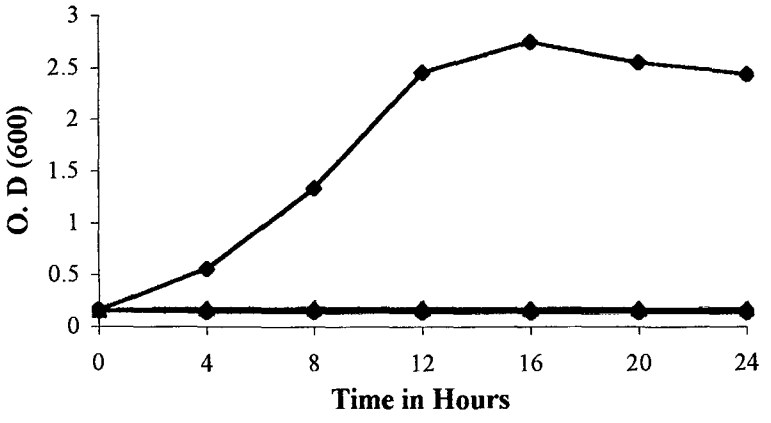


Fig. 4.7 Growth profile of SB-D in presence or absence of Na^+ in Horikoshi I medium.

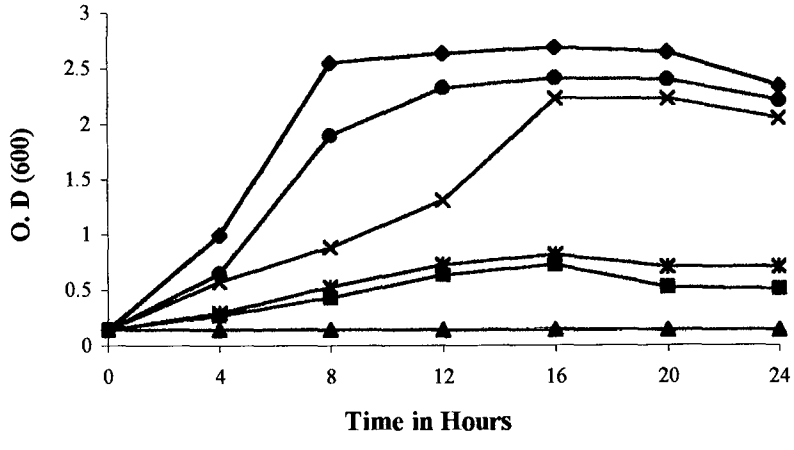
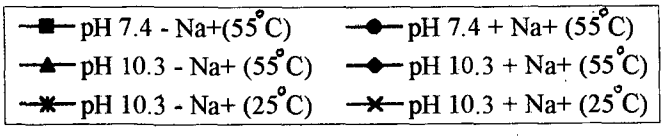


Fig.4.8 Growth profile of SB-W in presence or absence of Na+ in Horikoshi I medium.



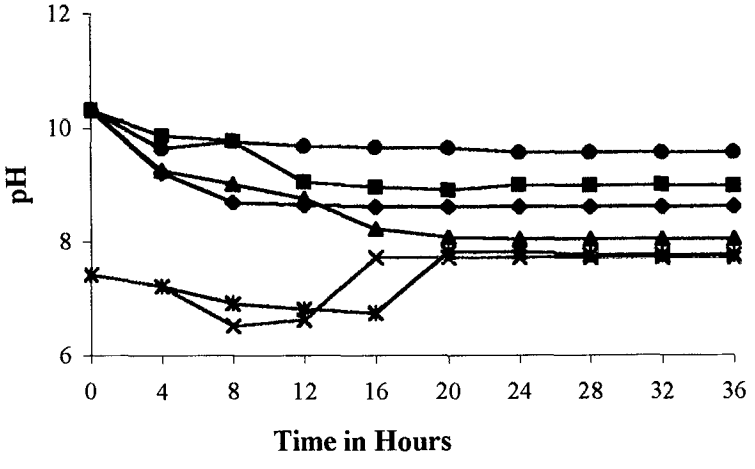


Fig 4.9 Change of external pH during growth of SB-D & SB-W in Horikoshi medium.

Table 4.1 Intracellular pH of SB-D & SB-W.

Organism (16 h in H I medium)	**pH of suspension medium**	**Intracellular pH.**
SB-D	7	8.02
SB-W	7	7.7

Table 4.2 Volume of KOH required for change of pH by 1 unit by alkaliphiles and neutrophiles.

	Volume of 0.05 M KOH reqd (µls).	
Organism	**Whole cells**	**Triton treated cells**
SB-D	920	350
SB-W	490	120
S. aureus	40	30
B. subtilis	85	40

Note: SB-D & SB-W- Alkaliphiles.

S. aureus & *B. subtilis* - Neutrophiles.

H I - Horikoshi I medium.

adaptations at high pH. That pH of the medium remains alkaline in short and long term cultivation of alkaliphiles and internal pH is maintained 1-2 pH units lower than the external pH, has also been reported (131,157,162,398). This ability of alkaliphiles to maintain optimum pH for their growth intracellularly / extracellularly is conferred on to the organisms by their buffering capacities.

In the present study, the buffering capacities of SB-D and SB-W and two neutrophilic isolates were determined.

It was interesting to note that the alkaliphiles, SB-D and SB-W required 920 and 490 µls of 0.05 M KOH respectively for change of 1 pH unit. Neutrophiles *S.aureus* and *B.subtilis* however, needed only 40 and 85 µls respectively for 1 pH unit change to occur. (Table 4.2& Figs.4.10& 4.11). These cultures however, were found to lose this property with the treatment of Triton X-100 with the requirement substantially coming down to 30-350 µls. (Table 4.2 & Figs.4.10& 4.11). Significantly, only culture SB-D showed the requirement of 350 µls of 0.05 M KOH for changing the pH by one unit even after triton treatment and exhibited the maximum cytoplasmic buffering capacity. (Table 4.3).

Comparison of buffering capacities of SB-D and SB-W in different media revealed a low Bo value (250) for obligate alkaliphile in alkaline mineral medium. It is interesting to note that the highest Bo value obtained is also for the same culture when grown in nutritionally rich Horikoshi I and II media (9200 & 9300 respectively). This indicates that the mediacomposition has a tremendous effect on overall buffering capacity of the culture (Table 4.4). However, the age of the culture did not influence a

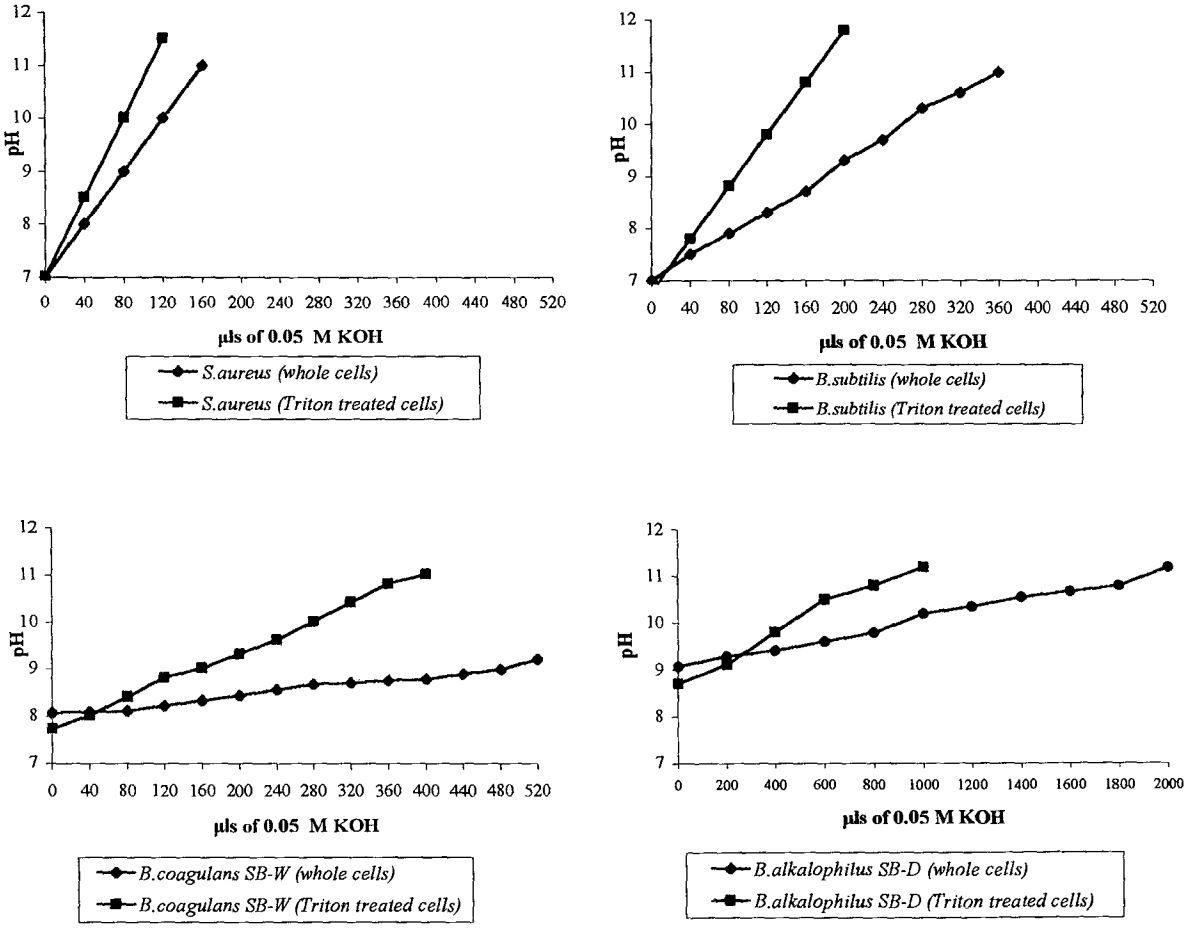


Fig 4.10 Volume of KOH required for change of one pH unit by Alkaliphiles & Neutrophiles.

Note: *S. aureus & B. subtilis* - Neturophiles.
B. coagulans SB-W - Facultative alkaliphile.
B. alkalophilus SB-D - Obligate alkaliphile.

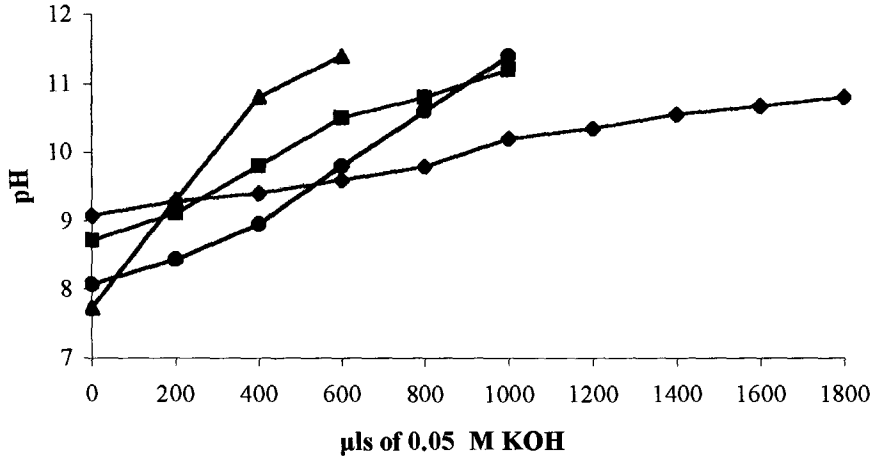


Fig 4.11 Volume of KOH required for change of one pH unit by facultative & obligate alkaliphile.

Note: *B. alkalophilus* SB-D - Obligate alkaliphile.
B. coagulans SB-W - Facultative alkaliphile.

Table 4.3 Buffering capacities of Alkaliphiles & Neutrophiles.

Culture	Growth medium & pH	Group	Buffering capacity		
			Whole cells (Bo)	Treated cells (Bt)	Cytoplasmic (Bi)
SB-D	Horikoshi I (pH 10.3)	OA	9200	3500	5700
SB-W	Horikoshi I (pH 10.3)	FA	4900	1200	3700
S. aureus	Horikoshi I (pH 7.4)	N	400	300	100
B. subtilis	Horikoshi I (pH 7.4)	N	850	400	450

Note: O.A - Obligate Alkaliphile

F.A - Facultative Alkaliphile

N - Neutrophile

Table 4.4 Effect of medium composition on buffering capacities of SB-D & SB-W.

		Buffering capacity		
Culture	**Medium**	**Whole cells (Bo)**	**Treated cells (Bt)**	**Cytoplasmic (Bi)**
SB-D	Mineral medium	250	200	50
	Mineral medium + Yeast extract	2200	800	1400
	Horikoshi I	9200	3500	5750
	Horikoshi II	9300	3500	5800
SB-W	Mineral medium	400	200	200
	Mineral medium + Yeast extract	1300	500	800
	Horikoshi I	4900	1200	3700
	Horikoshi II	4900	1200	3700

variation on the whole cell buffering capacity of the isolates (Table 4.5) indicating the adaptation of cells to the extreme pH of the culture medium during growth itself. High and stable values of cytoplasmic buffering capacities have also been reported for alkaliphiles (217) and it has been postulated that the elevation of cytoplasmic buffering capacity at highly alkaline pH has an important role in alkaliphily.

Chemical analysis of the triton treated extracts showed the presence of proteins by BCA method in both extracts, higher being in SB-W(Table 4.6). Further, the Triton treated extracts of SB-D isolate also revealed the presence of sugars and lipids (Table 4.7). Proteins on account of their ability to exist in zwitterionic state are known to be best buffering molecules and the stability against the alkaline environment has been assigned to the presence of cell envelope components such as proteins (397), cytochromes (220) and Na^+/H^+ antiporters (101) in *Bacillus* strains.

Scanning electron micrographs of the cells after triton treatment revealed a variation in the size of the cells, being reduced as compared to their normal size (Plates 4.1.1-4.1.4). Some filamentous exopolymeric substance surrounding the SB-D cells was found to be removed on triton treatment (Plate 4.1.2)

Triton X-100, a non ionic detergent is known to permeabilise the cell membrane resulting in the removal of the surface associated proteins without affecting the normal morphology of the cells (217,299,320,321). SDS-PAGE is a convenient and valuable tool for the separation of these proteins.

Table 4.5 Effect of incubation period on buffering capacities of SB-D & SB-W.

		Buffering capacity		
Culture	**Age**	**Whole cells (Bo)**	**Treated cells (Bt)**	**Cytoplasmic (Bi)**
SB-D	12 h	9100	3400	5700
	16 h	9200	3500	5750
	20 h	9200	3500	5750
SB-W	12 h	4900	1200	3700
	16 h	4900	1200	3700
	20 h	4900	1200	3700

Note : Growth medium : Horikoshi I.

Table 4.6 Protein content of SB-D & SB-W.

Culture	Concn. Of protein (µgm/ml)		
	Whole cells	**Triton treated cells**	**Permeabilised extract**
SB-D	1000	796	204
SB-W	1000	235	765

Table 4.7 Chemical Composition of permeabilisation extracts.

Culture	Triton X 100 extracts tested for:		
	Proteins	**Sugars**	**Lipids**
SB-D	+	+	+
SB-W	+	-	-

Note: Methods used for the determination of:

1. Proteins- BCA method
2. Sugars - Phenol - Sulphuric acid method
3. Lipids - TLC

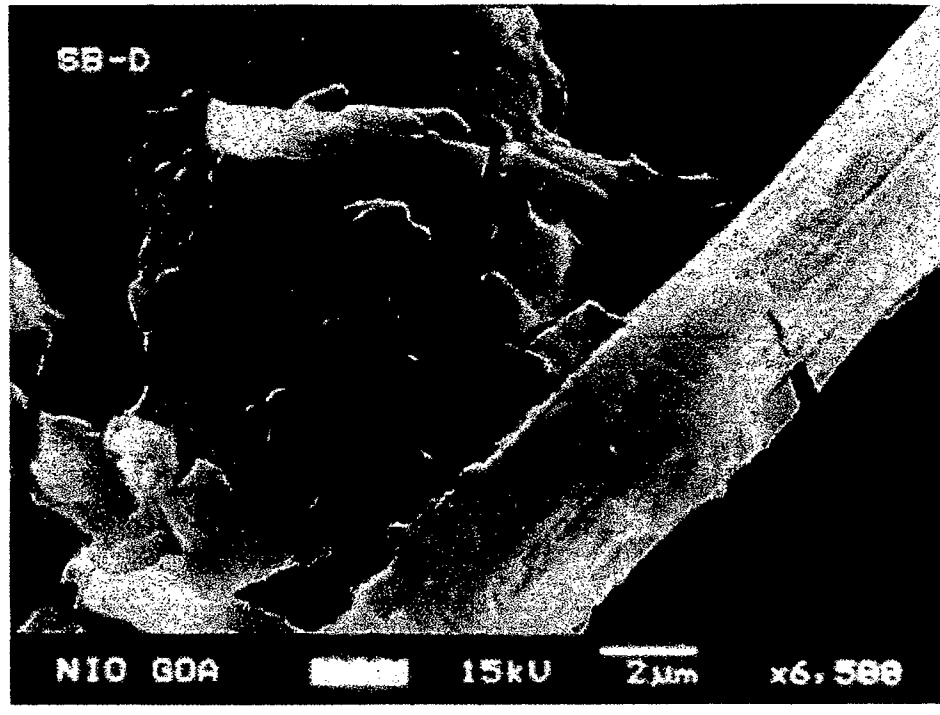


Plate 4.1.1. SEM of Triton untreated *B. alkalophilus* SB-D

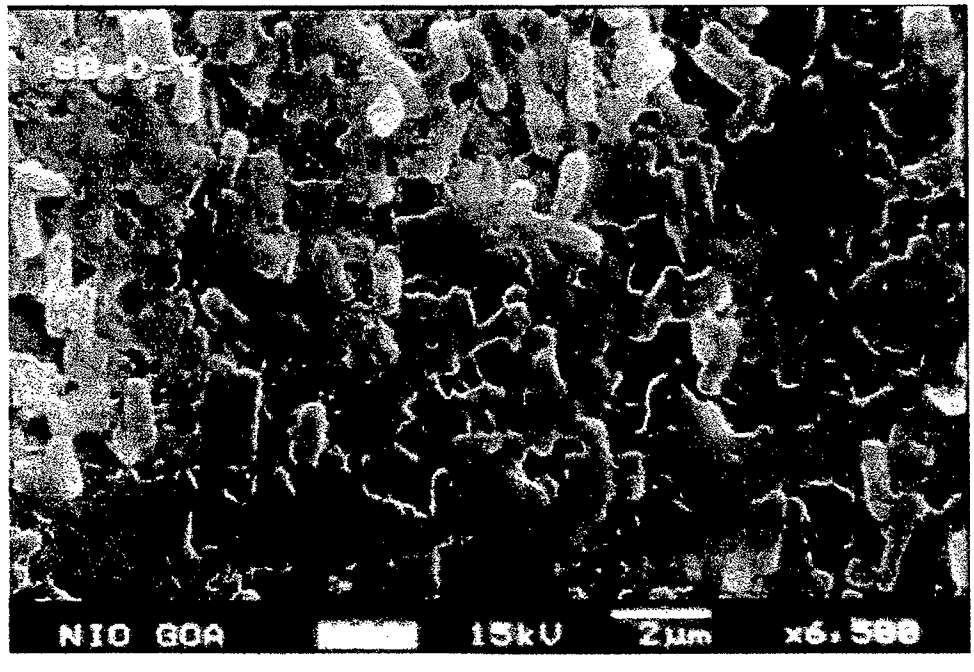


Plate 4.1.2. SEM of Triton treated *B. alkalophilus* SB-D

Space between the cells contain filamentous polymer.

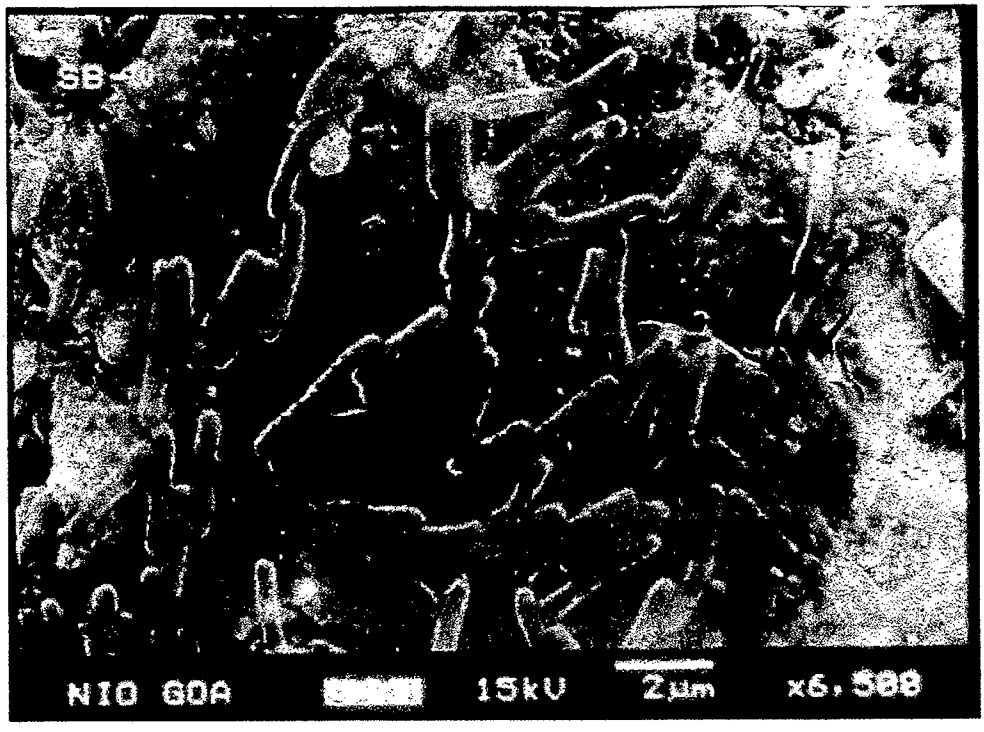


Plate 4.1.3. SEM of Triton untreated *B. coagulans* SB-W

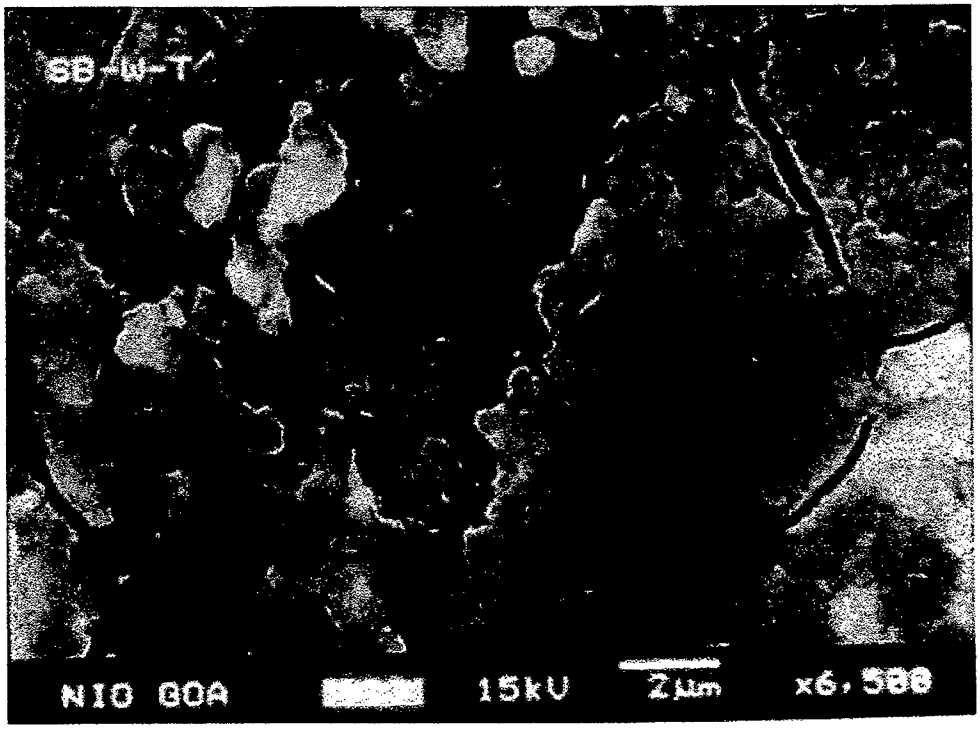


Plate 4.1.4 SEM of Triton treated *B. coagulans* SB-W

Reduction in cell size observed after triton treatment.

Electrophoretic patterns of Triton treated and untreated cells of SB-D and SB-W showed an appreciable loss of proteins from the cell membrane (Plate 4.2) increasing the protein content of permeabilised extracts as shown by chemical analysis (Table 4.7). Further, protein profiles of whole cells of SB-D culture showed no significant difference with reference to the complex medium (Plate 4.3). However, the profiles in mineral medium and mineral medium supplemented with yeast extract showed less number of bands (Plate 4.4). Protein profiles of SB-W grown at pH 7.4 and 10.3 significantly showed the absence of bands as marked on Plate (4.3). Koyama *et al* have also indicated a difference in patterns of membrane proteins in facultative alkaliphile grown at near neutral vs. alkaline pH. (213)

Electrophoresis has been broadly applied to prokaryotes for proteome characterisation under diverse conditions and SDS-PAGE protein patterns have been exploited for measuring systematic relationship between microorganisms (333). In addition, a battery of biochemical tests and chemotaxonomic studies also help in the placement of the isolate to a specific category and play an important role in classification, nomenclature and identification.

The two isolates SB-D and SB-W were found to be spore forming, (Plate 4.5) catalase positive, motile and aerobic rods. The presence of spores was confirmed by testing the survival of cultures to heating temperature of 70-80°C for 10 minutes, followed by cultivation on Horikoshi I medium. Both the strains showed acid production from glucose, arabinose, xylose and mannitol, hydrolysed starch and casein and were oxidase positive test (Table 4.8).

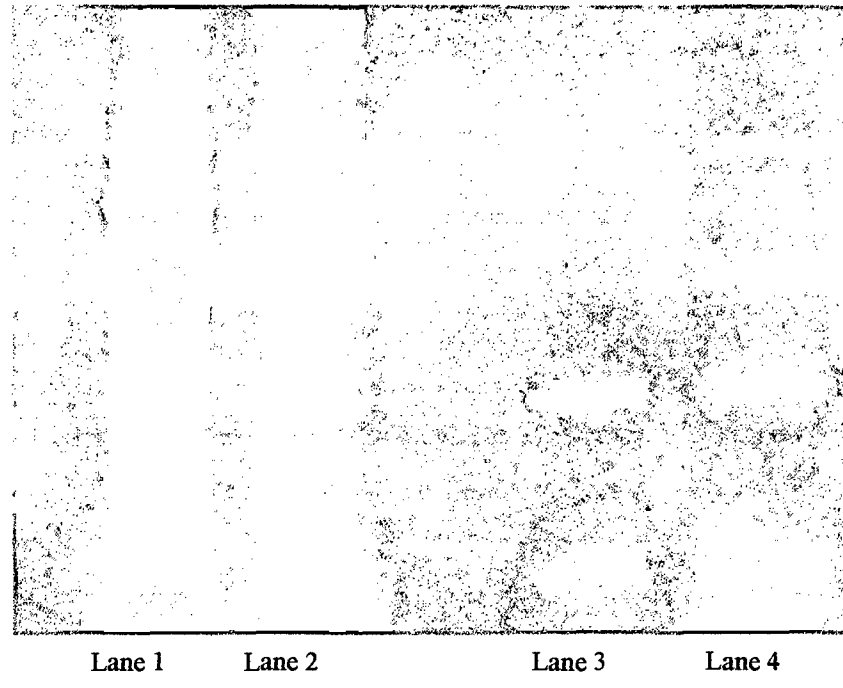


Plate 4.2. SDS-PAGE profile of whole cell proteins extracted from *B. coagulans* SB-W and *B.alkalophilus* SB-D before and after triton treatment

Lane 1: Triton untreated *B. alkalophilus* SB-D
Lane 2: Triton untreated *B. coagulans* SB-W
Lane 3: Triton treated *B. coagulans* SB-W
Lane 4: Triton treated *B. alkalophilus* SB-D

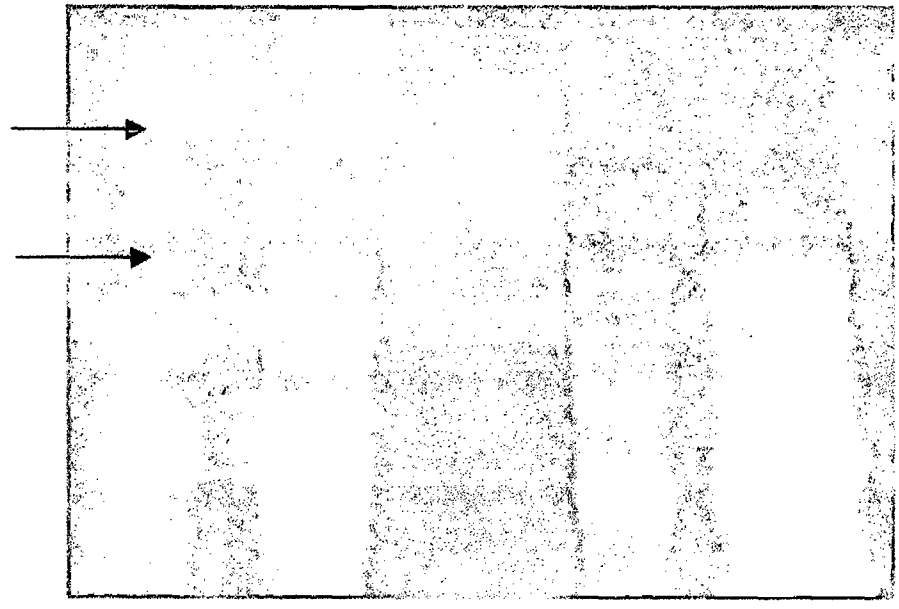
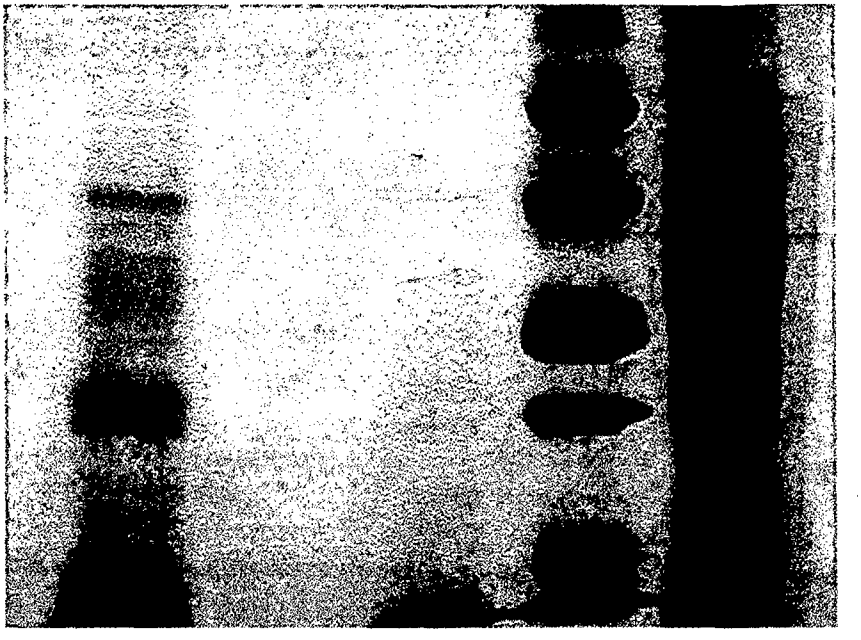


Plate 4.3. SDS-PAGE profile of whole cell proteins extracted from *B. coagulans* SB-W and *B.alkalophilus* SB-D.

Lane 1: *B. coagulans* SB-W at pH 7.4 (Horikoshi I medium)
Lane 2: *B. coagulans* SB-W at pH 10.3 (Horikoshi I medium)
Lane 3: *B.alkalophilus* SB-D at pH 10.3 (Horikoshi I medium)
Lane 4: *B.alkalophilus* SB-D at pH 10.3 (Horikoshi II medium)



Lane 1 Lane 2 Lane 3 Lane 4

Plate 4.4. SDS-PAGE profile of whole cell proteins extracted from *B. alkalophilus* SB-D grown on different media

Lane 1: cells grown in MM + 0.05% yeast extract
Lane 2: cells grown in MM
Lane 3: Marker
Lane 4: Cells grown in Horikoshi I medium.

Further, identification of Gram positive organisms is based on chemotaxonomic tools differing in different genera, (118), for eg. cell wall components, menaquinones and phospholipids. Studies of the cell surface components of the two isolates revealed the presence of diaminopimelic acid, arabinose, galactose, MK7 as isoprenoid quinones and phosphatidyl ethanolamine and diphosphatidyl glycerol as phospholipids in the cell wall. Based on Bergey's Manual of Systematic Bacteriology (116,333), the organisims were identified as *Bacillus.* Members of this genus have been reported to possess the property of alkaliphily and have been widely used as biochemical tools to understand the mechanisms for survival under high pH conditions (10,117,126,157,219).

The isolate SB-D showed capsules with needle like crystals (Plate 4.6), similar to those as observed by Bretzel *et al* during riboflavin fermentation by *B. subtilis* (44). SB-D was found to be a mesophilic, obligate aerobic alkaliphile, showing growth only at high pH. It could grow in presence of NaCl ranging from 2% to 7%. Based on these characters the organism was identified as *Bacillus alkalophilus.* The growth on different concentrations of yeast extract of this isolate was accompanied with pigment formation ranging from buff-yellow-orange. Pigment was found to be water-soluble with absorption maxima at 429 and 451 nm and shoulders at 409 and 480 nm. This spectrum suggests the presence of carotenoid components similar to that reported by Souza *et al* (338) and Kimura *et al* (191). The SB-D cells as observed in electron micrographs revealed pallisade arrangement morphology, the cells interconnected by fibrous amorphous material. (Plates 4.1.1 & 4.1.2).

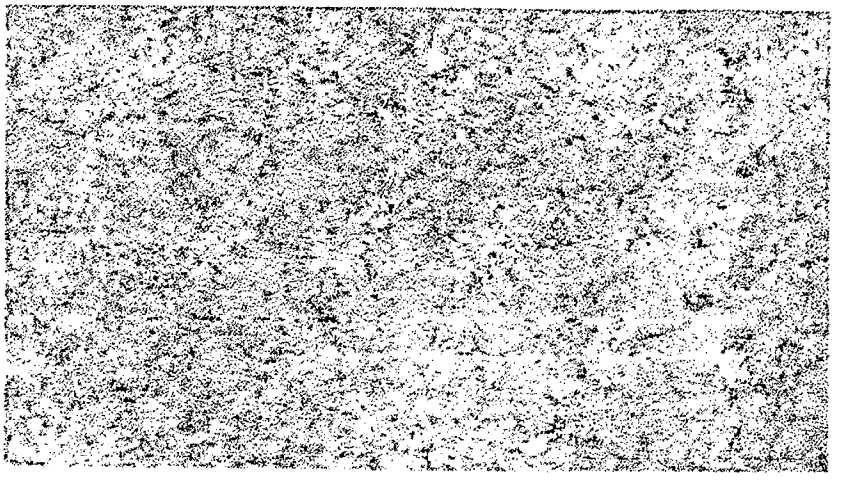


Plate 4.5. Endospores of *B. alkalophilus* SB-D

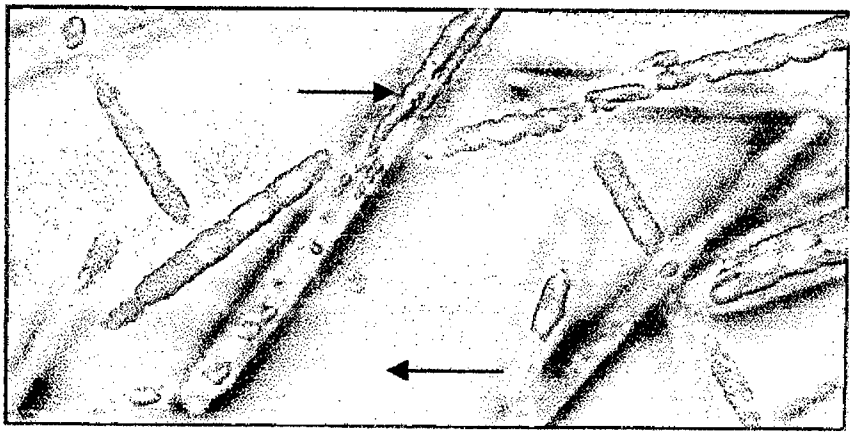


Plate 4.6. Needle like crystals (→) and capsules (←) of *B. alkalophilus* SB-D.

The presence of *Bacillus alkalophilus* has been noted in ecosystems like indigo dye balls, soda lakes etc. (41,127,233,353,377). In addition, the other *Bacillus* sps, which can also grow at pH 10.5, have also been isolated from varied ecosystems for example *B. pasteuri* (110), *B. circulans* (57), several strains of *B. firmus* RAB (69, 128, 144, 198, 219), *B.licheniformis* (55, 111, 403), *Bacillus thermoalkalophilus* (301), *B. stearothermophilus* (139), *B. subtilis* (232) and other *Bacillus* sps. (98,133,145,262), with reports also on new genus *Exiguobacterium aurantiacum* (62). Interestingly, the second organism SB-W, a facultative alkaliphile was a facultative anaerobe showing positive V P reaction, but no hydrolysis of gelatin (Table 4.8). On the basis of these properties strain SB-W resembles *Bacillus coagulans,* first isolated by Allen in 1953 (333). The characteristic feature of this isolate was its ability to grow at high temperature between 55°C-65°C in alkaline conditions. The culture is therefore designated as *B. coagulans* nov. var. *thermophilus.* The cells of this isolate appeared to be adhering at the ends occuring in pairs as seen in electeron micrographs (Plate 4.2.2).

Bacillus coagulans growing at alkaline pH and high temperature has been reported by Medda and Chandra (252). The other reported facultative alkaliphilic bacteria include *B.firmus* OF4, OF6, OF3, OF1 (112,129), *B. lentus,* C-125 (14,15,117,140, 227), *B. pseudofirmus* OF4 (113), *B. Polymyxa* (256) and other *Bacillus* sps. (34,213, 227,299) with reports also on alkalitolerant organisms (37,80,86).

Table 4.8 Cultural, Biochemical and Chemotaxonomic characteristics of SB-D and SB-W.

Strain no.	Gram stain	Cultural characteristics	Growth temp.			Motility	Endospore formation	Resistance to NaCl	Frementation				NO_3 reduction	Indole production	MR reaction	VP reaction	Citrate use	Catalase activity	Oxidase activity	Gelatin liquefaction	Starch hydrolysis	Casein hydrolysis	Egg yolk lecithinase	Growth with lysozyme	Chemotaxonomic analysis	Identification
			Maximum	Optimum	Minimum				Glucose	Arabinose	Xylose	Mannitol														
SB-D	Rod +	Buff yellow, Opaque, Slimy, Convex, with smooth margin.	40°C	28°C	16°C	Motile	Endospore former, swollen sporangium central spore.	grows in 2%,5% & 7% but not in 0% &10% NaCl	A	A	A	A	-	-	-	-	-	+	+	+	+	+	-	-	**Murein type** *meso*-DAP **Main menaq.** MK 7 **Phospholipids** PE & PG **C.W.sugars** Ara,Gal.	***Bacillus alkalophilus***
SB-W	Rod +	White, Opaque, glossy, Convex, with smooth margin.	60°C	55°C	30°C	Motile	Endospore former, central spore.	grows in 2%,5%, 7% & 10% but not in 0%NaCl	A	A	A	A	-	-	-	+	-	+	+	-	+	+	-	-	**Murein type** *meso*-DAP **Main menaq.** MK 7 **Phospholipids** PE & PG **C.W.sugars** Ara,Gal.	***Bacillus coagulans*** var. ***thermophilus***

Note: (A):Acid, (+): Positive test, (-): Negative test.

Meso-DAP *Meso*-Diaminopimelic acid.

MK 7 Menaquinone with 7 isoproene units.

PE Phosphatidylethanolamine.

PG Phosphatidylglycerol.

Ara,Gal. Arabinose, Galatose.

The significant points of this study are:

1) Rich growth of the isolates was obtained in complex media with pH 10.3. However, the synthetic mineral salt medium resulted in poor growth.

2) The requirement of sodium was found to be necessary for the growth of both the cultures at extreme pH. For the facultative alkaliphile, the requirement of sodium was also seen at neutral and high pH at growth temperature being 25°C / 55°C.

3) The internal pH of both the isolates was found to be 2 units less as compared to external pH that showed no significant difference after growth of the organisms.

4) The buffering capacity of obligate alkaliphile SB-D was not affected much on triton treatment. However, SB-W showed a significant drop in the buffering capacity.

5) The scanning electron micrographs have confirmed a change in the cell size of *Bacillus coagulans* as comapred to *Bacillus alkalophilus* on Triton treatment. The electron micrographs of SB-D have shown the presence of a filamentous polymer which was also found to be released when the cells were treated with Triton X-100. The chemical analyses of the Triton extracts have shown the presence of sugars and lipids besides proteins in SB-D. The isolation and characterisation of this polymer is discussed in Chapter VI.

6) Protein profiles of the whole cells and triton treated cells showed wide variations in the pattern indicating the role of proteins in the maintenance of buffering capacities.

7) The organisms SB-D and SB-W belong to the *Bacillus* genus with the obligate alkaliphile being identified as *Bacillus alkalophilus* and facultative alkaliphile identified as *Bacillus coagulans* nov.var. *thermophilus.*

The following chapter is concentrated on the "Amylase production" by SB-D and SB-W at pH 10.3. Isolation, purification and characterisation were undertaken and are detailed out in Chapter V.

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CHAPTER V

Production and Characterisation of alkaline amylase(s) from B.alkalophilus SB-D and B.coagulans SB-W.

Extracellular enzymes of bacteria of the genus *Bacillus* have several applications in paper, textile, food, starch, adhesive and sugar industries (94). Among the various hydrolytic enzymes, α-amylase is the most studied and commercially exploited enzyme.

The demand for, α-amylase for use in industry as detergents has also been growing for several years. Such amylases not only need to have an alkaline pH optimum but also must withstand oxidative degradation caused by bleach components in the detergent formulation and be stable at moderately high temperature. Alkaliphilic microorganisms are found to have enormous potential to produce such thermostable alkaline amylases, which can be exploited for industrial uses. Further, high value is placed on extreme alkaliphilic thermostable enzymes as these enzymes reduce bacterial contamination, increase substrate solubility and enhance enzymatic reactions. So, there is a considerable commercial demand to develop thermostable enzymes as biocatalysts in modern biotechnology.

Although, amylase is produced by different organisms, their production and activity is optimum under different physico-chemical and nutritional factors. Carbon, nitrogen, phases of growth, extent of aeration and agitation, temperature and pH have been found to influence the production as well as activity. During the studies on enzyme production by alkaliphiles, 83 amylolytic bacterial strains were isolated from different samples on Horikoshi II agar (Chapter III). Amongst these, bacterial strains, SB-D 56 and SB-D 57 efficiently hydrolysed starch and were identified as *B.alkalophilus* and *B. coagulans* respectively (Chapter IV). Production and

characterisation of alkaline amylase(s) from *B.alkalophilus* SB-D and *B.coagulans* SB-W is discussed here with.

Materials and Methods:

5.1 Spot inoculation test:

A loopful of the culture from 18 h-24 h old slant was spot inoculated on Horikoshi II medium at alkaline and neutral pH with 1% soluble starch (Appendix A). The plates were incubated at R.T. and 55°C for 48-72 h, flooded with 1% iodine in 2% potassium iodide solution and the zones of hydrolysis of starch were measured.

5.2 Ability of SB-D and SB-W to utilise a natural starch rich substrate:

The cultures were streaked on SB medium prepared from the gruel of boiled rice (Appendix A) and incubated over night. Changes in colony characters of the organisms and amylase production were observed as described.

5.3 Starter culture:

Inoculum for each organism was prepared by transferring a loopful of culture from Horikoshi II agar plate/slant to a 100 ml Erlenmeyer flask containing 20 ml of liquid Horikoshi II medium. The flasks were incubated at 25°C in a controlled environment incubator shaker for 24 h at 200 rpm.

5.4 Relation of time to growth and starch utilisation:

Sets of 50 ml of sterile Horikoshi II medium (pH 10.3) in 250 ml Erlenmeyer flasks were inoculated with 1% starter culture and agitated at 200 rpm at 25°C for SB-D and 25°C and 55°C for SB-W. The flasks were removed after every four hours of incubation, and the growth was measured in terms of absorbance at 600 nm and residual starch determined by the iodine blue value.

5.5 Enzyme assay:

a. Harvesting: The culture broth/liquor was centrifuged at 10,000 x g for 20 minutes at 4°C using Remi RC 24 centrifuge. The clear cell free supernatant was treated as the crude enzyme source.

b. Amylase activity:

i) Qualitative analysis by gel diffusion method: The supernatants obtained at different time intervals were tested for their ability to hydrolyse starch. Wells (6 mm) were bored on starch agar plates (pH 10.3) with the help of sterilised cork borer. To each well, 50 µls of the crude enzyme sample was added. The plates were incubated at room temperature for 12 h. and hydrolysis of starch observed as described.

ii) Quantitative amylase assay:

a) Preparation of reaction mixture: Amylase activity was measured in a 2 ml reaction mixture that contained 1.2 ml of 1% starch solution (w/v) prepared in carbonate bicarbonate buffer (0.2 M pH 10) (Appendix B.2), 0.6 ml 0.2 M carbonate-

bicarbonate buffer pH10 and 0.2 ml of the crude enzyme. The reaction mixture was incubated for 30 minutes.

b) Residual starch by iodine blue value: 0.1 ml of the reaction mixture was treated with 2.4 ml of 0.3 % iodine in 3% KI solution further diluted to 4%. Readings were taken at 620 nm against iodine solution blank. The iodine blue value was calculated by dividing the O.D. of test with O.D. of blank and multiplying by 100.

c) Specific activity by DNSA method: 1 ml of reaction mixture after 30 minutes of incubation at appropriate temperature was added to a test tube containing 2 ml water and 1 ml DNSA reagent (Appendix D). The tube was heated for 10 minutes in boiling water bath and cooled in running tap water. Absorbance at 540 nm was then used to estimate units of enzyme activity from the maltose standard Protein was determined by BCA/Folin Lowry's method (Appendix D) with bovine serum albumin as the standard.

One unit of enzyme activity was defined as the amount of enzyme that produced 10 μgms of reducing sugar estimated by DNSA using maltose standard curve at 25°C / 55°C for 30 minutes.

5.6 Optimisation of culture conditions for growth:

For all the optimisation experiments the culture SB-D at pH 10.3 was incubated at 25°C for 24 h while SB-W was incubated at 55°C for 16 h unless otherwise stated. Growth was monitored by measuring the turbidity at 600 nm, residual starch by iodine blue value method and amylase activity by DNSA method.

a) Effect of physical parameters: The cultures were grown at different 1) Temperatures (5°C-75°C), 2) pH (4.3-13.3), 3) Agitation rates (100, 150, 200, 250 and 300 rpm) and 4) Inoculum volumes (1-8%) at appropriate conditions of incubation.

b) Effect of nutritional parameters: In order to optimise medium for maximum growth and enzyme production, components of Horikoshi II medium were varied by (1) Substituting soluble starch with different carbon sources (1%) (2) Replacing yeast extract with organic and inorganic nitrogen sources (0.5%) and (3) Supplementing with different concentrations of yeast extract (0%-1.1%) and starch (0%-7%). Cultures were incubated at optimum conditions.

5.7 Isolation, Purification and characterisation of amylase enzyme:

a) Isolation: The cultures were grown under optimised conditions and the culture broth was centrifuged at 10,000xg for 20 minutes at 4°C. Supernatants obtained were treated with ammonium sulphate with constant stirring until the saturation point reached 80% with SB-D and 70% with SB-W. The saturated supernatants were kept over night at 4°C and precipitates obtained were collected by centrifugation.

b) Purification: The precipitates were dissolved in small volume of carbonate bicarbonate buffer (pH 10.0) and the solutions were dialysed twice over the course of 10 h against the same buffer to remove the ammonium salt. Dialysis was carried out in sigma cellulose tubing (molecular weight cut off = 12 kDa). Protein concentration and amylase activity was determined before and after dialysis.

c) Characterisation of the purified amylase :

i) Type of amylase: The enzyme assay for the purified sample was carried out as described earlier. The relation of residual starch concentration to sugar formed was plotted to determine the type of amylase.

ii) Effect of various parameters/factors on enzyme activity: The activity of the partially purified enzyme was assayed under various factors such as:

a) pH ranging from (4.3-12.3) (Appendix B).

b) Temperature ranging from 5°C to 75°C.

c) In presence of **organic solvents** - Methanol, butanol, Ethanol, Isopropanol, chlorofom and diethyl ether at concentration of 50% (v/v).

d) In presence of **Detergent additives** - Triton X-100, Tween 80, SDS and EDTA in 1% concn.

e) In presence of **Metal ion** solutions-$CaCl_2$, $MgCl_2$, $CuSO_4$ and KCl. (0.1mM / L).

The amount of reducing equivalent released was measured by the DNSA method after 60 minutes. Activity was expressed as % relative activity with respect to maximum activity.

iii) Stability of the enzyme: The pH and temperature stability profile of the enzyme(s) were determined by preincubating the enzyme at different pH (4.3-12.3) and at various temperatures (25°C-80°C). The enzyme activity was assayed.

iv) Electrophoretic analysis: Sodium dodecyl sulphate polyacrylamide gel electrophoresis was done using purified enzymes as described (Chapter IV) (Appendix E.3). Samples were stained for protein with coomassie brilliant blue R-250.

Results and Discussions: The greatest enzymatic diversity on earth resides in microorganisms. Amongst the bacteria, organisms belonging to the genus *Bacillus* have been widely used for the commercial production of multiple enzymes (94).

During the screening of alkaliphiles for enzyme activity, several alkaline amylase producing bacteria were isolated on Horikoshi II agar and it was established that SB-D and SB-W produced maximum amylase activity in comparison with other isolates. Qualitative analysis of the amylolytic potential indicated maximum zone at 25°C for SB-D and at 55°C for SB-W at pH 10.3 (Plate 5.1, Table 5.1). Starch agar assay is found to be a convenient method for the screening of amylase activity. During the monitoring of growth of SB-D and SB-W in various formulations of Horikoshi II medium, it was interesting to note that the isolates could grow in the absence of starch utilizing peptone and yeast extract as carbon and nitrogen sources (Table 5.2). However, poor growth was observed in a medium containing only starch or peptone. Further, several carbohydrates were effective for growth of both the isolates (Table 5.3). Starch as a source of carbon was found to exhibit good growth and initiate amylase production. Amongst the nitrogen sources checked, organic nitrogen sources such as polypeptone and growth factors like yeast and meat extracts were essential for growth. Inorganic nitrogen sources such as ammonium nitrate, diammonium sulphate and sodium nitrite did not support growth (Table 5.4). An attempt was also made to grow both the isolates in SB medium prepared from boiled rice natural starch (gruel) as the sole source of carbon and nitrogen excluding bacteriological peptone and yeast extract at alkaline pH.

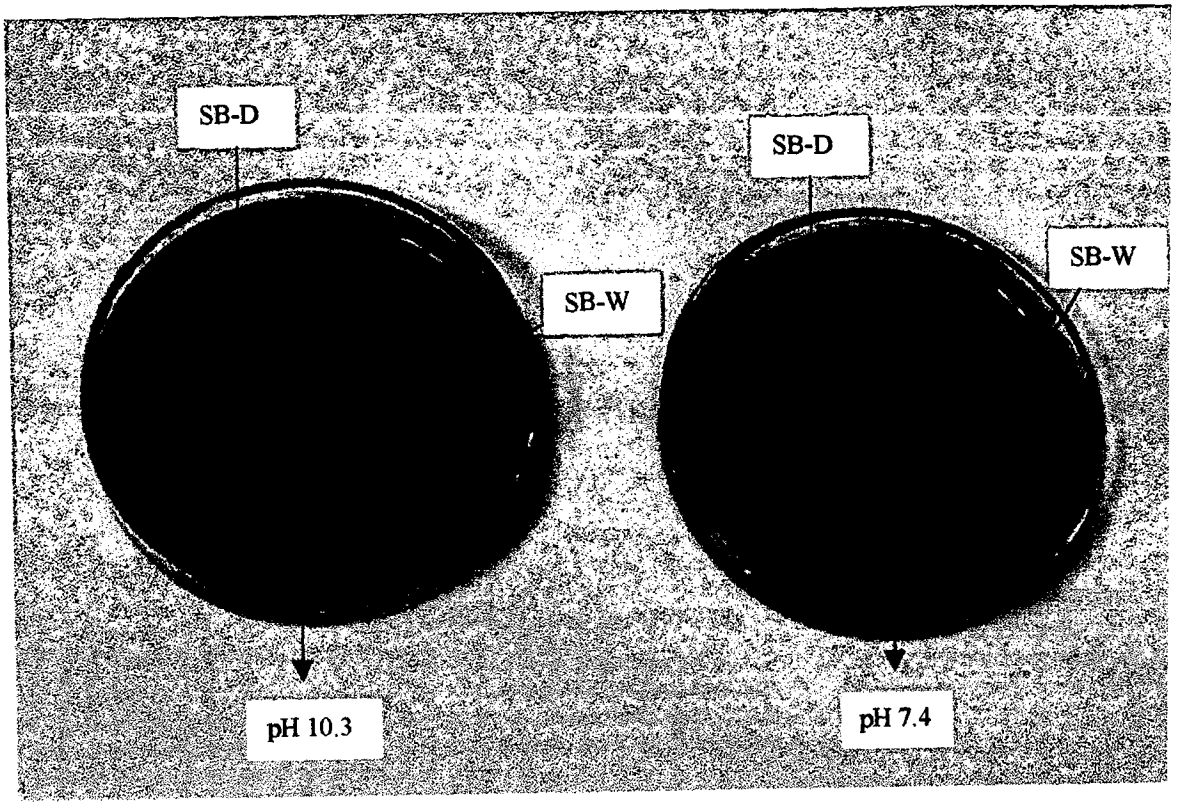


Plate 5.1. Amylase activity at pH 10.3 and 7.4.

Table 5.1 Qualitative analysis of the amylolytic potential of SB-D & SB-W on Horikoshi II medium.

	Zone size (mm)			
	pH 7.4		pH 10.3	
Culture	**25 ° C**	**55 ° C**	**25 ° C**	**55 ° C**
SB-D	-	-	40	-
SB-W	20	38	22	45

Table 5.2 Monitoring of growth of SB-D & SB-W in various formulations of Horikoshi II medium.

	Medium			(O.D. 600) (20 h, 25°C)	
No	**Starch**	**Yeast extract**	**Peptone**	**SB-D**	**SB-W**
1	+	+	+	2.799	2.59
2	-	+	+	2.03	1.85
3	+	-	+	2.5	2.2
4	+	+	-	2.38	2.1
5	+	-	-	1.62	1.42
6	-	+	-	2.42	2.18
7	-	-	+	1.43	0.84

Table 5.3 Effect of different carbon sources (1%) on growth of SB-D & SB-W.

Carbon sources	O.D (600)	
	SB-D	SB-W
Glucose	2.64	2.34
Fructose	2.34	2.13
Rhamnose	0.35	0.32
Xylose	2.23	2.13
Lactose	0.64	0.34
Sucrose	2.00	2.06
Maltose	0.34	0.45
Glycerol	2.80	2.60
Sorbitol	2.77	2.45
Xylitol	1.33	1.24
Mannitol	2.81	2.34
Chitin	0.43	0.32
Starch	2.79	2.63
Chitosan	0.74	0.34
Tributyrin	1.18	2.30
Tween 80	1.90	2.42
Coconut oil	2.24	2.73
Longifolene	2.66	2.72

Table 5.4 Effect of different nitrogen sources (0.5%) on growth of SB-D & SB-W.

Nitrogen source	O.D (600)	
	SB-D	SB-W
Control ($-N_2$)	1.35	1.25
Organic Nitrogen Source		
Peptone	2.28	2.16
Yeast extract	2.50	2.23
Meat extract	2.49	2.32
Soya bean meal	1.54	1.25
Casein	1.45	2.21
Chitosan	0.75	0.34
Urea	1.08	1.23
Peptone+Yeast extract	2.80	2.60
Inorganic Nitrogen Source		
Ammonium nitrate	0.19	0.05
Diammonium sulphate	0.27	0.15
Potassium nitrate	1.57	0.34
Sodium nitrate	1.12	1.13
Sodium nitrite	0.89	0.45
Diammonium hydrogen ortho phosphate	1.75	1.62
Ammonium dihydrogen phosphate	1.34	1.23

Interestingly, the isolates SB-D and SB-W could grow on agar plates prepared using this medium and exhibited amylase activity.

Growth profiles and percentage residual starch in culture supernatants at different time intervals in Horikoshi II medium was further studied. The culture SB-D showed a continuous growth profile in Horikoshi II medium. However, utilisation of starch, and hence enzyme production was found to be initiated only after 8 hours of incubation (Plate 5.2, Figs. 5.1, 5.2 & 5.3). Utilization of starch was observed with increase in growth upto 24 hours. It appears that the culture utilizes other available carbon sources for initial growth till the enzyme production was induced. (Table 5.2).

The growth rate of SB-W was found to be faster at 55°C, also showing a sharp reduction in residual starch within 8 hours. The utilization of starch at 25°C, corresponding with the increase in turbidity was also depicted from the time of incubation till 20 hours. (Figs. 5.1, 5.2 & 5.3). The temperature therefore appears to have a significant effect on the utilization of starch and growth of SB-W.

The temperature profile of culture SB-D showed optimum growth and least residual starch in the supernatant of the culture broth grown at 25°C (Fig.5.4) However, SB-W showed growth over a wide range of temperature between 25°C-65°C with least residual starch in the supernatant being shown by the culture grown at 55°C (Fig. 5.4). Utilization of starch by *Bacillus coagulans* at 55°C has also been reported (20-22,252).

The effect of pH on growth, measured at 25°C after 24 hours for SB-D and 55°C after 16 hours for SB-W by varying the pH of the assay medium was further studied.

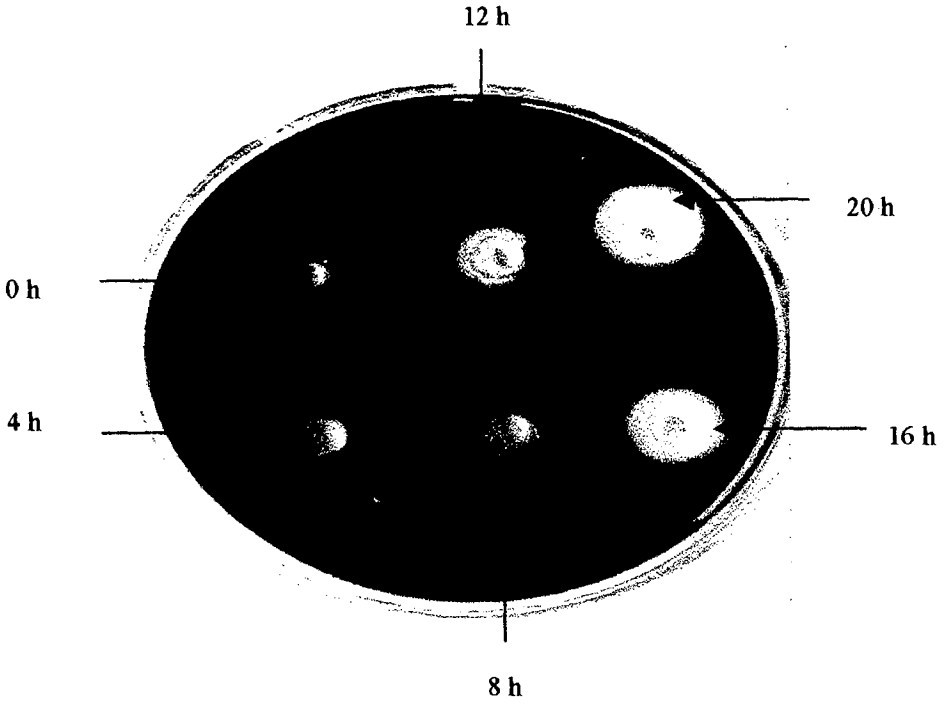


Plate 5.2. Amylase activity of culture supernatant of *B. alkalophilus* on starch agar during growth (h).

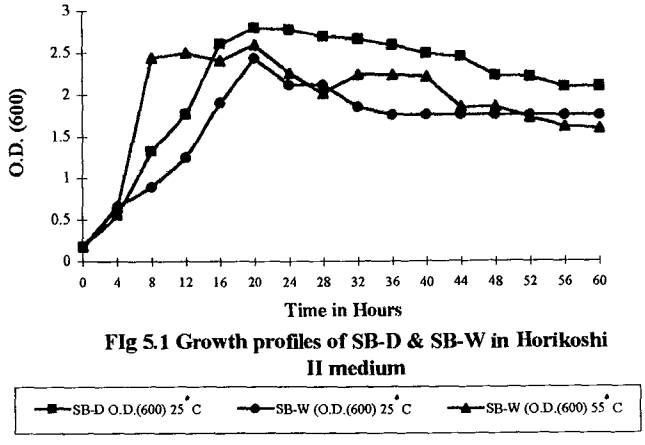


FIg 5.1 Growth profiles of SB-D & SB-W in Horikoshi II medium

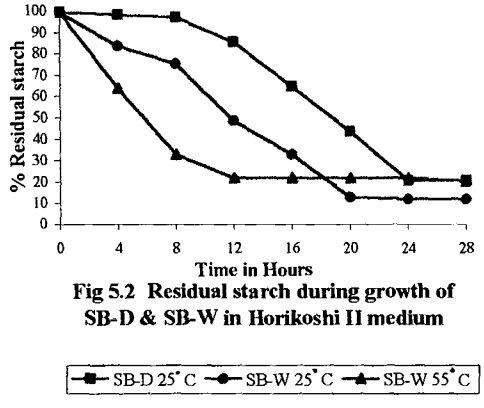


Fig 5.2 Residual starch during growth of SB-D & SB-W in Horikoshi II medium

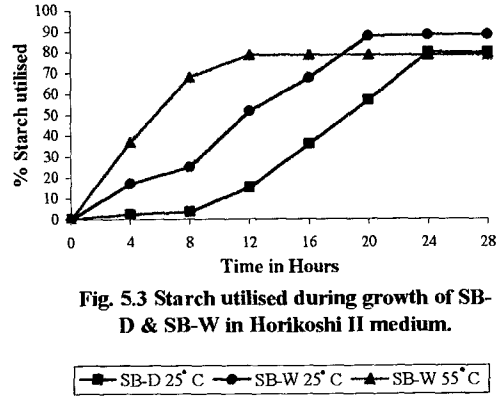


Fig. 5.3 Starch utilised during growth of SB-D & SB-W in Horikoshi II medium.

Note: Method used for estimation of starch: Iodine blue value.

It was interesting to note that the growth and starch utilization showed a distinct relationship at different pH for SB-W isolate, maximum growth and starch utilization seen at pH 10.3 (Fig. 5.5). However with SB-D the growth was initiated at pH 8.3 but there was no utilization of starch at this pH. Above pH 8.3, the growth and starch utilization were linearly related with optimum being at pH 10.3 (Fig. 5.5).

Following the parametric optimisation for maximum growth, hence amylase production, it was observed that an agitation rate of 200 rpm and 250 rpm were best for growth of SB-D and SB-W respectively (Fig. 5.6). Further an inoculum size of 2% was required for maintaining least residual starch in the culture supernatant by both the isolates (Fig. 5.7) although 3% inoculum size was optimum for growth of the cultures. Such positive noticeable effect of inoculum level with high inoculum densities inhibiting amylase production has also been reported (20).

With respect to nutritional factors, the medium supplemented with 0.5% yeast extract supported maximum growth and utilization of starch declined sharply (Fig. 5.8). With SB-W, optimum growth was observed at 0.5% yeast extract that declined with increasing concentration of yeast extract. However, the conversion of starch continued up to 0.7% yeast extract. Further, increase in yeast extract showed a sharp inhibition of utilization of starch (Fig. 5.8). This sharp inhibition of utilization of starch on increasing the yeast extract concentration is perhaps due to competitive utilization of the carbon source. This also is reflected on the extent of growth of the culture on yeast extract alone (Table 5.2).

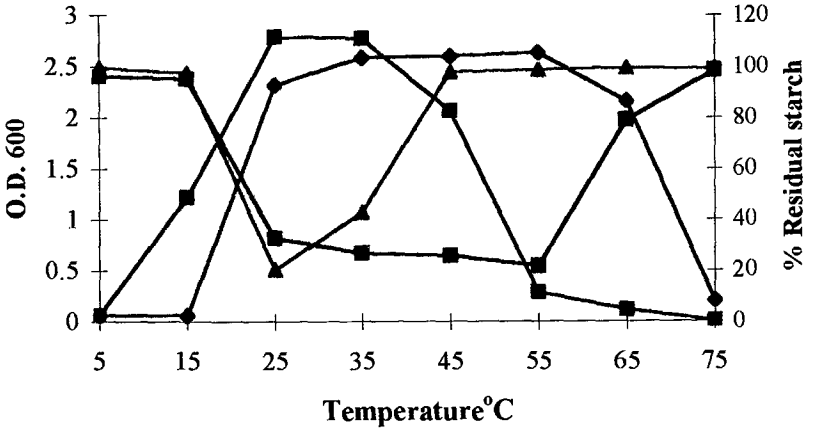


Fig. 5.4 Effect of temperature on growth of SB-D & SB-W & residual starch.

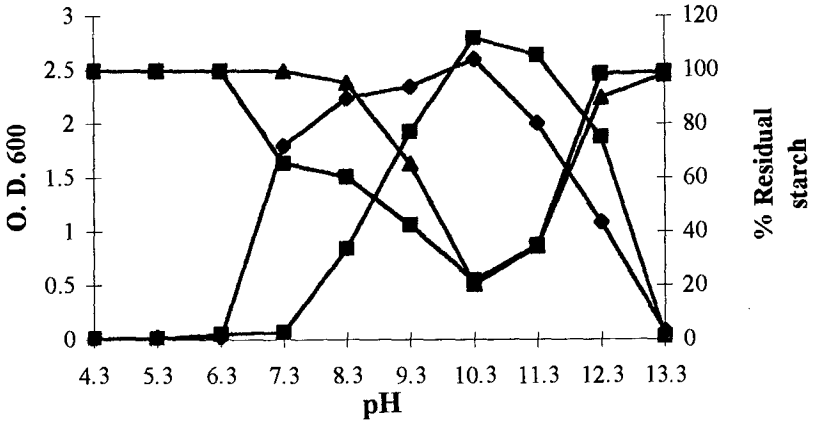
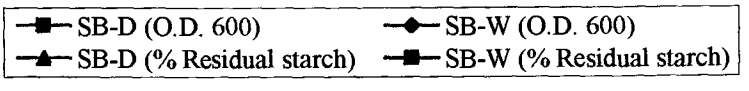


Fig 5.5 Effect of pH on growth of SB-D & SB-W & residual starch.



	Growth conditions	Culture	
Note:	Incubation	SB-D	SB-W
Fig.5.4	pH	10.3	10.3
	h	24	16
Fig.5.5	Temperature °C	25	55
	h	24	16

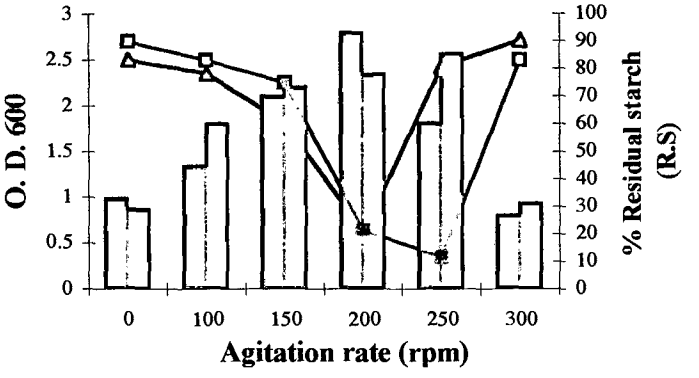


Fig 5.6 Effect of agitation on growth of SB-D & SB-W & residual starch.

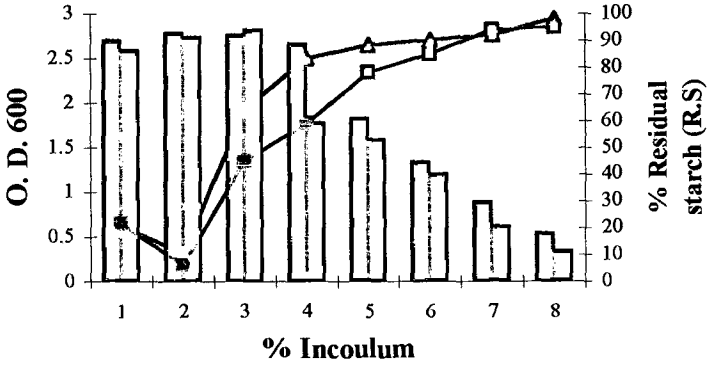
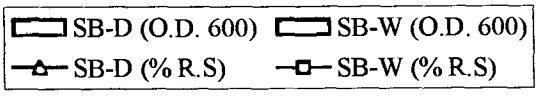


Fig. 5.7. Effect of Inoculum density on growth of SB-D & SB-W& residual starch.



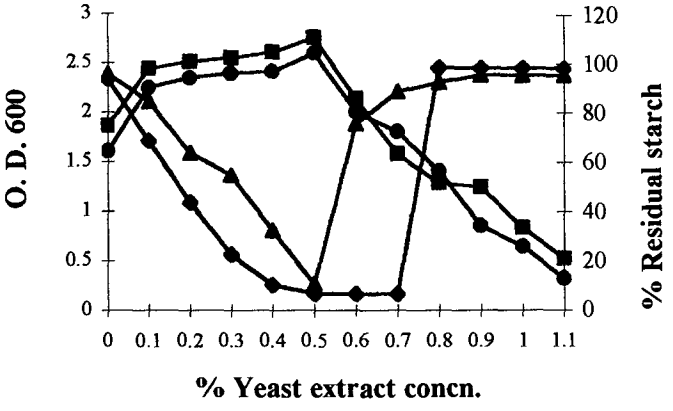


Fig 5.8 Effect of yeast extract concn. on growth of SB-D & SB-W & residual starch.

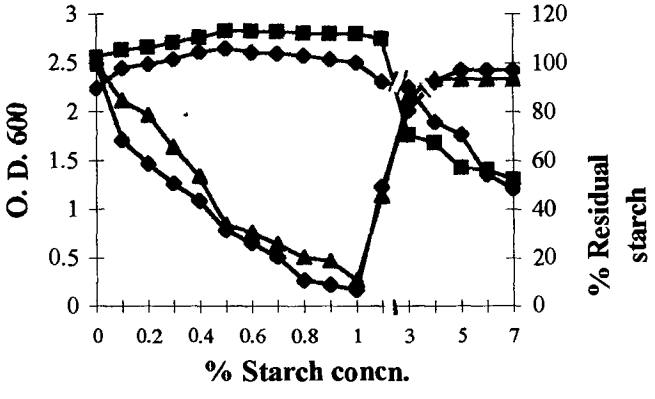
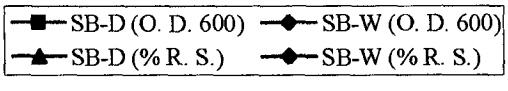


Fig 5.9 Effect of starch concn. on growth of SB-D & SB-W.



Growth under varying concentrations of starch was found to be optimum at 0.5% for both the cultures. SB-D however, showed no reduction in growth with increase up to 1% and the residual starch was also found to be least. However, SB-W although showed least residual starch at 1% concentration, growth was found to be reduced above 0.5% of starch (Fig. 5.9). Such effect of the carbon source on the extent of growth of gram positive cultures is related to the stress due to excess of nutrients and induction of sporulation (333).

It has been reported that the cultural conditions have a profound influence on amylase production (20,88,392). Bacilli sps. have been reported to secrete alkaline amylases in alkaline media (40,41,142,152,161,186). Further, Ohta *et al* (278) have demonstrated that intracellular enzymes of alkaliphilic members of the genus *Bacillus* do not possess unusual alkalistable properties. In contrast, their extracellular enzymes exhibit maximum activity at pH 10 or above and are unusually alkalistable. This difference in pH optima between intracellular and extracellular enzymes suggests that alkaliphiles are able to maintain a neutral internal environment in the presence of high external pH (157).

On optimisation (Table5.5) and ammonium sulfate precipitation of the culture supernatant with subsequent incubation in the refrigerator resulted in precipitate, which on dialysis showed the amylase activity. The activity was found to increase with purification by 2.91 and 1.84 fold with SB-D and SB-W respectively as compared to crude enzyme (Table 5.6).

Table 5.5 Optimised cultural conditions for growth of SB-D & SB-W in Horikoshi II medium.

Condition	Strain SB-D	Strain SB-W
Medium composition	Same as for screening	Same as for screening
Initial pH	10.3	10.3
Incubation time (h)	24	16
Temperature $^{\circ}$ C	25	55
Agitation	200	250
Inoculum density	2%	2%

Table 5.6 Effect of purification on amylase activity of *B. alkalophilus* SB-D & *B. coagulans* SB-W.

Fraction	*B. alkalophilu* s SB-D					*B. coagulans* SB-W				
	Protein (mg/ml)	Reducing sugar DNSA(mg/ml)	Enzyme Units	Specific activity (U/mg protein)	Fold Purification	Protein (mg/ml)	Reducing sugar DNSA(mg/ml)	Enzyme Units	Specific activity (U/mg protein)	Fold Purification
Crude liquor	0.506	0.24	24	47.43	1	0.597	0.35	35	58.62	1
$(NH_4)_2 SO_4$ ppt before dialysis	0.67	0.58	58	86.56	1.82	0.742	0.65	65	87.6	1.49
$(NH_4)_2 SO_4$ ppt after dialysis	0.47	0.65	65	138.2	2.91	0.724	0.78	78	108.33	1.84

The purified enzyme was used for further characterization. It was observed that the relationship between iodine blue value and reducing sugar was similar to α-amylase as seen in fig. 5.10. This indicates that both the enzymes belong to the group of α-amylases (351). Alkaline α-amylases of genus *Bacillus* (40,41,141,142,161,190,252) and *Pseudomonas* (306) have been reported.

pH of the purified enzyme revealed 2 pH optima of 7 and 10.3 with SB-W and only 1 pH optimum of 10.3 with SB-D (Fig 5.11). The pH optimum of the enzyme obtained from SB-W confirms its facultative nature.

Enzyme of SB-D showed an optimum temperature of 25°C for its activity while for the enzyme from SB-W, the activity was maximum at 55°C irrespective of the pH being either 7 or 10.3 (Fig 5.12). Studies on the growth of the culture have also confirmed the thermostable property of the enzyme.

The stability of the enzyme exposed to different pH and temperature for 60 minutes was further studied. Interestingly, the enzyme from SB-W was found to be stable between pH 7.3 and 10.3 and temperature up to 65°C (Figs 5.14 & 5.16), while SB-D had more thermolabile enzyme being stable only up to 35°C and pH between 9.3-10.3 (Figs 5.13 & 5.15).

Further the activity of the enzymes in presence of organic solvents was monitored. It was interesting to note that the purified enzyme of SB-W retained almost 80 % activity in butanol, isopropanol and diethyl ether (Fig. 5.17). However, α-amylase of SB-D showed no activity in presence of organic solvents. Both the enzymes obtained from SB-D and SB-W were found to be stable in the presence of detergent

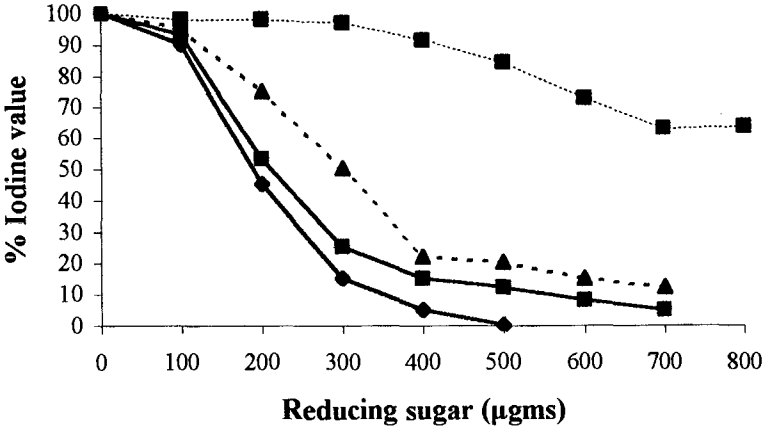


Fig. 5.10 Relation between residual starch & reducing sugar.

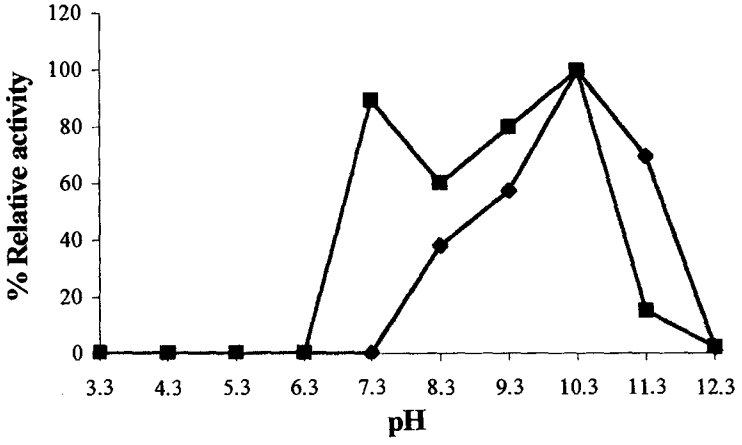


Fig. 5.11. Effect of pH on purified amylase activity of SB-D & SB-W.

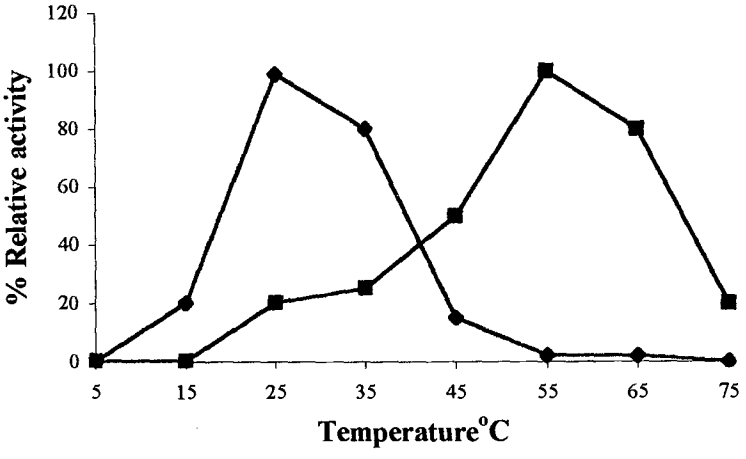
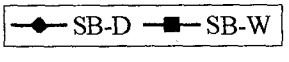


Fig. 5.12 Effect of temperature on purified amylase activity of SB-D & SB-W.



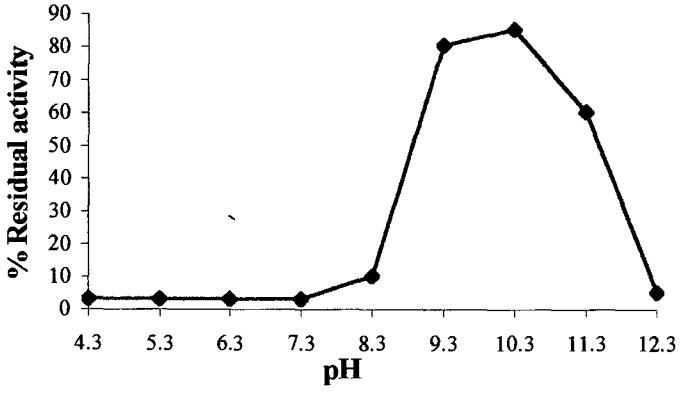


Fig 5.13. pH stability of SB-D amylase after 1 hour at 25°C.

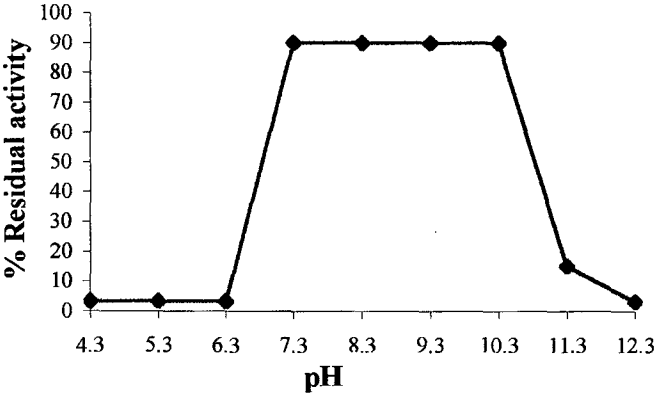


Fig 5.14. pH stability of SB-W amylase after 1 hour at 55°C.

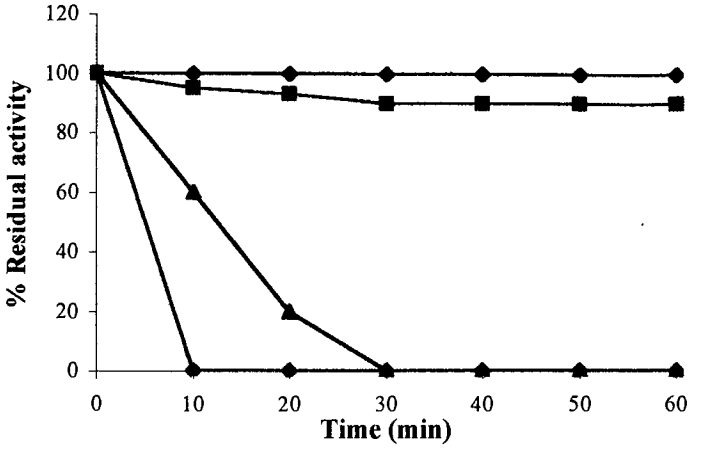
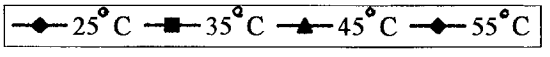


Fig. 5.15 Thermostability of SB-D amylase.



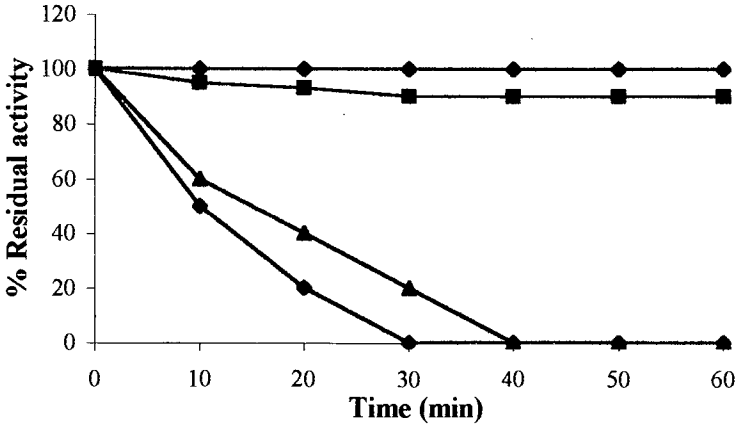
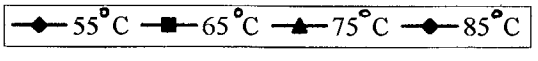


Fig. 5.16 Thermostability of SB-W amylase.



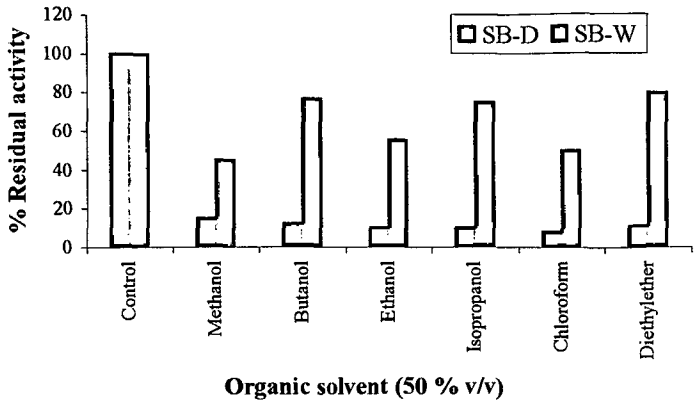


Fig 5.17. Activity of amylase from SB-D & SB-W in presence of organic solvents.

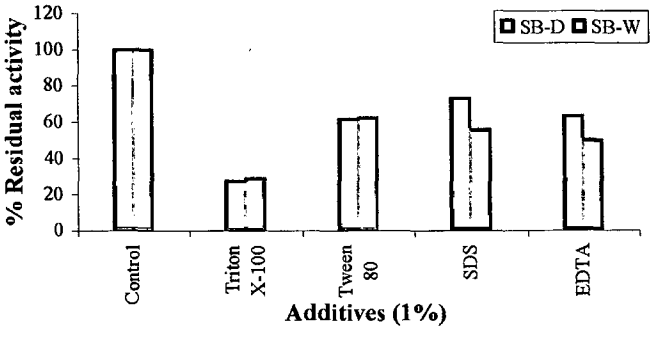


Fig 5.18. Activity of amylase from SB-D & SB-W in presence of detergent additives.

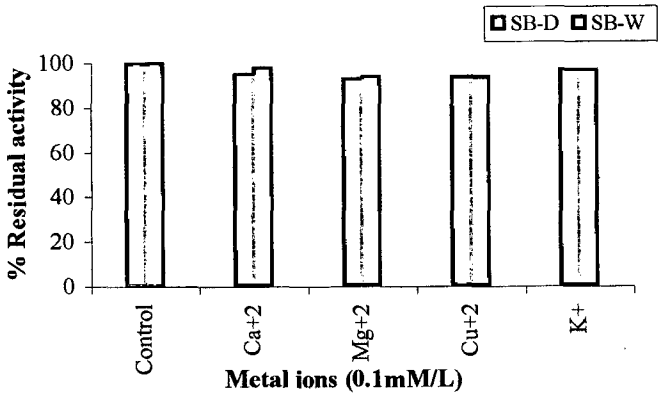


Fig 5.19. Activity of amylase from SB-D & SB-W in presence of metal ions.

additives (Fig 5.18) and were not influenced in their activity by the presence or absence of metal ions like Ca^{+2}, Mg^{+2}, Cu^{+2} and K^{+}(Fig5.19).

Purified enzymes on electrophoretic mobility showed the presence of 4 bands in SB-D whereas multiple units were observed with SB-W (Plate 5.3) indicating multiple amylolytic proteins as also observed with *Bacillus* and *Lactobacillus* (114). Kim *et al* (190) have also reported the production of five alkaline amylases in the culture broth.

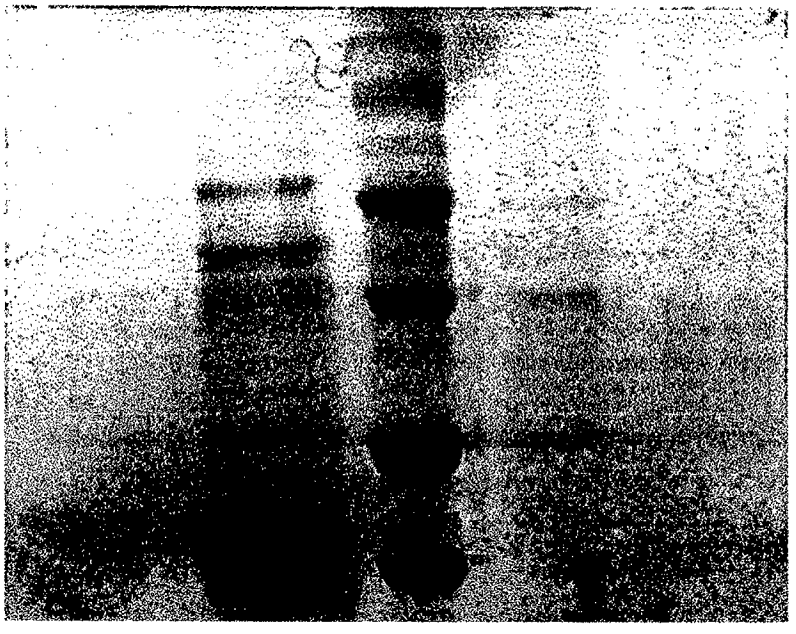
Salient features of this study are:

1. **The cultures SB-D and SB-W showed the production of amylase at 25°C at pH 10.3, SB-W giving a better activity at 55°C.**

2. **The optimum conditions for growth and enzyme production were found to be 2% inoculum, 10.3 pH, with incubation time of 24 h at 25°C and 200 rpm for SB-D, while for SB-W it was 16 h, 55°C and 250 rpm.**

3. **The amylase from SB-W was highly tolerant to organic solvents and temperature showing the stable nature of the enzyme and activity over a wide range of pH of 7.3-10.3.**

4. **The amylase from SB-D functioned between pH 9-11 at temperature below 35°C and was highly sensitive to organic solvents.**



Lane 1 Lane 2 Lane 3

Plate 5.3. SDS-PAGE profile of Amylase enzyme of:

Lane 1: *B. coagulans* SB-W.
Lane 2: Marker.
Lane 3: *B. alkalophilus* SB-D.

During the studies on purification of amylase from SB-D it was found that the viscosity of Horikoshi II medium increased indicating the production of exopolymer. This was also depicted due to the increase in total sugars as measured by phenol sulphuric acid method. The conditions for production, isolation and characterisation of this polymer are discussed in the following chapter.

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CHAPTER VI

Studies on Viscous Exopolymer (EP) of B. alkalophilus SB-D.

Microbial exopolymers are high molecular weight secretions of bacteria, yeasts and microalgae. While polymers are quite stable, their synthesis is influenced by a wide variety of conditions such as culture medium composition, environmental factors, phases of growth, etc. (347-349).

There are a number of reports on the production of such exopolymers at neutral pH. However, studies on exopolymer production at alkaline pH are reported with few organisms. (71,380).

In the course of our study on alkaliphiles in diverse econiches, an amylase producing bacterium identified as *B. alkalophilus* (SB-D), was isolated from spent water samples of an Agrochemical factory. This culture was found to increase the viscosity of the culture broth grown on shaker for 16 hours and when kept stationary for 4 hours in Horikoshi I medium. It was therefore envisaged to standardise the conditions such as medium composition, age of culture, inoculum size, aeration and agitation for optimum production, isolate and characterise the EP using analytical methods.

Materials and methods:

6.1 Media and growth conditions: A loopful of *B. alkalophilius* SB-D from slant was inoculated into sterile Horikoshi I/II media (pH 10.3) (Appendix A). The culture was incubated at 25°C on a rotary shaker at 200 rpm over night and used as inoculum for all the experiments.

6.2 Alcian blue adsorption assay for cell free/cell bound exopolymers (26,376):

The 24 hour old culture broth was centrifuged and the pellet was resuspended in 4 ml carbonate-bicarbonate buffer (0.2 M, pH 10.0). The assay was carried out by adding 1% aqueous alcian blue solution (8µls) (8G x Sigma) to 4ml of 24 hour old culture broth and the cell suspension. The mixture was centrifuged after 5 minutes. The color retained in the supernatant was measured at 606 nm. Aqueous and carbonate-bicarbonate controls without cells and negative control of cells were also tested by the assay method. The decrease in the reading of the absorbance in the supernatant after the cells were removed by centrifugation determined the amount of the dye bound/adsorbed to the cells/exopolymer.

6.3 Factors affecting EP production:

6.3.1 Effect of growth conditions: 250 ml Erlenmeyer flasks containing 100 ml of Horikoshi I / II media were inoculated with 2% (v/v) of an overnight grown culture and incubated at static, shaker and combined shaker + static conditions. Growth, change in pH, appearance of viscosity and yield of the polymer in Horikoshi medium were determined.

Growth was measured at 600 nm after every 4 hours of incubation upto 60 hours and change in pH was recorded using pH meter (Labindia). The culture broth was centrifuged at 10,000 X g for 10 minutes in a Remi 24 C centrifuge at 4°C. Wet weight of cell pellet was determined on Dhona 200 D balance. Viscosity of the cell free supernatant was measured using Brookfield Digital viscometer model DV-111+

Programmable rheometer by taking 2 ml of the solution in the sample cup with spindle 40 at 25 rpm and 25°C. 1:1 volume of cold isopropanol was added to the cell free viscous supernatant, and the weight of the spoolable precipitate (G1) was determined (Scheme 2 pg. No.163).

6.3.2 Effect of holding time under static conditions on cell mass, pH, viscosity and polymer production: The culture was grown on shaker at 200 rpm and 25°C for 16 hours in Horikoshi I and 20 hours in Horikoshi II medium, followed by incubating at static conditions for 0, 2, 4, 6 and 8 hours. Growth, change in pH, viscosity and yield of the polymer were recorded as above.

6.3.3 Process optimisation for maximum EP (G1) production:

i Physical parameters:

(a) Effect of rate of agitation: The culture was grown in Horikoshi I medium for 16 hours at different agitation rates of 100,150,200 and 250 rpm at 25°C, pH 10.3 followed by incubating the flasks at stationary conditions for 0,2,4,6 and 8 hours. Cell mass, pH change, viscosity and polymer yield was determined.

(b) Effect of flask size to volume ratio: The culture was inoculated in different capacity of Erlenmeyer flasks so as to attain the flask size to volume ratio as 1:7.5, 1:5, 1:4, 1:2.5 and 1:1 by varying volume of Horikoshi I medium (pH 10.3). The flasks were incubated at 200 rpm for 16 hours on shaker and 4 hours at stationary conditions. Cell mass, pH change, viscosity and polymer yield was determined as described.

(c) Effect of Inoculum size: Horikoshi I medium was inoculated with different volumes of the inoculum (0 %-5%) and incubated at 200 rpm for 16 hours followed by 4 hours at static conditions. Growth, viscosity and polymer yield were monitored.

ii Nutritional parameters:

Effect of different concentrations of yeast extract and glucose: 4% 18 hour old culture was inoculated in Horikoshi I medium and flasks incubated at 25°C for 16 hours on shaker (200 rpm) followed by 4 hours of static conditions. The concentrations of yeast extract and glucose were varied in the medium and the effect on growth, viscosity and polymer yield was studied.

6.4 Isolation, purification and characterization of G1 & G2 exopolymer:

A Isolation:

I. G 1:

i. From Cells: The cell pellet obtained after centrifugation of the culture broth (10 gms) grown in Horikoshi medium for 16 hours on shaker and 4 hours of static condition was suspended in deionised water, phosphate buffer saline and 10% Triton X-100 and kept on shaker for 4 hours for release of the polymer. The content was centrifuged at 6000 rpm for 10 mins. and the polymer was spooled on a glass rod and the yield of the spoolable polymer (G 1) (Scheme 2 pg. No.163) was noted when the cells were suspended in water, PBS and the detergent.

ii) From viscous culture supernatant: Different methods were used for the extraction of exopolymer viz. precipitation with various solvents such as cold ethanol,

acetone, isopropanol, methanol, chloroform methanol mixture in the ratio of 2:1, N, N, N cetyltrimethyl ammonium bromide/cetavlon, salt precipitation using ammonium sulphate, acidification of the supernatant followed by salt and solvent precipitation. The precipitate obtained on addition of isopropanol was purified.

II. G 2:

The culture was inoculated in Horikoshi I/II medium on shaker and was centrifuged at 1000 X g for 10 minutes after every 4 hours upto 24 hours. The precipitate (G2) obtained after centrifugation (Scheme 2 pg. No.163) was washed twice with d/w and wet weight of the precipitate to relationship of incubation period and cell mass determined.

B Product purification:

i) Dialysis of the polymers: The polymers G1 and G2 were transferred into a pretreated dialysis tubing with a cut off of 12,000 and were dialysed three times against double distilled water for 24 hours until the odor of isopropanol could no longer be detected. The concentrated polymers were resuspended in minimum volume of d/w and lyophilised.

ii) Selenisation of the glassware used for lyophilisation to prevent the adherence of G1 to the glass surface (393): Since G1 was found to stick to the glass surface which could interfere with the yield of the polymer and scraping of the polymer from the tube was difficult, glassware used before lyophilisation were made hydrophobic.

Tubes were washed in detergent, rinsed with ethanol (95%) and double d/w, dried at 450°C for 4-6 hours and siliconised in 10 % dichlorodimethylsilane prepared in methylene chloride with subsequent washings in methylene chloride, ethanol and finally in double d/w. These siliconised tubes were used for the lyophilisation.

iii) Lyophilisation of G1 and G2: The tubes containing the polymer suspensions were frozen, connected to Labconco Lyophiliser and dried to a powder form. The purified, lyophilised polymers were stored at -20 °C for subsequent analysis.

Schematic representation of the protocol for isolation and Purification of spoolable polymer (G1) and needle like crystals (G2) from Horikoshi I medium is as represented in Scheme 2 pg.no 163.

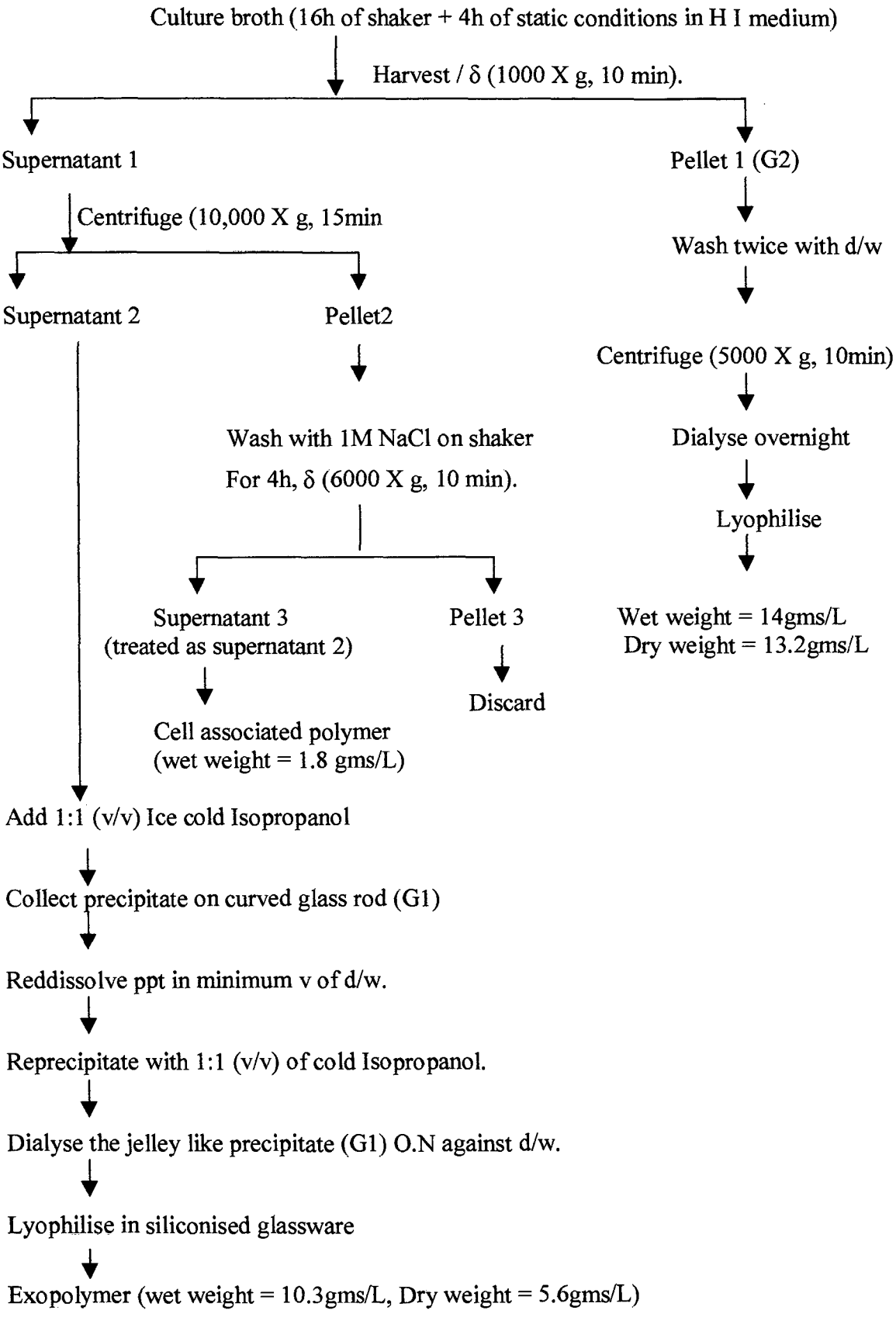
C. Characterisation of the Polymers:

a) Physical Properties: Wet weight/dry weight, color, pH were determined. Solubility of G1 and G2 in d/w, hot d/w, 0.1 N NaOH, 1.5 M NaCl, 1 N HCl, chlorofom, methanol, pyridine, acetone, ether, was checked. Melting point was recorded using Centrofix melting point instrument.

a) Chemical analysis:

1) Analysis of unhydrolysed polymer: Inorganic and organic content of the polymer was determined by ashing a known quantity of the polymer in a muffel furnace at 400°C for 4 hours. The residue left was weighed as inorganic content.

Scheme 2. Schematic representation for the isolation and purification of G1 and G2.



The carbon and nitrogen content of G 1 and G 2 was determined using NCS-2500 Elemental analyser (C. E. instruments, Italy).

Total carbohydrate was determined by the Phenol H_2SO_4 method of Dubois et al (312) using glucose as the standard. Protein was determined by the method of Lowry et al (1951) (239) and Bicinchoninic acid method of Smith et al (1985) (332) using Bovine serum albumin as the standard. Lipid content was estimated by the method outlined by Pande, Khan and Ventikasubramaniam (1963) (284) using stearic acid standard. Uronic acids were determined by both Carbazole reaction of Bitter (1962) (36) and Sulfamic acid-metahydroxy biphenyl method (Filisetti and Nicholas 1991) (92) using glucuronic acid as the standard. Methyl pentoses were determined using the 10 min version of the cysteine-hydrochloride reaction of Dische-Shettle's method (1948) (81) using rhamnose as the standard. Inorganic phosphates were estimated by Fiske-Subbarow's method using KH_2PO_4 as the standard (173). Estimation of phenolic acid was carried out by Marambe and Ando's method (1992) (245) using catechol as the standard (Appendix D).

2) **Analysis of hydrolysed polymer:** The samples were hydrolysed as described in Appendix D. The released components such as glucose was determined by glucose oxidase method (312) with glucose as the standard. Pyruvate was determined colorimetrically by the 2,4-dinitrophenyl-hydrozone-method (Slonecker and Orentas 1962) (331) using pyruvic acid as the standard. Sulphates were determined by Dodgson and Price (1962) (82) method using K_2SO_4 as the standard (Appendix D).

c) Chromatographic Analysis:

i) Hydrolysis of the sample: 1 ml of 2 N HCl was added to 1 mg of the sample. The ampule was flushed with nitrogen gas, sealed and hydrolysed at 100°C for 2 hours (137). The contents were washed twice with d/w after neutralisation of HCl with 1 ml of 2 N NaOH and evaporated using vacuum evaporator. The sample was reconstituted in 1 ml double d/w.

ii) Identification of Monosaccharides :

a) By Thin Layer Chromatography: The hydrolysed and unhydrolysed samples were spotted on the TLC plates with standards.

The chromatograms were developed in five different solvent systems using specific spray reagents (Appendix E.1).

b) By Gas liquid chromatography: For GC analysis, the released sugars after hydrolysis were derivatised as alditol acetates (Appendix E.2) and identified by capillary gas chromatography (GC equipped with CP sil-88 25 mm, id = 0.32 mm, df = 0.12, Chrompack, Model CP 9002 Middleburg, the Netherlands).

D. Detection of Lipids (180): Three chambers were presaturated with chloroform, methanol and acetone separately and another chamber containing chlorofom: Methanol: water (65:35:5) was prepared. Samples were spotted on the plate and developed as follows:

1. In Chloroform only.
2. First in Chloroform, dried, and developed in methanol.
3. In Chloroform, dried, developed in methanol, dried and developed in acetone.
4. In chloroform: methanol: water (65:35:5).

The plates were dried and stained by spraying with α-naphthol-sulphuric acid reagent and anthrone reagent; (Appendix E.1) further dried in an oven for 10 minutes at 100°C for the visualisation of spots. The spots were identified by comparing the Rf values of the spots in different solvent systems and comparing the colour obtained using Tripalmitin as standard.

6.5 Characteristic properties of the polymer

i) Emulsification (64):

a) Emulsification properties of the fermentation broth: 5 ml of benzene was added to 5 ml of the supernatant obtained after centrifugation of culture broth at 16 h and at 16 + 4 h in Horikoshi I medium. The tubes were vortexed vigorously for 10 minutes and left undisturbed for 1 hour. Absorbance of the emulsion formed was measured at 610 nm using uninoculated Horikoshi medium as blank. Increase in turbidity was taken as a measure of emulsifying activity. The dye-oil red *O* was added to monitor the visual distinction between the oil and water phases and determine the type of emulsification.

b) Emulsifying property of the cell bound exopolymer: The cell pellet obtained after the centrifugation of culture broth at 16 h and 16+4 h from Horikoshi I

medium and 20 h and 20 + 4 h from Horikoshi II medium was suspended in phosphate buffer saline (PBS) (Appendix B.2) so as to give an absorbance of 0.54. To 5 ml of this suspension was added 5 ml of benzene and emulsification property determined as above. The cell pellet washed and suspended in PBS and the washings of the cell pellet were also used to determine the release of cell bound EP into the supernatant. A gram negative *Sphingomonas* culture was used as a positive control and haloalkaliphilic *Natronococcus*, alkalphilic *Bacillus* sps were also used for comparative study.

c) **Emulsifying activity of G1 and G2:** Lyophilised and nonlyophilised samples (5 mg) were suspended and dissolved in 1 ml deionised water by heating in a boiling water bath for 30 minutes. PBS was added to the sample to make up the volume to 5.0 ml and emulsifying activity determined as described.

ii) **Metal adsorbtion by G1 and G2:** 10 mg of purified polymer were suspended for 1 h at room temperature in 10 mM $CuSO_4$ solution (1 mg/ml). After 1 h of equilibration at room temperature, the polymer-metal mixture was observed for the metal bound to the polymer. A process control was maintained and monitored as described.

iii) **Adhesive properties of G1 and G2:** Adhesive strength of Lyophilised and nonlyophilised G1 and G2 polymers were determined on 2.5 x 7.5 cm surfaces such as clean glass slides and wood pieces. The polymers were also used to stick stamps on to envelopes and placed on butter paper to note the adhesive property.

Results & Discussions:

The culture SB-D during its growth showed an increase in the viscosity of Horikoshi I and II media. It was therefore envisaged that the viscosity may be due to the presence of the polymer which may either be attached to the cells or released extracellularly. Such polymers have been found to confer the viscosity and can be checked using various methods. One of the simple semiquantitative assay methods is based on the adsorption of Alcian blue, a dye known to bind the polyanionic compounds (26). It was interesting to note that the alcian blue was adsorbed on the cells obtained from the media indicating the presence of cell associated exopolymer. As seen in Table 6.1 and Plate 6.1, the absorbance of the supernatants of SB-D cells/entire culture broth showed a marked reduction from 0.5 to 0.05. However, with SB-W the absorbance remained as 0.48 similar to control. Further, SEM of the culture SB-D (Plate 4.1.2, Chapter IV) also indicated the presence of this polymer as fibrils, also reported (189, 235) with other isolates.

Having seen the presence of the polymer, the growth conditions required for the release of the polymer into the extracellular medium were studied. The culture was grown at 3 different conditions in Horikoshi II medium and only condition III in Horikoshi I medium, as explained in Materials and methods.

From Fig. 6.1, it was observed that the culture when incubated under stationary conditions in Horikoshi II medium showed very slow growth with the stationary phase reaching after 36 hours. The growth on shaker conditions, was found to be faster, reaching stationary phase after 16 hours in Horikoshi I and 20 hours in Horikoshi II

Table 6.1 Alcian Blue Adsorption Assay for Exopolymer.

Sample analysed	O.D. at 606 nm	
	SB-D	SB-W
A 1	0.05	0.48
A 2	0.05	0.5

Note : A 1 - Treated culture broth system.

A 2 - Treated cell pellet system.

Control

Carbonate- Bicarbonate Buffer (pH 10) - O.D. 0.5

Water - O.D. 0.5

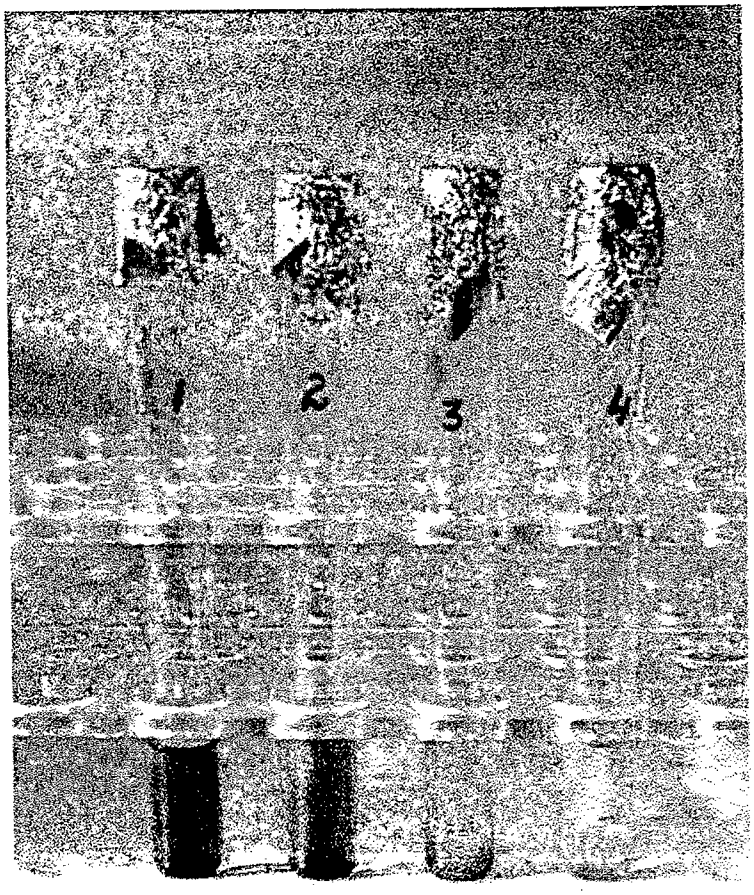


Plate 6.1. Alcian Blue Adsorption Assay for cell bound/cell free exopolymers.

1. Control.
2. *B. coagulans* SB-W cells.
3. *B. alkalophilus* SB-D cells.
4. Supernatant after centrifugation of 3.

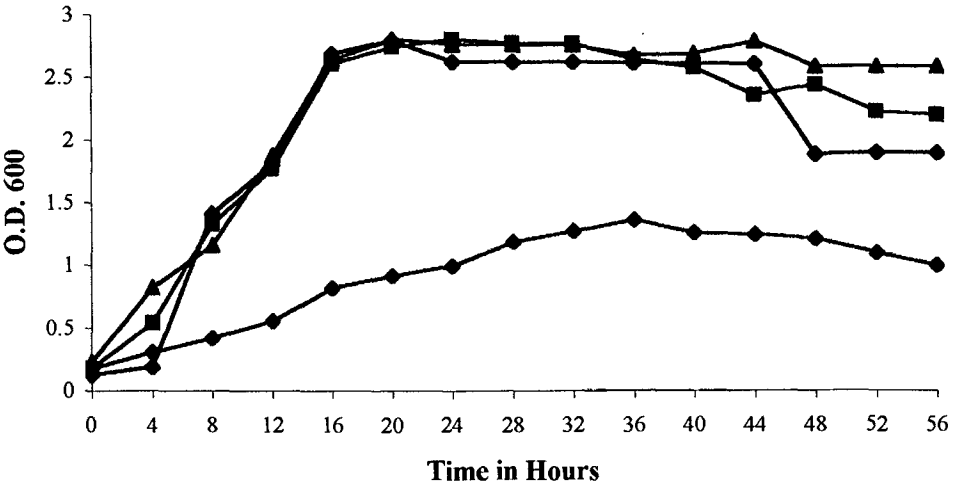


Fig. 6.1

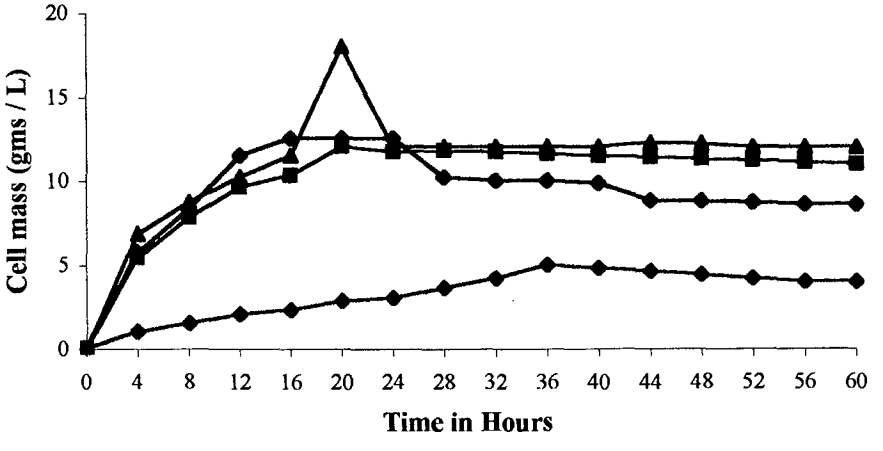
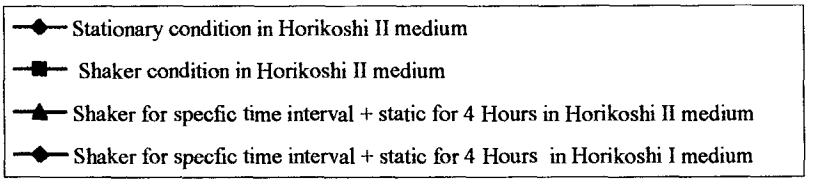


Fig. 6.2



Effect of static, shaker & shaker + static conditions on:

Fig 6.1 Growth of SB-D.

Fig 6.2 Cell mass of SB-D.

media. In the third condition, the effect of static conditions on the culture grown at different time intervals was determined by growing the culture for 4,8,12, upto 60 hours and keeping them at stationary conditions for different intervals. It was interesting to note that the subsequent removal of the flask from shaker and keeping under stationary conditions resulted in the release of the polymer and increase in the viscosity of the culture broth (Plate 6.2.1-6.2.4). Further, the time required for the viscosity to be produced when maintained on static conditions after growth was found to be 4 hours. The culture also produced the viscosity even when kept at static conditions after growth of 8 hours, although the holding time of static conditions remained 4 hours. The incubation of flask in the refrigerator during static conditions also increased the viscosity.

It was also observed that the absorbance of such flasks did not show any increase in the turbidity as seen in fig. 6.1. Similar results were also observed when the culture was grown in Horikoshi I medium showing the increase in viscosity after growing the culture for 16 h and keeping stationary for 4 hours (Fig. 6.1). Further, the incubation of cells after 16 hours of shaker and 4 hours of static condition resulted in the isolation of EP, which was separable as a spoolable material on the glass rod on addition of isopropanol in concentration of 1:1. This polymer is now referred as G1.

The SB-D culture therefore exhibited a unique behavior of releasing the polymer in the culture broth under both shaking and stationary conditions compared with either the conventional shaking or stationary conditions.

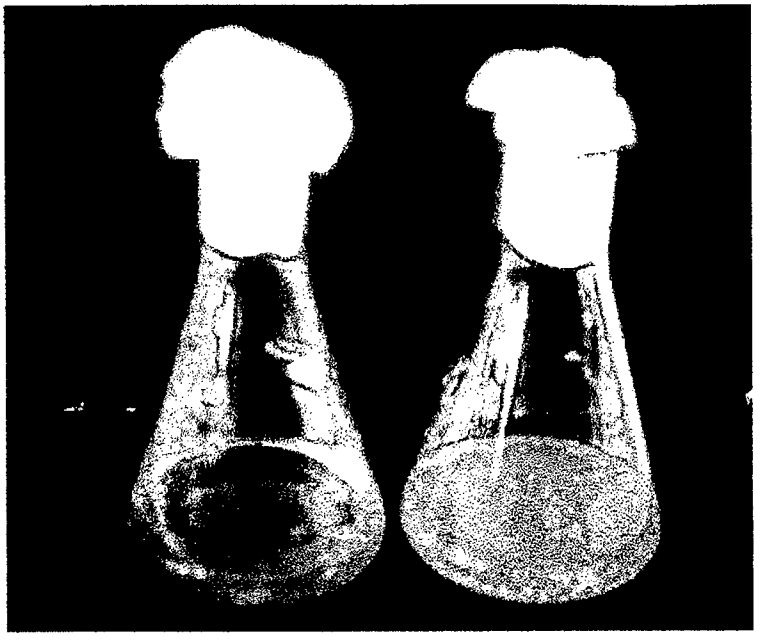


Plate 6.2.1.

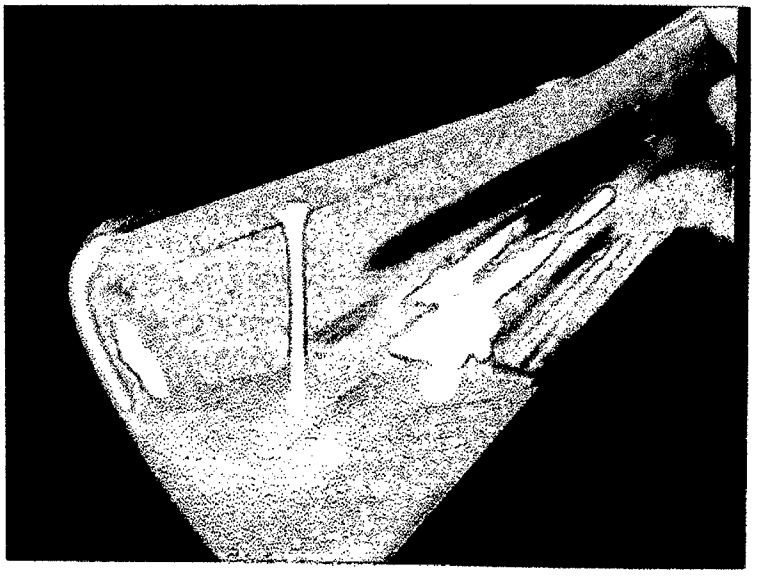


Plate 6.2.2.

Viscosity of culture broth of *B. alkalophilus* SB-D under unoptimized conditions.

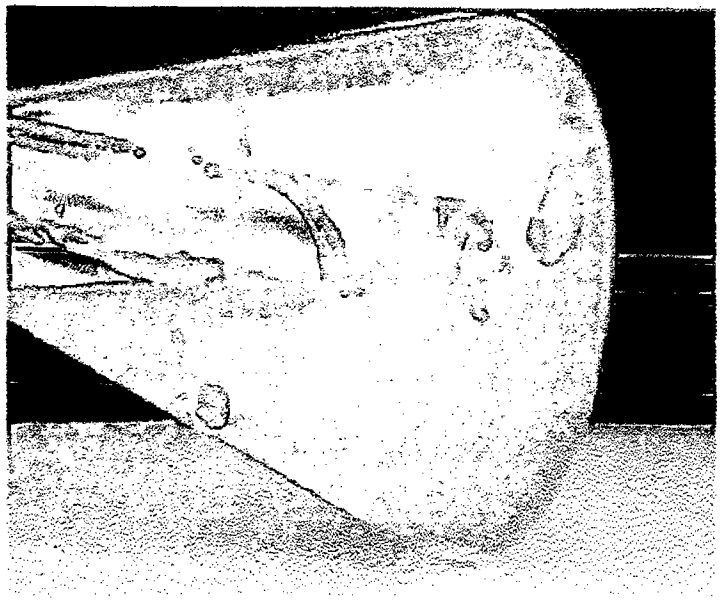


Plate 6.2.3.

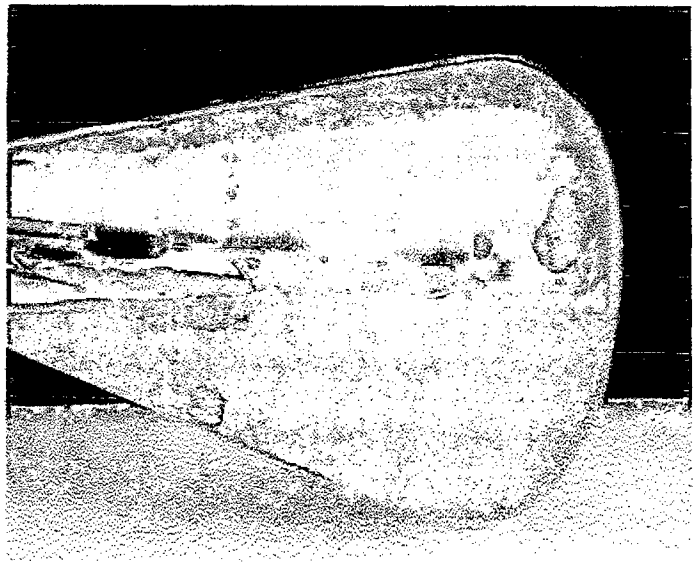


Plate 6.2.4.

Viscosity of culture broth of *B. alkalophilus* SB-D under optimized conditions.

Further, the effect of the growth conditions on cell mass, pH, viscosity and total yield of the polymer was monitored. From Fig. 6.2 it was observed that the biomass rose rapidly in condition II grown cells and reached a peak after 16 hours in Horikoshi I and 20 h in Horikoshi II medium. This yield in biomass is comparable to that of *Bacillus subtilis* at pH 7. This indicates that the obligate alkaliphile SB-D is not simply oxidising large amounts of substrate in order to grow at very high pH but is utilizing the substrates as efficiently as comparable aerobes at conventional pH values. An interesting observation made in condition III of Horikoshi II medium was a peculiar rise of cell mass to 18 gms/ltr at 20 + 4 hours of incubation. Further, it was interesting to note that the maximum viscosity was obtained during the third condition in Horikoshi I medium when the 16 hour old culture was kept under stationary condition for 4 hours (Fig.6.3). This was also confirmed with the yield of the polymer G1 showing highest in this particular condition in Horikoshi I medium (Fig. 6.4). Significant change in pH was also noted in this condition where there was a reduction of pH from 10.3 to 9.0 (Fig.6.5).

Although, the holding time for release of the polymer was observed to be 4 hours, the effect of different time intervals was monitored to standardise the conditions for isolation of the polymer. As seen in Table 6.2, it was noted that although the cell mass increased to 20 gm/l after 2 hours, the yield of the polymer was better after 4 hours in Horikoshi I medium. However, in Horikoshi II medium, no significant difference was seen with 2 and 4 hours.

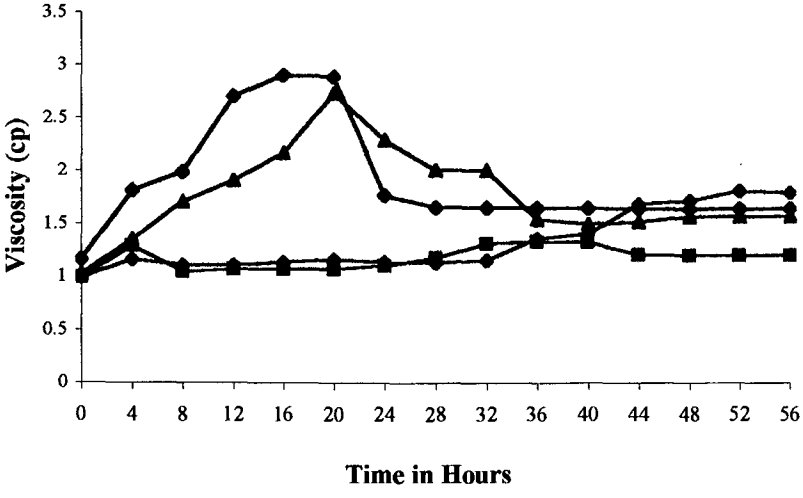


Fig. 6.3.

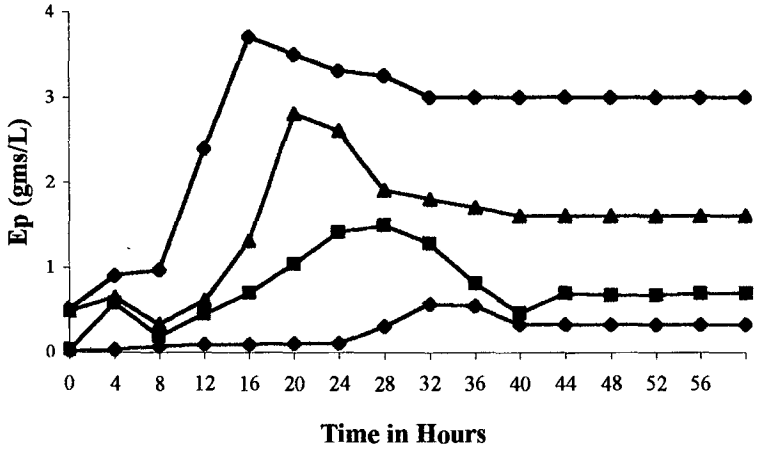


Fig. 6.4

- Stationary condition in Horikoshi II medium
- Shaker condition in Horikoshi II medium
- Shaker for specfic time interval + static for 4 Hours in Horikoshi II medium
- Shaker for specfic time interval + static for 4 Hours in Horikoshi I medium

Effect of static, shaker & shaker + static conditions on:
Fig 6.3 Viscosity during growth of SB-D.
Fig 6.4 Yield of EP (G1) during growth of SB-D.

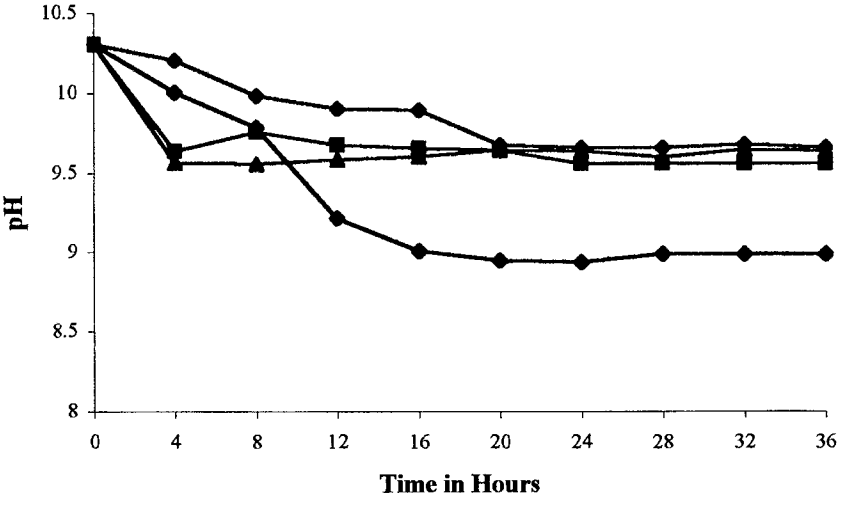


Fig. 6.5 Effect of static, shaker & shaker + static conditions on change in pH during growth of SB-D.

Table 6.2. Effect of holding time of static conditions on cell mass, pH change, viscosity & Polymer production before optimisation.

Hours	Cell mass (gms/L)	pH	Viscosity (cp)	EP G1 (gms/L)
Horikoshi I				
16+0	12.5	8.9	1.1	0.5
16+2	20	8.76	1.7	2
16+4	11	8.58	2.9	3.7
16+6	10	8.69	2.7	3
16+8	10	8.78	2.7	3
Horikoshi II				
20+0	12	9.64	1.1	0.4
20+2	22	9.5	2.64	3.1
20+4	18	9.57	2.73	2.8
20+6	12	9.48	2.65	2.8
20+8	12	9.48	2.65	2.8

For the isolation and purification of the polymer, it was felt that Horikoshi I medium was giving a better yield. Besides, when the dissolved polymer from Horikoshi II medium when put on starch agar medium showed starch hydrolysing activity as seen from zones in plate 6.3. It was therefore confirmed that the precipitate from Horikoshi II medium is also associated with the amylase enzyme, which may interfere during purification. Therefore, further studies for the optimisation were done with Horikoshi I medium.

Culture broth supernatants and pelleted cells before the addition of isopropanol also depicted strong water in oil emulsification activity (Table 6.8) (Plates 6.4.1 & 6.4.2) observed with only 16+4 hour supernatant, indicating that this activity is associated with the release of the polymer. However, the emulsification activity was almost similar with the cells of 16 hours and 16+4 hours. There was a decrease in the emulsification activity of PBS washed cells indicating the release of the polymer by saline. Rapid drop collapsing test (168) for biosurfactant producing organisms was also performed by placing a drop of viscous supernatant on a glass slide containing a drop of oil followed by mixing of the contents, which showed the collapsing of the drop of oil.

Microorganisms generally produce surface active compounds also known as surfactants or emulsifiers when grown on hydrocarbons to facilitate the uptake of insoluble substrates (79,146,147,308). These emulsifiers are found to be complex lipids or polymeric compounds. However, there have been several examples of the

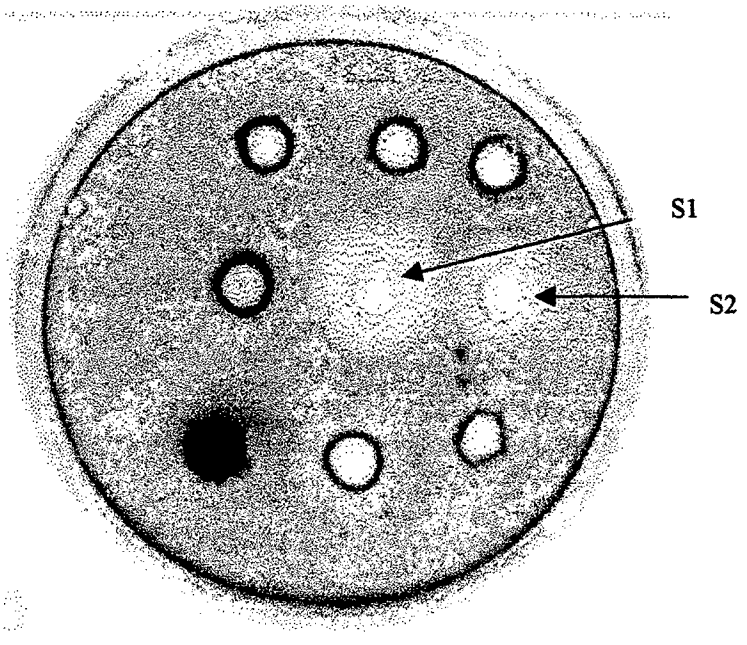
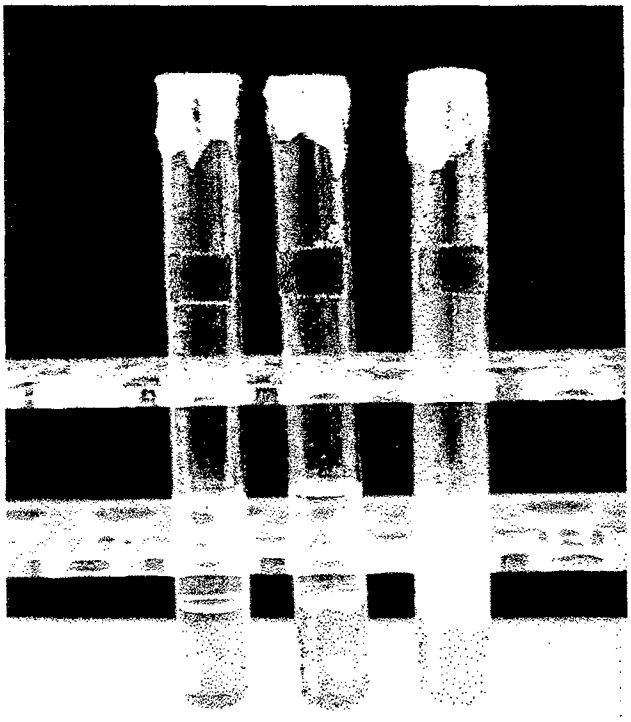


Plate 6.3. Zones of S1 and S2 on starch agar indicating amylase activity

S1-EP obtained from Horikoshi II medium.
S2-Needle like crystals obtained from Horikoshi II medium



1 2 3

Plate 6.4.1. Emulsification activity of culture supernatant of *B. alkalophilus* SB-D

1. Control.
2. 16 h supernatant.
3. 16 + 4 h supernatant.

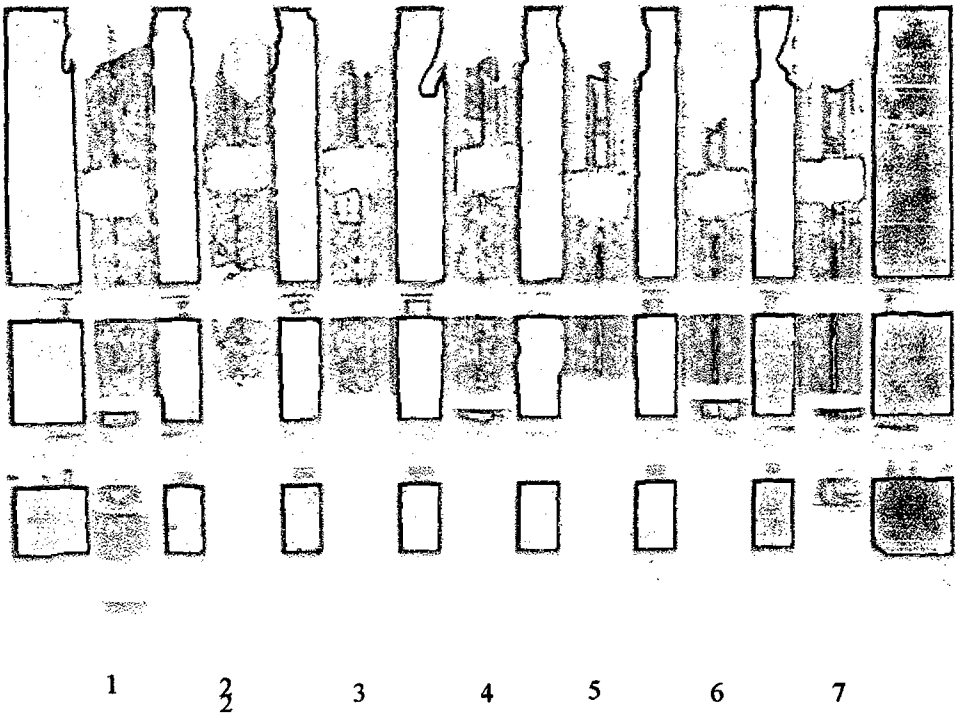


Plate 6.4.2.

Emulsification activity of cells of *Bacillus alkalophilus* SB-D

1. Control.
2. 16 h cells of *B. alkalophilus* SB-D.
3. 16 + 4 h cells of *B. alkalophilus* SB-D.
4. PBS treated cells of *B. alkalophilus* SB-D.
5. *Sphingomonas* cells.
6. *Bacillus* sp.– Alkaliphile.
7. *Natronococcus* cells.

production of these compounds during growth of the organism in water soluble substances such as glucose, sucrose, glycerol, ethanol etc. (53,100,259).

It has been reported that the massive release of the exopolymer into the liquid medium makes the culture broth progressively more viscous (74) and can be recovered by various methods (Table 2.4.C). Further, the organisms may synthesise and secrete the polymer continuously during the growth phase, and is considered as a primary metabolite (30,59,352), while other organisms produce polymer in stationary phase, considered as a secondary metabolite (288,347). Their appearance in the culture broth is described as resulting from a "sloughing off" process from the cell surface. Further, the polymer may be firmly or loosely associated with the cell surface (74) and can be isolated as per Scheme No. 1, pg. No. 58. Isolate SB-D could produce the EP even on natural source of carbon such as rice gruel. EP production using industrial waste products such as cheese whey has been reported (24).

Results from earlier studies have shown that a number of physiological conditions including concentration and source of carbon and nitrogen, temperature and aeration influence exopolymer production in vivo (124,288,330,378,391). Hence the factors controlling the production of the polymer at optimum growth pH and temperature were studied.

Although the cell mass at the agitation rate of 200 rpm was found to be maximum with 16 hours of incubation and 2 hours of static conditions (Fig.6.6), maximum viscosity, and amount of the polymer was released when the culture was agitated at 200 rpm and held at stationary conditions for 4 hours (Figs. 6.7 & 6.8). This reflects that the

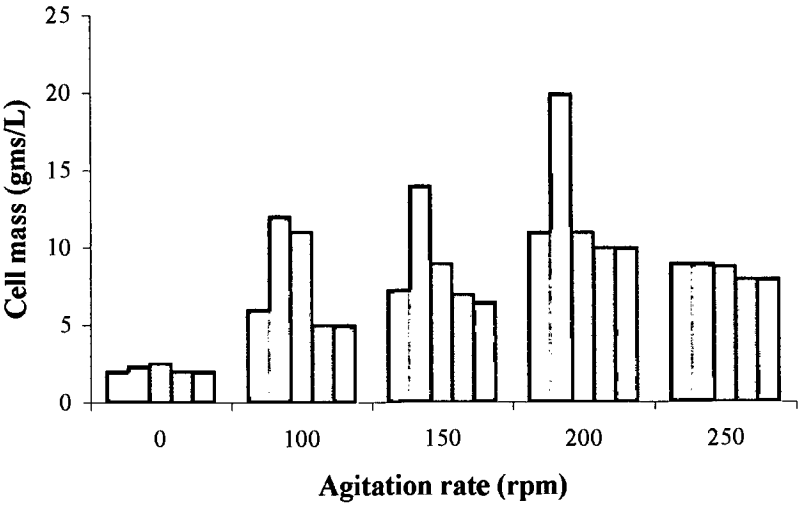


Fig.6.6

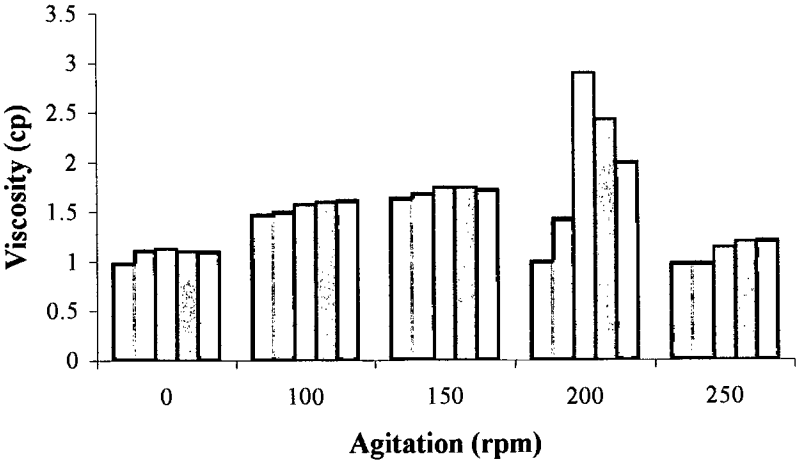
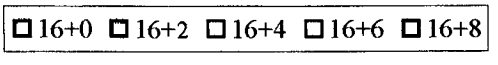


Fig 6.7



Effect of agitation rate and incubation at different time intervals under static conditions on:

Fig. 6.6 Cell mass.

Fig. 6.7 Viscosity

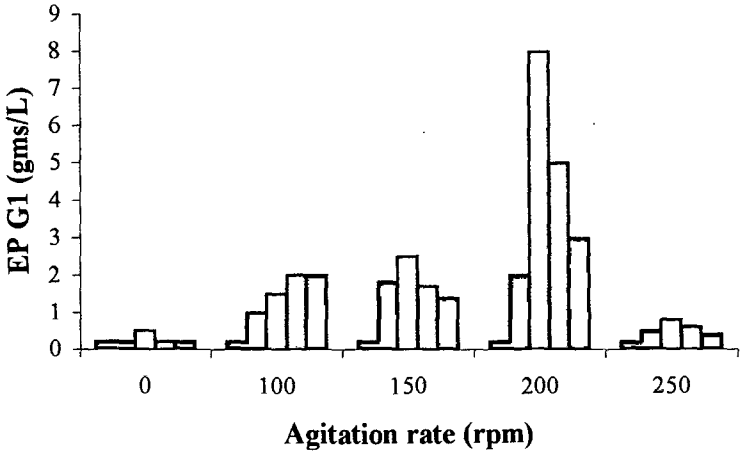


Fig 6.8.

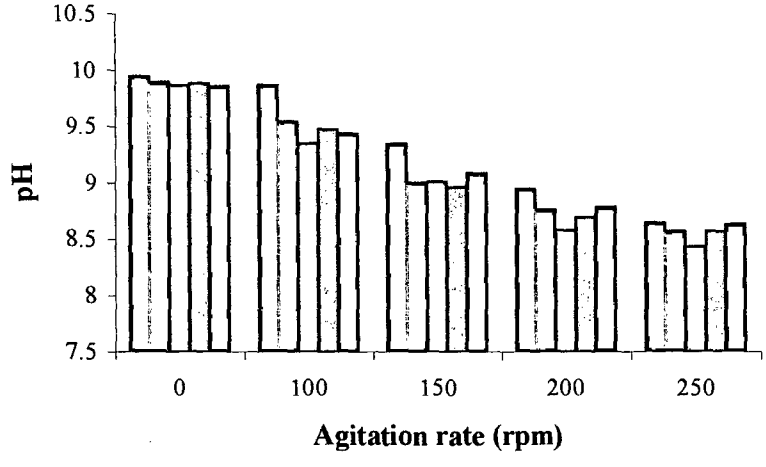
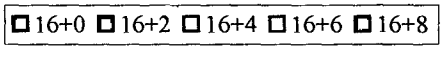


Fig.6.9.



Effect of agitation rate and incubation at different time intervals under static conditions on:

Fig. 6.8 Yield of EP G1.

Fig. 6.9 pH.

polymer is associated with the cells at 16 +2 hours resulting in the increase of cell mass which is subsequently released into the extra cellular medium showing an increase in viscosity and thereby giving better yield. The cell mass of 16 + 2 h when washed with PBS has also shown the presence of EP in the extracellular washings (Table 6.8). The increase in agitation rate showed a significant effect on the pH of the medium (Fig,6.9).

The alkaliphiles are known to maintain the external pH, which is optimum for their growth. During the growth of this culture, the pH was found to reduce from 10.3 to 9.7 within 8 hours. This decrease was associated with the increase in cell mass of 8.3 gm/L and production of needle like crystals in the medium, the yield of which was 9.5 gms/L at 8 hours, increasing to 14 gms at 12 hour and subsequently decreasing after 16 hours to 8 gms at which time the culture is entering the stationary phase. Thus, increase in the weight of needle like crystals were in relation with the change in pH and the exponential growth phase. The decrease in pH appears to be associated with the production of these crystals, designated as G2 in the study (Fig.6.10) (Plate 4.6 ChapterIV).

Further studies on the effect of flask and inoculum size and nutritional conditions showed that the the best production of EP G1 under optimum conditions was when:

(1) The ratio of flask size to medium volume is maintained at 1:2.5. (Fig. 6.11) This also gave the best viscosity and higher cell mass.

(2) The inoculum size was 4 % (Fig. 6.12).

(3) Substrate concentration in terms of glucose was 3 % (Fig 6.13).

(4) Yeast extract concn. in the medium was 0.1 % (Fig. 6.14).

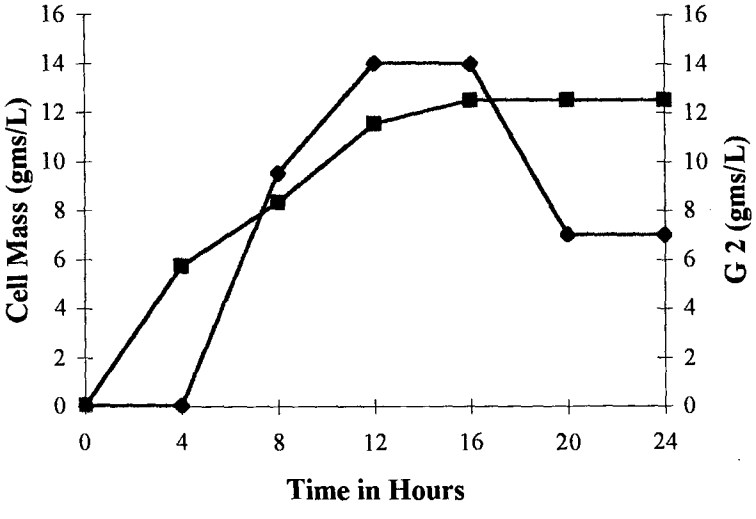


Fig 6.10 Relationship of incubation period, cell mass production & appearence of G2 in SB-D.

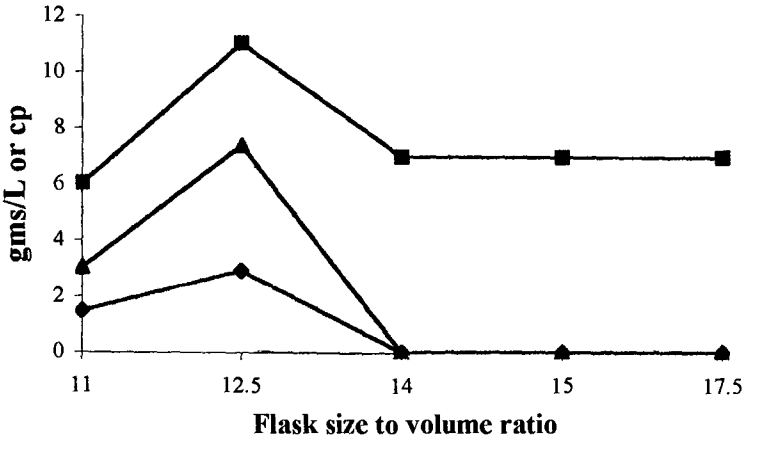


Fig 6.11

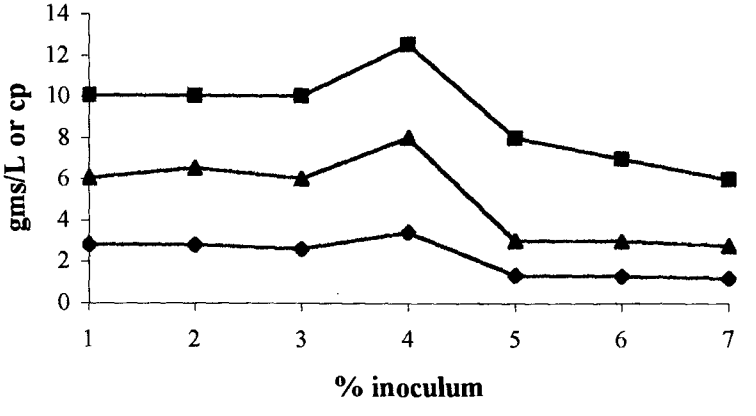


Fig 6.12

Viscosity (cp) — Cell mass (gms/L) — G 1 (gms/L)

Effect of :

Fig 6.11 - flask size to volume ratio

Fig 6.12 - inoculum size

on cell mass, viscosity & G1 production

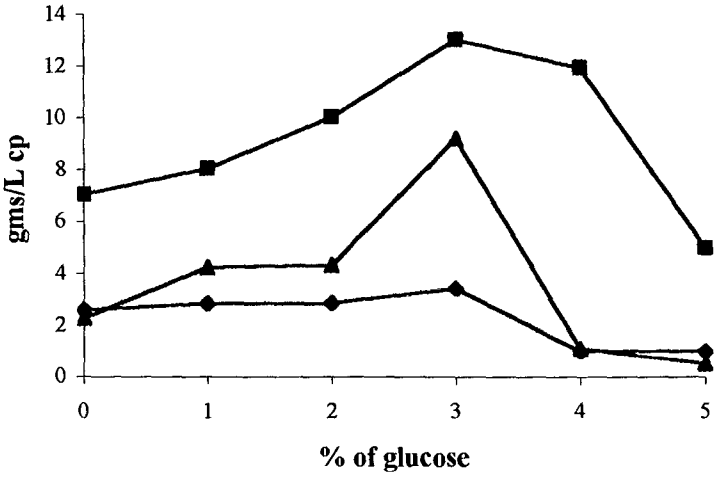
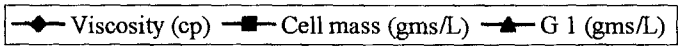


Fig.6.13.



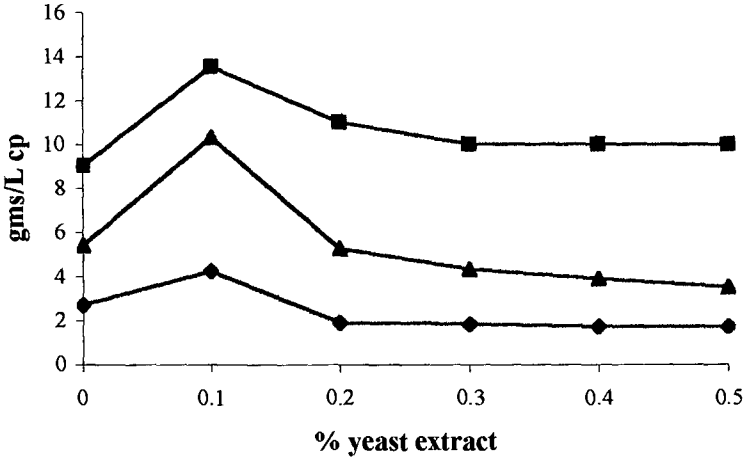
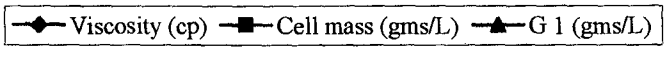


Fig 6.14.



Effect of :

Fig.6.13 - glucose concn.

Fig.6.14 - yeast extract concn.

on cell mass, viscosity & G 1 production.

For many bacteria, well aerated conditions with 2% inoculum size (378) and a glucose concentration of 1-4% (w/v) yields the greatest exopolymer production. 4% glucose concentration yielding highest xanthan titres (30 g/kg culture broth) has been reported (337) while 5% sucrose was found to be essential for EP production by *Azotobacter* (378).

For the isolation and purification of the polymer, culture was grown under optimised conditions as listed in Table 6.3. The isolation carried out as per the flow chart (Scheme 2 Page No. 163) resulted in the polymer G1 of 10.3 g/ltr wet weight and 5.6 dry weight and the needle like crystals (G2) of 14 g/ltr wet weight and 13.2 g/ltr dry weight. It was observed that the yield of G1 as spoolable precipitate was better under optimised conditions giving an increase of 2.78 fold (Table 6.4). However the increase in yield of G2 was only by 1.7 fold. Further, the yield of the spoolable and cell associated polymer was found to be 10.3 g/ltr and 1.8 gms/L respectively. (Table 6.5) The yield of exopolymers as reported ranges from 400 mg (244), 3 g (6), 1.7 g (64), 12 g (276) and 30 g (337).

The Physico-chemical analysis of EP G1 and G2 was carried out with the lyophilised sample. Both the polymers were found to be soluble in HCl and possessed melting point above 200 (Table 6.6).

G2 contained 56.67 % of organic content with 187.43 µgms/mg of proteins with no lipids. (Table 6.7) The inorganic content was found to be 43.36% that may be due to the presence of higher content of phosphates in the polymer. In contrast, G1 contained 76% of organic content with 212 µgms/mg of carbohydrates and 130

Table 6.3 :Optimised conditions for maximum GI production by *B.alkalophilus* SB-D.

Parameter	Horikoshi I
Incubation period	16 h of Shaker and 4 h of static conditions.
pH	10.3
Temperature	25°C
Agitation rate	200 rpm
Inoculum density	4%
Flask size: Volume ratio	2.5:1
Glucose	3%
Peptone	0.5%
Yeast extract	0.1%

Table 6.4 :Comparison of Polymer Production in Horikoshi I medium by SB -D under optimised and unoptimised conditions.

Polymer	Yield (gms/L) Condition		FOLD INCREASE
	Unoptimised	Optimised	
G 1	3.7	10.3	2.78
G 2	8.2	14	1.7

Table 6.5 :Yield of soluble & cell associated polymer (G1) in Horikoshi I medium on optimisation.

Yield (gms/L)	
Soluble[a] G 1	Cell associated[b] G 1
10.3	1.8

a. G1 recovered from viscous culture supernatant.

b. G1 recovered from cell pellet on PBS extraction.

Table 6.6 : Physical characteristics of G 1 and G2

Parameter	G 1	G 2
Wet weight (gms/L)	10.3	14
Dry weight (gms/L)	5.6	13.2
Colour	White	White
pH	Alkaline	Alkaline
Solubility	1 N HCl	1 N HCl
Melting point	> 200	> 200

Table 6.7 : Chemical analysis of G 1 and G2

Component analysed	Method used	Concentration (µgms/mg)	
		G 1	G 2
Inorganic content	Ashing	23.38	43.36
Organic content	Ashing	76.62	56.67
Carbon content	NCS Analyser	91.385	89.279
Nitrogen content	NCS Analyser	8.615	10.721
Total sugars	Phenol sulphuric acid	212.4	27.94
Glucose	Glucose oxidase	110	20
Proteins	Bicinochoninic acid	86.16	187.43
Lipids	Acid Dichromate	130	-
Uronic acids	Carbazole	92.56	20.57
	Metahydroxybiphenyl	90.36	18.28
Methyl pentoses	Cysteine Hydrochloride	75.78	19.79
Inorganic Phosphate	Ammonium Molybdate	50	80.34
Organic acids	Dinitrophenylhydrazine	40.02	51.5
Sulphates	Barium-chloride gelatin	13.18	9.28
Phenols	Folin-Ciocalteau	108	65

μgms/mg of lipids. The compound also contained uronic acids, pentoses, phosphates and pyruvate. The inorganic content was found to be 23.38 % (Table 6.7). The product appears to be complex with the major components being carbohydrates and lipids and therefore a glycolipid complex molecule. The confirmation that the molecule is a glycolipid was obtained with TLC and visualising with α-naphthol which gave a purple colored spot with Rf value of 0.89. Such complex polymers with carbohydrate and lipid component have been reported (26,146,167,405).

On hydrolysis and chromatographic analysis, G 1 was found to have glucose with Rf value of 0.54 as detected by TLC. The GC analysis of the alditol acetates of the components of G1 showed the presence of glucose and manose in equal proportion (Fig. 6.16) while G2 showed the presence of minute quantities of glucose and manose (Fig. 6.17).

Emulsification, metal adsorption and adhesive property of G1 and G2 was tested. It was observed that G2 possessed none of the properties. It was also interesting to note that although the viscous supernatant of SB-D culture possessed strong emulsification property (Plate 6.4.1 & 6.4.2), G1 EP when removed by the glass rod on addition of isopropanol resulted in the loss of emulsification activity of the supernatant as well as the precipitated polymer (Table 6.8). The low activity of the polymer but high activity of the supernatant appears to indicate the requirement of water soluble component for optimum activity as also reported (64,178).

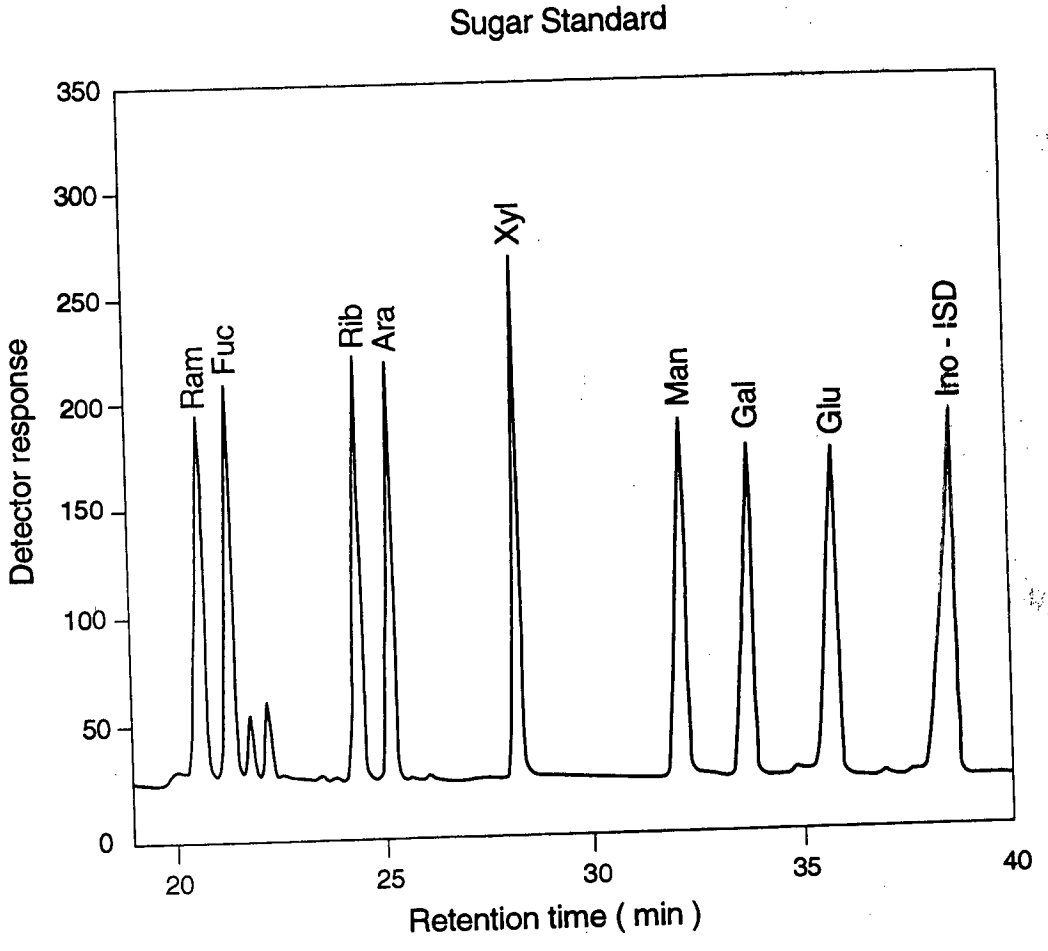


Fig. 6.15 Gas chromatogram of standard sugars.

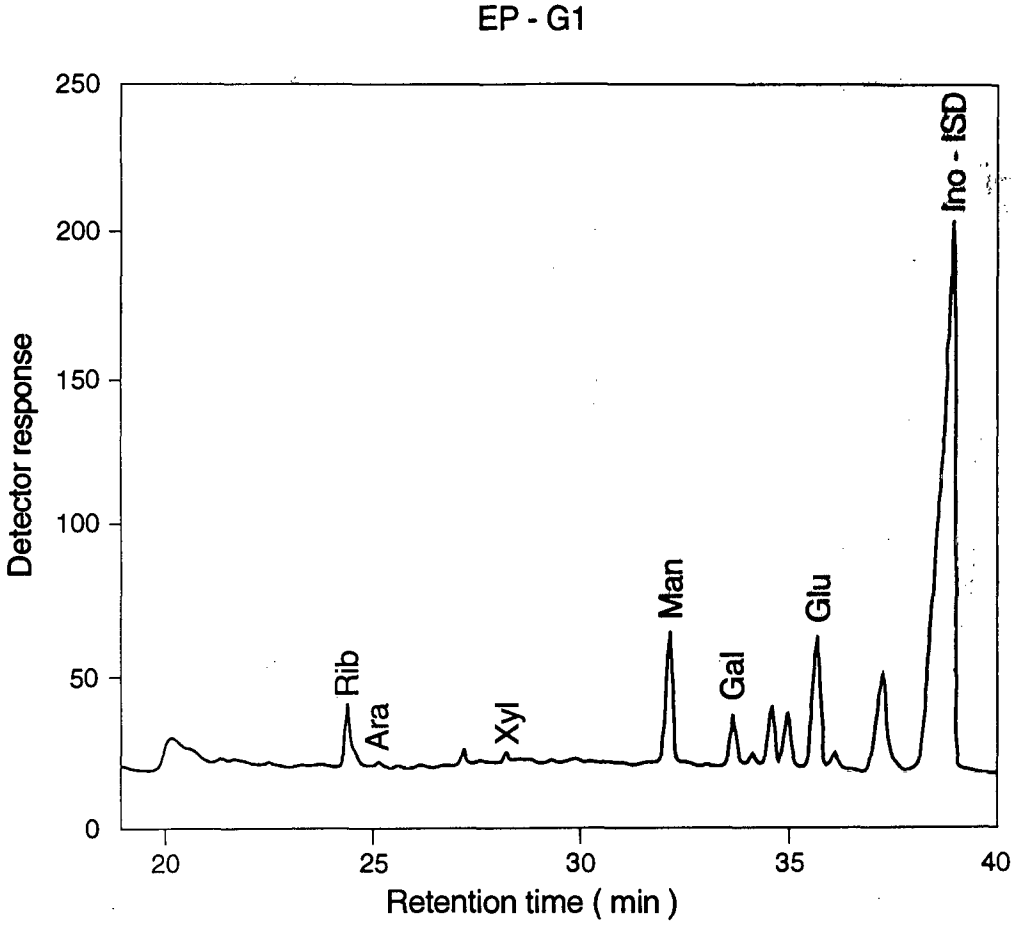


Fig. 6.16 Gas chromatogram of alditol acetate derivatives of hydrolysed EP (G1) of *B.alkalophilus* SB-D.

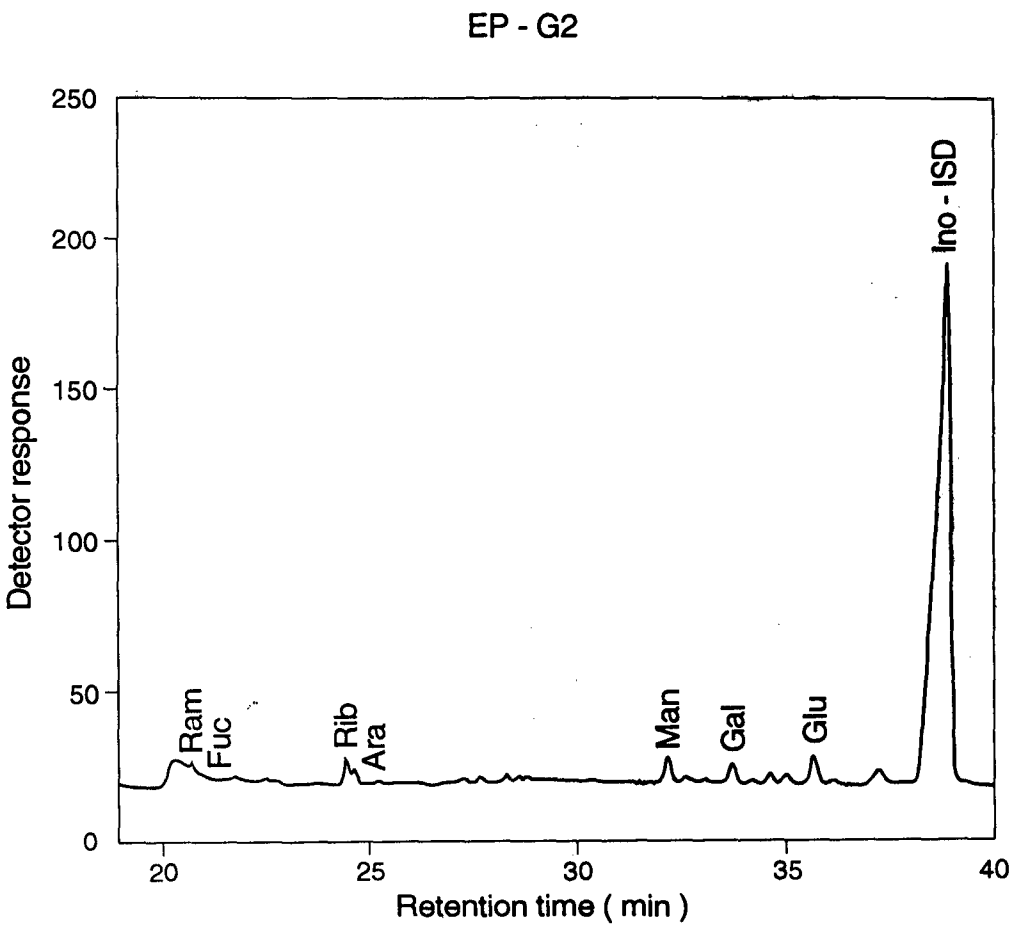


Fig. 6.17 Gas chromatogram of alditol acetate derivatives of hydrolysed EP (G2) of *B.alkalophilus* SB-D.

Table 6.8 : Emulsifying activity of supernatant, cells, G1 & G2 of SB-D culture in Horikoshi I medium.

Sample	Emulsifying activity
Control	0.08
16 h supernatant	0.221
16+4 h supernatant	2.022
16 h cells (O.D. 0.54)	2.962
16+4 h cells (O.D. 0.54)	2.872
16+4 h PBS washed cells (O.D. 0.54)	1.665
PBS washing	0.749
G 1	0.363
G 2	0.242
**Sphingomonas*	2.824
**Bacillus* (Alkaliphile)	0.234
**Natronococcus* (Haloalkaliphile)	0.245

Note: * Used as control.

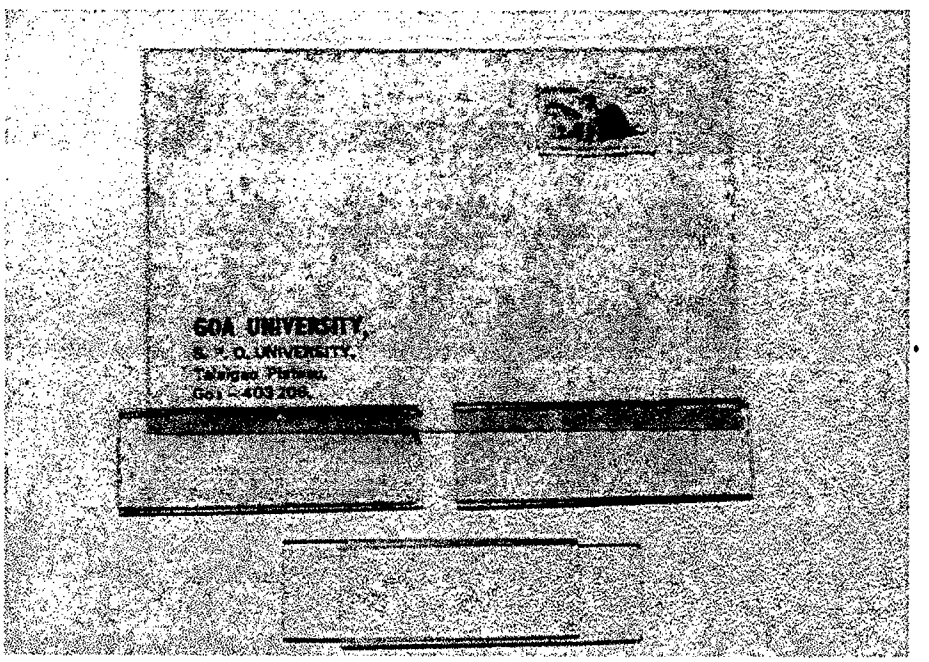


Plate 6.5. Adhesive property of viscous exopolymer G1

G1 possessed strong metal binding affinity towards copper, as reported for EP (90,105,177,249,255,323.) The most significant feature of EP G1 was that this polymer possessed strong adhesive property, which could be demonstrated by applying on glass plates and paper as seen in plate 6.5. Such adhesive polymers of microbial origin have also been reported (29,230,265-268,303). Since SB-D has maintained its ability to release the polymeric material into the medium even after seven years of repeated culture in Horikoshi medium, it may therefore be regarded as a steady source of a potentially useful biopolymer.

The salient features of this study are:

1. ***B.alkalophilus* SB-D produced a viscous EP under 16 h of shaker and 4 h of static incubation conditions.**
2. **EP G1 was separable as a spoolable material on a bent glass rod on addition of isopropanol, thereby resulting in the loss of viscosity of culture broth.**
3. **Culture broth and cells of SB-D isolate showed strong emulsification property.**
4. **Yield of G1 could be increased to as high as 10.3 g/ltr on optimization.**
5. **G1 was found to be a complex EP with carbohydrates and a lipid moiety.**
6. **G1 possessed strong metal adsorption and adhesive property for glass and paper and can therefore be used as a water-soluble microbial gum.**

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CHAPTER VII

Summary & Future Prospects.

SECTION A – SUMMARY

Studies on alkaliphiles-an industrially significant group of extremophiles were undertaken during the course of this work realizing their immense potential in various areas of research and industry.

A large number of ecological niches ranging from non-alkaline to natural and manmade alkaline environments were sampled for the isolation of alkaliphiles. All the samples, including acidic samples, were found to yield the presence of alkaliphilic bacteria. Isolates obtained belonged to the Gram-positive group with diverse variations in cultural and morphological features. These isolates were found to have potential alkaline enzyme activity on varied substrates like cellulose, starch, casein and tributyrin.

Two of the organisms which showed prominent amylase production were selected for further studies and characterised as *Bacillus alkalophilus* SB-D and *Bacillus coagulans* SB-W nov. var. *thermophilus.* These isolates were found to be obligate and facultative alkaliphiles respectively with SB-W growing at high pH as well as high temperature of 55°C. Both the isolates had their growth optimum at pH 10.3, requiring the presence of sodium ions when grown at this pH. Their cytoplasmic pH was found to be 2 pH units less as compared to external pH, which showed no significant change during growth. The isolates possessed dramatically high cytoplasmic buffering capacities, absent in neutrophilic cultures. Permeabilisation with Triton X-100 was found to solubilise and extract proteins as indicated by chemical analysis and

SDS-PAGE profiles. Scanning electron micrograph of the isolate SB-D revealed the release of a fibrous polymer after Triton treatment.

The amylase(s) produced by the isolates SB-D and SB-W were purified and found to be α-amylase group of enzymes. The starch water obtained from rice (gruel) was an economical and easily available substrate for the growth of these organisms. α-Amylase produced by SB-W was found to be stable at high temperature as high as 65°C and active in presence of organic solvents. α-Amylase(s) from both the isolates were found to be calcium independent retaining more than 50% activity in the presence of additives such as Tween 80, SDS and EDTA and hence posses potential applications as detergent additives.

The culture SB-D was found to produce a viscous exopolymer under shaker and static conditions. The polymer was spooled on a glass rod and identified to be a complex polymer, comprising of carbohydrates such as glucose, mannose and lipids. Yield of the spoolable polymer after optimisation was found to be 10.3 gms/L. The polymer had metal adsorption and adhesive property. It could be used as a water-soluble gum for sticking paper and glass.

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SECTION B - FUTURE PROSPECTS

From the results of the present study, it can be conclusively inferred that the alkaliphiles are a diverse group of organisms present in varied econiches. They exhibit an interesting phenomenon of maintaining their internal pH more than 2 units less than the external pH and possess high cytoplasmic buffering capacity. Besides, they are a potential source of several enzymes not only active at pH 10.3 but also other harsh conditions such as high temperature, presence of organic solvents and detergent additives, therefore having wide industrial applications. They are also a versatile source of other new metabolites / bioactive compounds.

Hence future research on this group of organisms may be directed towards:

1) **Role of membrane proteins/components in the mechanism of pH tolerance.**

2) **Studies on hydrolytic extracellular enzymes such as cellulase, amylase, protease, lipase, xylanase, etc. of industrial application for potential use in organic matter degradation and bioremediation of toxic effluents.**

3) **To study the biopolymer obtained from the *Bacillus alkalophilus* SB-D, improvise its production, adhesive and metal adsorption property for industrial exploitations.**

Thus, alkaliphiles, by virtue of several unique characteristics, hold immense potential for the production of the enzymes active at alkaline pH with wide applications in various industries. Since alkaliphilic bacterial strains are more tolerant to toxic compounds and have better bioavailability, this can improve the efficiency of biodegradation in control and removal of pollutants and in waste treatment systems.

The five major extreme groups of microorganisms i.e. **Thermophiles, Halophiles, Acidophiles, Barophiles and Alkaliphiles** have been rightly termed by Horikoshi in 1991, as **Super Bugs** on account of their potentiality. Basic research, directed towards isolation of extremophiles producing unique extremozymes and bioactive products need to be continued, strengthened, and pursued with renewed scientific vigor so that their potentials are revealed and exploited.

****

CHAPTER VIII

Appendices.

APPENDIX – A

Media composition:

A.1. Mineral medium (MM) (Double Strength)

a. $FeSO_4.7H_2O$	60mg, Dissolved in 250ml d/w
b. Stock solutions added as follows:	
K_2HPO_4(12.6%)	50ml
KH_2PO_4 (18.20%)	10 ml
NH_4NO_3(10%)	10 ml
$MgSO_4$(1%)	10 ml
$Na_2MoO_4.2H_2O$(0.6%)	0.1 ml
$MnSO_4.H_2O$(0.6%)	0.1 ml
$CaCl_2.2H_2O$(1%)	7.5 ml

Added $CaCl_2$ drop by drop and stirred.

Made volume to 500ml with d/w.

Stored the medium in brown bottle.

Sterilized the medium in pressure cooker for 10 minutes.

pH of the medium adjusted to 10.3 using 10% Na_2CO_3 solution, sterilised separately.

Supplemented the medium with 0.05% yeast extract when the cells were grown at pH 10.3 with 1% glucose or starch as carbon source.

A.2. Basal media for Alkaliphilic Microorganisms (157).

Ingredients	Horikoshi - I	Horikoshi -II
	g/L	g/L
Glucose	10	-
Starch	-	10
Poly peptone	5	5
Yeast extract	5	5
KH_2PO_4	1	1
$MgSO_4.7H_2O$	0.2	0.2
Na_2CO_3	10	10
Agar	20	20
d/w	1000ml	1000ml
Final pH	10.3	10.3

A.3. Polypeptone Yeast Extract Glucose Agar (PPYG) (106).

Ingredients	g/L
Peptone	5
Yeast extract	1.5
Glucose	5
$Na_2HPO_4.12H_2O$	1.5
NaCl	1.5
$MgCl_2.6H_2O$	0.1
Na_2CO_3	5.03
Agar	15
Final pH	10.3

Solutions of glucose, starch and Na_2CO_3 sterilised separately as 10% solutions and then added to the cold basal molten medium to avoid precipitation of salts.

A.4. Media used for enzyme activities for Alkaliphiles (157).

Ingredients g/L	**Protease**	**Amylase**	**Cellulase**	**Lipase**
Soluble starch	-	10	-	-
CMC	-	-	10	-
Tributyrin/Tween 80	-	-	-	10
Skimmed milk	20	-	-	-
Polypeptone	-	5	5	5
Yeast extract	-	5	5	5
KH_2PO_4	1	1	1	1
$MgSO_4.7H_2O$	0.2	0.2	0.2	0.2
Na_2CO_3	10	10	10	10
Agar	20	20	20	20
d/w	1L	1L	1L	1L
Final pH	10.3	10.3	10.3	10.3

10% Na_2CO_3 and 10% carbon source sterilised separately and added just before pouring the medium as plates.

For cellulase activity, the plates were stained for 15 min by flooding with 0.1% aqueous solution of congo red. Unbound dye molecules were removed by reflooding the plates with 1M NaCl. A clear zone surrounding the colony indicated the production of Carboxy Methyl Cellulase by the test strain.

A.5. SB medium for Amylase activity

Ingredients	g/L
*Rice gruel	1L
Agar	20g
Na_2CO_3	10g

* Rice was cooked in water, filtered and the filtrate was used as a source of starch.

A.6. Medium for Bacterial Lipases by plate assay method (212,314).

Ingredients	g/L
Nutrient broth	8
NaCl	4
Agar	10
Na_2CO_3	10
Tween 80	25ml
Rhodamine B	10 ml of 0.001% (w/v) of Rhodamine B solution.
Final pH	10.3

Plates after growth were exposed to U.V light.

A.7. Basal medium for Haloalkaliphiles (363).

Ingredients	g/L	g/L
Polypeptone	1	5
Tryptone	-	1
Tri sodium citrate	0.3	0.3
KH_2PO_4	-	13.7
K_2HPO_4	-	0.05
$MgSO_4$	2.0	-
KCl	0.2	-
Na_2CO_3	5	5
NaCl	25	25
Agar	20	20
Final pH	10.3	10.3

10% Na_2CO_3 and 25% NaCl were separately autoclaved and added.

25% NaCl added to PPYG for isolation of Haloalkaliphiles.

A.8 Effect of sodium carbonate concentration on pH of Horikoshi medium:

Concn. of Na_2CO_3 %	pH of Horikoshi medium
0	7.16
0.05	8.01
0.1	8.91
0.2	9.51
0.3	9.93
0.5	10.20
1.0	10.3
1.5	10.3
2.0	10.3

Appendix B

Composition of stains and buffers.

B.1. Stains

Gram stain reagents

Crystal violet
Solution A - 2g of crystal violet dissolved on 20ml ethanol.
Solution B - 0.8g ammonium oxalate dissolved in 80ml d/w.
Mixed solution A and B and filtered through Whatman paper No.1.

Gram's iodine
Dissolved 1g iodine and 2g potassium iodide in 300 ml d/w. Filtered through Whatman filter paper N0.1 (diameter =12.5cm).

Safranin
2.5g of Safranin *O* dissolved in 10ml ethanol made the volume to 100ml with d/w and filtered through Whatman filter paper No.1.

Procedure: Prepared smear of the organism on a slide and heat fixed it. Flooded the smear with crystal violet for a min. Washed with tap water and flooded with gram's iodine for a min. Washed with tap water and decolorized with 60% ethanol prepared in d/w. Counter stained with safranin for 45 seconds. Washed with tap water, blot dried with tissue paper and examined under oil immersion.

2. Endospore staining [Schaeffer and Fulton's method]:

Malachite green solution:
Dissolved 5g of malachite green in 100ml d/w. Filtered through Whatman filter paper No.1.

Safranine:
Dissolved 1g of Safranine in 100ml d/w. Filtered through Whatman filter paper No.1.

Procedure: Prepared smear of the organism and heat fixed it. Flooded the smear with 5% malachite green solution. Held the slide over a boiling water bath for 15 min to allow the penetration of the stain through calcium dipicolinate of spores. Washed with tap water and counterstained with safranine for 2min. washed with tap water and blot dried with tissue paper and examined under oil immersion objective.

Endospores appeared green with cells colored red.

Capsule staining

1. Negative staining using India Ink

Procedure: Prepared a smear of the culture in a drop of India Ink by using another slide so as to prepare a thin film. Air dried the smear and examined under oil immersion objective.

Background stained dark blue with colorless haloes around capsule producing bacteria.

2. Alcian blue method

0.02% alcian blue solution (pH 2.5)

0.02g of alcian blue 8X (Sigma Co.) dissolved in 0.06% of glacial acetic acid

Procedure: Heat fixed smear of the organism flooded with 0.02% alcian blue solution for 15 seconds. Counterstained with safranine for one second. Blot dried and examined under oil immersion objective/phase contrast.

Green colored haloes observed around capsule producing bacteria.

1.Congo red staining (137):

Saturated congo red solution: Dissolved congo red in 10% (v/v) Tween 80 solution to obtain saturation.

Ziehl carbol fuchsin: Dissolved 1.2g of basic carbol fuchsin in 100ml of 95% alcohol and mixed well until the dye completely dissolved.

Procedure: Heat fixed smear of the organism flooded with saturated congo red solution for 2min. Washed the slide with tap water and further stained with carbol fuchsin for 2min. Washed with tap water, blot dried and examined under oil immersion objective.

Red colored haloes seen around the capsule producing bacteria and the cells stained pink.

B.2. Buffers (173,290,312)

(i) Citrate - phosphate buffer (0.05M).

Solution A: (0.05 M citric acid): Dissolved 10.2g of citric acid in 1000ml d/w.

Solution B: (0.05 M dibasic hydrogen phosphate): 7.1g Na_2HPO_4 dissolved in 1000ml d/w.

Xml of A+Yml of B mixed to obtain buffers of the desired pH

X	Y	pH
85.0	15.0	2.0
79.0	21.0	3.0
61.4	38.6	4.0
24.3	25.7	5.0

(ii) Phosphate buffer (0.05M).

Solution A: (0.05M monobasic hydrogen phosphate): 6.0g of NaH_2PO_4 dissolved in 1000ml d/w.

Solution B: (0.05M dibasic hydrogen phosphate): 7.1g of Na_2HPO_4 dissolved in 1000ml d/w.

Xml of A+Yml of B mixed to obtain buffers of the desired pH

X	Y	pH
87.7	12.3	6.0
39.0	61.0	7.0
5.3	94.7	8.0

(iii) Tris-HCl buffer (0.05M).

Solution A: (0.05 M Tris): 6.0g of Tris dissolved in 1000ml d/w.
Solution B: (0.05M HCl): 4.4ml of 11.35 N HCl added to 1000ml d/w.

70ml of A and 30ml of B mixed to obtain buffer of pH 9.0.

(iv) Glycine-NaOH (0.05 M)

Solution A (0.05 M Glycine): 3.75g of glycine dissolved in 1000ml d/w.

Solution B (0.05 M NaOH): 2.0g of NaOH added to 1000ml d/w

62ml of A and 38ml of B mixed to obtain buffer of pH 10.0.

(v) Phosphate buffer (0.05M):

Solution A (0.05 M dibasic hydrogen phosphate): 7.1g of Na2HPO4 dissolved in 1000ml d/w.

Solution B (0.05M Sodium hydroxide): 0.2g of NaOH dissolved in 1L d/w

91ml of A and 9ml of B mixed to obtain buffer of pH 11.0.

(vi) Hydroxide -Chloride buffer 0.05M

Solution A (0.05M potassium chloride): 3.75g of KCl dissolved in 1L d/w.

Solution B (0.05M potassium hydroxide): 2.8g of KOH dissolved in 1L d/w

82ml of A and 18ml of B mixed to obtain buffer of pH12.0.

(vii) Carbonate-bicarbonate buffer (0.2 M)

Solution A (0.2 M anhydrous sodium carbonate): 21.2g of anhydrous sodium carbonate dissolved in 1000ml d/w

Solution B (0.2 M sodium bicarbonate): 16.8g of sodium bicarbonate dissolved in 1000ml d/w

27.5ml of A +22.5ml of B, diluted to a total volume of 200ml with d/w to obtain buffer of pH 10.0.

Carbonate-bicarbonate buffer (pH10) was sterilized by autoclaving for serial dilution technique.

(viii) Carbonate-bicarbonate buffer (0.1 M)

Solution A (0.1 M anhydrous sodium carbonate): 10.6g of anhydrous sodium carbonate dissolved in 1000ml d/w

Solution B (0.1 M sodium bicarbonate): 8.3g of sodium bicarbonate dissolved in 1000ml d/w.

51.0ml of A +49.0ml of B, mixed to obtain buffer of pH 10.0.

(ix) Phosphate buffer (0.2 M)

Solution A (0.2 M Monobasic hydrogen phosphate): 27.8g of NaH_2PO_4 dissolved in 1000ml d/w.

Solution B (0.2 M dibasic hydrogen phosphate): 53.65g of Na_2HPO_4. $7H_2O$ or 71.7g Na2HPO4 .12H2O dissolved in 1000ml d/w.

28ml of A +72ml of B diluted to a total of 200ml with d/w to obtain buffer of pH 7.2.

9.6mM Phosphate buffer saline (PBS)

9.6ml of 0.2 M phosphate buffer (pH 7.2) taken in a volumetric flask and volume made to 200ml with d/w followed by the addition of 1.7g NaCl.

Appendix C - Biochemical and chemotaxonomic studies, morphological features of the isolates and buffering capacities.

C.1. Biochemical media used for identification of SB-D & SB-W (333).

Carbohydrate fermentation

Inoculated sugar broth containing a carbohydrate source (0.5%) viz arabinose, glucose, mannitol and xylose containing *O*-Cresol red indicator. Incubated the tubes at R.T. for 24-48h. Then examined the tubes for change in color and presence or absence of gas bubble. Positive reaction was indicated by a change in color of the indicator and by appearance of air bubble in the Durham tube. Uninoculated tubes served as the control.

Sugar broth

Peptone	5.0g
Beef extract	3.0g
Sugar	0.5g
d/w	to make 1L
O-Cresol red	0.01g
pH	9.5 adjusted by using 10% Na_2CO_3 solution.

Autoclaved at 15 psi for 20 minutes.

Nitrate reduction test

Inoculated nitrate broth and incubated at R.T. for 24-48 h. After incubation, added 5 drops of sulfanilic acid and then 5 drops of α-naphthylamine. Red coloration indicated a positive test while in a negative test, red coloration is observed after addition of 5mg of zinc. Uninoculated tubes served as the control.

Nitrate broth

Peptone	5g
Beef extract	3g
KNO_3	1g
d/w	to make 1L
Na_2CO_3	10g
pH	10.3

Indole production

Indole production was checked in SIM agar tubes by stab inoculating the organism and then incubating at R.T. for 24-48 h. After incubation, 10 drops of Kovac's reagent were added and it was examined for colouration. The red reagent layer was a positive indication of indole production. Uninoculated tubes served as the control.

SIM agar

Peptone	5g
Beef extract	3g
Ferrous ammonium sulphate	0.2g
Sodium thiosulphate	0.025g
Agar	10g
d/w	to make 1L
Na_2CO_3	10g

Methyl red test

Inoculated MR-VP broth and incubated at R.T. for 24-48 h. Then added 5 drops of methyl red indicator and observed for the color change. A red color was indicative of a positive test. Uninoculated tubes served as the control.

MR-VP broth

Peptone	7g
Dextrose	5g
Potassium phosphate	5g
d/w	to make 1L
Na_2CO_3	10g
PH	10.3

Voges - Proskauer test

Inoculated MR-VP broth and incubated at R.T. for 24-48 h. Then added 10 drops of Barrit's reagent A, vortexed and added 10 drops of Barrit's reagent B, vortexed and recorded the color change. Development of a deep pink color was indicative of a positive test. Uninoculated tubes served as the control.

Citrate utilization test

Inoculated Simmon's citrate agar slants by means of a stab-and-streak inoculation and incubated for 24-48 h at R.T. Following incubation, observed the slant for presence or absence of growth. Citrate utilizers were indicated by the presence of growth on the slant. Uninoculated tubes served as the control.

Simmons citrate agar

Ammonium dihydrogen phosphate	1g
Diammonium phosphate	1g
Sodium chloride	5g
Magnesium Sulphate	2g
Sodium carbonate	10g
Agar	20g
d/w	to make 1L
pH	10.3

Catalase test

Inoculated Horikoshi I agar slants and incubated for 24-48 h at R.T. Then added three or four drops of 3% (v/v) hydrogen peroxide to the slant. Appearance of gas was indicative of catalase positive organisms. Uninoculated tubes served as the control.

Oxidase test

Inoculated Horikoshi I agar plates and incubated for 24 h at R.T. Then added two or three drops of *p*-aminodimethylaniline oxalate to the plate and observed for color change. Development of pink, then maroon and finally black coloration on the surface of colonies was indicative of presence of cytochrome oxidase and thus a positive test. Uninoculated plates served as the control.

Gelatin liquefication

Inoculated Gelatin deep tubes and incubated at R.T. for 24-48 h. Following growth, the tubes were refrigerated for 30 min and the medium was observed. Liquid medium after refrigeration was a positive test. Uninoculated tube served as the control.

Nutrient Gelatin

Peptone	5g
Beef extract	3g
Gelatin	120g
d/w	to make 1L
Sodium carbonate	10g
pH	10.3

Starch hydrolysis

Inoculated starch agar plates by streak inoculation. Incubated the plates at R.T. for 24-48 h. Then flooded the plates with Gram's iodine for 1 min. and poured off the excess of stain. Clear zone surrounding the organism was a positive test. Uninoculated agar plate served as the control.

Starch agar

Peptone	5g
Beef extract	3g
Soluble starch	2g
Agar	20g
d/w	to make 1L
Sodium carbonate	10g
pH	10.3

Casein Hydrolysis

Inoculated milk agar plates by streak inoculation and incubated at R.T. for 24-48 h. Then examined the plates for the presence or absence of a clear area around the organism. A clear area around the bacterial growth was indicative of positive proteolysis. Uninoculated agar plate served as the control.

Milk agar

Skim milk powder	100g
Peptone	5g
Agar	20g
Sodium carbonate	10g
d/w	to make 1L
pH	10.3

Resistance to lysozyme reaction: Inoculated a loopful of broth culture into a tube of resistance to lysozyme reaction and into a control tube of nutrient broth at high pH. Growth or its absence observed after incubation of 48 hours at room temperature. Uninoculated tube served as the control.

Resistance to lysozyme medium

Lysozyme solution- 10,000 enzyme units /ml of d/w sterilized by filtration

Peptone	5g
Beef extract l	3g
Lysozyme solution	10ml
Sodium carbonate	10g
d/w	to make 1L
pH	10.3

Egg yolk reaction: Inoculated the culture into an egg yolk broth and a tube of broth without egg yolk. Incubated the tubes for 48 hours at 28°C and observed for the appearance a heavy white precipitated in or on the surface of egg yolk containing medium. Uninoculated tube served as the control.

Egg yolk reaction medium

Tryptone	10g
Disodium hydrogen phosphate	5g
Potassium dihydrogen phosphate	1g
Sodium chloride	2g
Magnesium sulphate	0.1g
Glucose	2g
Sodium carbonate	10g
d/w	to make 1L

Egg yolk - 15ml added to the medium after sterilization at 121°C for 20 min and allowed to stand in a refrigerator over night before inoculation.

Hugh and Leifson's test

Inoculated young culture in the medium dispensed into two tubes. After inoculation, the medium of one tube was overlayered with sterile liquid paraffin. Growth and color change of the indicator dye was noted in the two tubes. Strict aerobes grow only in aerobic conditions. Facultative anaerobes grow in both aerobic and anaerobic conditions. The anaerobic organisms grow only in anaerobic conditions.

Hugh and Leifson's medium :

Peptone	2g
NaCl	5g
K_2HPO_4	0.3g
Glucose	10g
O-cresol red	0.01g
Sodium carbonate	10g
d/w	to make 1L
pH	10.3

Reagents for biochemical tests

Reagents for nitrate reduction

Solution A (Sulfanilic acid)

Sulfanilic acid	8g
Acetic acid (5N)	One part of glacial acetic acid added to 2.5 parts of d/w.
d/w	to make 1L

Solution B (α-naphthylamine)

α-naphthylamine	5g
Acetic acid (5N)	1L

Kovac's reagent for Indole production

p-dimethylaminobenzaldehyde	5g
Amyl alcohol	75ml
HCl (concentrated)	25ml

Dissolved *p*-dimethylaminobenzaldehyde in Amyl alcohol and added hydrochloric acid.

Methyl red Indicator

Methyl red	0.1g
Ethyl alcohol	300ml
d/w	to make 500ml

Barrit's reagent

Solution A

α-naphthol	5g
Ethanol	95ml

Dissolved α-naphthol in Ethanol with constant stirring.

Solution B

Potassium hydroxide	40g
Creatine	0.3g
d/w	to make 1L

***P*-amino dimethylaniline oxalate.**

P-amino dimethylaniline oxalate.	0.5 g
d/w	to make 50 ml

The solution was warmed to completely dissolve the component.

C.2. Chemotaxonomic methods (118):

Chemotaxonomic analysis of cell wall by Alkali treatment method.

400 mgms wet weight of cells were dispersed in 4 ml of 600 mM KOH in a glass bijou bottle sealed with polypropylene cap. The suspension was autoclaved at 1 atmosphere for 5 minutes and the cooled suspension was diluted to 20 ml with 2 M HCl. The content was centrifuged at 3200 g for 20 min. The deposit was then washed twice with 10 ml of d/w and decanted. Cell deposit was resuspended in 1 ml of 10 M HCl, divided equally between 2 clean glass bottles and analysed for the presence of amino acids and sugars.

Amino acid analysis : The portion for amino acid analyses was diluted with twice its own volume of 6 mM HCl and hydrolysed at 100°C overnight. Hydrolysate was evaporated to dryness over boiling water bath. Residue was taken up in 0.05 ml of 10% (v/v) Isopropanol and stored at-8°C till further use.

Chromatography for amino acids: The hydrolysate was applied on paper chromatograms and the spots were subjected to 2 dimensional ascending chromatogrpahy on whatman filter paper no. 1. The first solvent system n-butanol-acetic acid-water (60:15:25 v/v) was run for 20 h at 20°C. After drying, the chromatogram was further run in the second dimension for 16 h at 20°C using as solvent phenol - H_2O (4:1 w/v) with 1 ml of NH_4OH per 200ml solvent. After drying, the chromatogram was dipped in 0.2 % (w/v) ninhydrin in acetone and heated at 100°C for 2 mts to locate the amino acid spots. A reference mixture of amino acids prepared by dissolving L -alanine L- aspartic acid, L- glutamic acid, glucosamine, glycine, L-lysine, L- ornithine and L-serrine prepared in 10% v/v isopropanol was applied in 3 µls amounts.

Sugar analysis: The portion to be used for sugar analysis was diluted with twice its own volume of d/w and hydrolysed at 100°C for 2.5 h. Hydrolysate was evaporated to dryness in vaccuo over silica gel in dessicator with small dish containing NaOH.

Chromatography of sugars: 2.5 µls was applied to Whatman filter paper No. 1 and run in upper phase of biphasic solvent system n-butanol - water - pyridine - toluene (5:3:3:4 v/v) and run for 36 h at 20°C. The spots were located by dipping the chromatogram in 200 mM Para anisidine + 100 mM phthalic acid in 96 % (v/v) ethanol and heating at 100°C for 2 min. To facilitate identification, a reference mixture of sugars containing arabinose, fructose, galactose, glucosamine, glucose, mannose, rhamnose, ribose and xylose at a concentration of 10 mM in 10 % (v/v) isopropanol was run.

Quinones - Extraction and analysis: 50 mgm dry bacterial cells were extracted with 30 ml of Chloroform: methanol (2:1) for 2 hours. The cell solvent mixture was filtered to remove cell debris, liquid collected in a flask and evaporated to dryness under reduced pressure. The pellet was resuspended in 1 ml Chloroform: methanol (2:1) and applied as a narrow band on to silica gel TLC plates. The plate was developed in hexane - diethylether (85:15 v/v) . Purified quinones were revealed as dark brown band on a green fluorescent background by brief irradiation with uv light at 254 nm.

Menaquinones and ubiquinones: The band of quinones obtained was scraped from the plate and mixed with 1 ml $CHCl_3$ and eluted through a sintered glass filter with chloroform. The purified quinones were evaporated to dryness under a stream of nigrogen gas.

Chromatographic analysis:
Reverse phase, partitioning, TLC for menaquinones: Samples in acetone were spotted on reverse phase thin layer plates. Reverse phase TLC plates were prepared by dipping the activated normal silica gel G plates into a solution of ether: paraffin (90:10). The plates were developed in a mixture of acetone:water (99:1).

Argenation (Ag^+) modified TLC of menaquinones: The menaquinone mixture was spotted on silver impregnated TLC plates, prepared in 5% aqueous $AgNO_3$ solution. Plates were developed in non polar solvent mixture hexane: Acetone (85:15). Menaquinones were revealed as blue spots on a yellow background by spraying with 10% dodecamolybdophosphoric acid in ethanol and heating at 140°C for 15 min.

Lipid extraction and analysis: 400 mg wet weight of cells were stirred for 1 min in 27 ml of 0.3 % aqueous methanol - NaCl solution (10 ml 0.3% w/v aqu NaCl in 100 ml CH_3OH) + 14 ml petroleum ether. The mixture after shaking for 20 min was transferred into a separating funnel to obtain 2 layers. The upper layer was collected and marked as I. To the lower layer was added 14 ml pet. Ether and separated into 2 layers again. Top layer was collected and marked as II. Fractions I and II were pooled, dried by evaporation of pet ether and weighed as non polar lipids.

The lower layer was kept in boiling water bath for 5 min and 30 ml of $CHCl_3$-CH_3OH - NaCl in the ratio of 90:100:30 was added. After shaking for 1 hr, mixture was centrifuged and the supernatant was collected. 10 m of $CHCl_3$ - CH_3OH - NaCl (50:100:40) was added to the residue and kept on shaker for ½ h, centrifuged and supernatants were pooled which contained polar lipids. Polar lipids were washed with a mixture of 18 ml $CHCl_3$ + 18 ml NaCl (0.3%) and after shaking, the layers were separated in a funnel. Lower layer containing purified polar lipids was collected and evaporated by a stream of oxygen free N_2.

The dried preparation of polar and non polar lipids was redissolved in small amount of $CHCl_3$ - CH_3 OH (2:1 v/v). Material was spotted on TLC and developed in a solvent system. Acetone: water:benzene (91:30:8) and visualised using uv/I2 chamber.

Detection of mycolic acids: This was done by whole organism acid methanolysis. 50 mg of dry bacteria were mixed with 3ml methanol, 3ml toluene and 0.1 ml concentrated H_2SO_4 in a capped tube. The contents were mixed thoroughly and methanolysis allowed to proceed for 16 h at 50°C. The reaction mixture was allowed to cool to room temp., 1.5 ml of hexane added, mixture shaken and then allowed to settle for the formation of two layers. Samples from the upper layer were spotted on TLC plates and the chromatograms developed in hexane:diethyl ether (85:15) (v/v). The lipids were revealed as blue spots on a yellow background by spraying with 10% dodecamolybdophosphoric acid in ethanol and heating at 140°C for 10 min.

Appendix C.3 Characteristics of the bacterial strains and enzyme production from diverse econiches.

Strain No.	Characteristics			Enzyme Activity			
	Morphological	Temp.	pH	C	A	P	L
Source of Isolation: Salt Pans							
1	Cocci in clusters	M	FA	+	-	+	-
2	Cocci in singles (P)	M	FA	+	-	+	-
3	Cocci in Pairs (P)	M	OA	-	+	-	-
4	Pallisade rods (P)	M	OA	+	+	-	-
5	Pleomorphic forms	T	OA	+	-	-	+
6	Filamentous forms (P)	T	OA	+	-	+	+
7	Filamentous forms (P)	T	OA	+	+	-	+
Source of Isolation: Mangrove sediments							
8	L-forms	M	FA	+	+	-	-
9	Pallisade rods (P)	M	OA	+	+	+	+
10	L-forms (P)	M	OA	+	+	-	-
11	Rods in Singles (S)	M	FA	-	+	-	-
12	Pallisade rods (P)	T	OA	+	+	-	-
Source of Isolation: Mangrove Water							
13	Pallisade rods (P)	M	OA	+	+	-	-
14	Cocci in clusters (P)	M	FA	+	+	-	-
Source of Isolation: Compost							
15	Rods in Chains (P) (S)	T	FA	-	+	-	-
16	Pallisade rods	M	FA	+	+	+	-
17	Rods in Pairs (P) (S)	M	FA	+	+	-	+
18	Long Rods	T	OA	+	+	-	-
19	Rods in Singles	T	OA	+	+	+	-
20	Rods in Singles (P)	T	FA	-	+	-	+
Source of Isolation: Cowdung							
21	Cocci in chains (P)	M	OA	-	-	-	-
22	Rods (S)	T	OA	-	-	-	+
23	**Cocobacilli (Gm-)**	**M**	**FA**	+	+	+	-
24	Filamentous forms (P)	T	OA	+	+	+	-
25	Rods in Chains (P) (S)	M	OA	+	+	-	-
26	Pallisade rods (P)	M	FA	+	+	-	-
27	Filamentous forms (P)	T	OA	+	+	+	+
28	Long Rods (P)	T	OA	-	+	+	-
29	Rods in Chains (P) (S)	M	OA	+	+	+	+

Strain No.	Characteristics			Enzyme Activity			
	Morphological	Temp.	pH	C	A	P	L
Source of Isolation: Droppings of Birds (Poultry)							
30	Cocci in chains	M	FA	-	-	-	-
31	Cocci in tetrads (P)	M	FA	+	-	-	-
32	Rods in Singles (P) (S)	M	FA	+	-	-	-
33	Cocci in Clusters (P)	M	FA	+	-	-	-
34	Rods in Chains (S)	M	FA	+	-	-	-
Source of Isolation: Piggery waste							
35	Cocci in chains	M	FA	-	-	-	-
36	Pallisade rods (P)(S)	M	OA	+	+	+	-
37	Cocci in Clusters (P)	T	OA	-	-	-	-
38	Cocci in pairs (P)	M	FA	-	-	-	-
39	Cocci in Clusters	M	FA	+	-	-	-
Source of Isolation: Coconut Rhizosphere							
40	Filamentous forms (P)	T	OA	+	-	+	-
41	Filamentous forms	T	OA	+	+	+	-
42	Filamentous forms	T	OA	-	+	+	+
43	Cocci in chains (P)	M	OA	-	-	-	-
44	Pallisade rods (P)	T	OA	+	+	+	-
45	Rods in Chains (P) (S)	M	OA	+	+	+	-
46	Rods (S)	T	OA	+	-	-	-
47	Pleomorphic forms	M	OA	+	-	-	-
48	Rods in chains (P) (S)	M	OA	+	-	-	-
49	Filamentous forms (P)	T	OA	+	-	-	+
50	Rods in Singles (P) (S)	M	OA	+	+	+	+
51	Pallisade rods	M	OA	+	+	-	-
52	Filamentous forms	T	FA	+	+	+	-
53	Cocci in Clustors (P)	M	FA	+	-	-	-
Source of Isolation: Alkaline soil							
54	Rods in chains (S)	M	OA	+	+	+	-
55	Cocci in Clusters (P)	M	FA	-	-	-	-
Source of Isolation: Agro-chemical factory effluent.							
56	**Pallisade rods (P)(S)**	**M**	**OA**	+	+	+	+
57	**Pallisade rods (S)**	**T**	**FA**	+	+	+	+
58	Rods in chains (S)	M	FA	+	+	+	+
59	Rods in chains (S)	T	FA	+	+	+	+
60	Thick rods (P) (S)	T	OA	+	+	+	+
61	Cocci in pairs (P)	T	FA	+	+	+	+
62	Cocci in Clusters (P)	M	FA	+	+	+	-

Strain No.	Characteristics			Enzyme Activity			
	Morphological	Temp.	pH	C	A	P	L
Source of Isolation : Mining effluent							
63	Pallisade rods (P)	M	FA	+	+	+	+
Source of Isolation : Dairy effluent							
64	Pallisade rods (P) (S)	M	FA	+	+	+	+
65	Rods in chains (P) (S)	M	FA	+	-	+	+
66	Cocci in chains	M	FA	+	-	+	+
67	Rods in chains (P) (S)	T	FA	+	-	+	+
Source of Isolation : Biogas digester sample							
68	Long rods (S)	T	FA	+	-	-	-
69	Rods in chains (S)	M	0A	+	+	+	-
70	Filamentous forms	T	FA	+	-	-	-
71	Cocci in singles	M	FA	+	-	-	-
72	Thick rods (P) (S)	T	FA	+	+	+	-
73	Cocci in clusters	M	OA	-	-	-	-
74	Pallisade rods (P) (S)	M	FA	+	-	-	-
75	Pallisade rods	T	FA	+	-	-	-
76	Pleomorphic forms (P)	M	OA	+	+	-	-
77	Rods in chains (S)	M	FA	+	+	+	-
Source of Isolation : Paper pulp							
78	Pallisade rods (P)(S)	M	OA	-	+	+	-
79	Cocci in clusters (P)	M	OA	-	+	+	-
Source of Isolation : Polyester dyeing sample							
80	Rods in chains (S)	M	FA	+	-	+	-
81	Cocci in clusters (P)	M	FA	+	-	+	-
82	Long rods	T	FA	+	+	-	-
83	Rods in chains (P) (S)	T	OA	-	+	-	-
84	Rods in chains (P) (S)	M	FA	-	+	+	-
Source of Isolation : Raw & treated sewage							
85	Rods in chains (S)	M	FA	+	+	-	-
86	Pallisade rods	M	OA	-	+	+	-
87	Rods in chains (S)	T	OA	+	+	-	-
88	Long rods (S)	T	FA	+	+	-	-
89	Rods in singles (S)	M	FA	+	+	-	-
90	Pallisade rods	M	FA	+	+	-	+

Strain No.	Characteristics			Enzyme Activity			
	Morphological	Temp.	pH	C	A	P	L
Source of Isolation : Sea water							
91	Pallisade rods	M	FA	-	-	-	-
92	Thick rods (P) (S)	M	FA	-	-	-	-
93	Rods	T	OA	+	+	+	+
94	Long rods (P) (S)	M	OA	+	+	+	+
95	Filamentous forms	T	FA	+	+	+	+
96	Rods in chains (S)	M	FA	+	+	+	+
Source of Isolation :Acidic Soil							
97	Rods in chains (P) (S)	M	FA	+	--	-	+
98	Cocci in clusters (P)	M	FA	+	-	-	+
99	Rods in chains (P) (S)	T	FA	+	-	-	+
100	L - forms (P)	M	FA	+	-	-	+
101	Pleomorphic forms (P)	M	FA	+	-	+	-
102	Rods in chains (S_	T	OA	+	-	+	-
103	Cocci in clusters (P)	M	FA	+	-	+	-
Source of Isolation :Garden Soil							
104	Pallisade rods (P) (S)	M	OA	+	+	+	-
105	Cocci in clusters	M	OA	+	+	+	-
106	Cocci in singles	M	OA	+	+	-	+
107	Pleomorphic forms (P)	M	OA	+	+	+	-
108	Pallisade rods (P) (S)	T	OA	+	+	+	-
Source of Isolation :Goat droppings							
109	Rods in chains (S)	M	OA	+	+	+	+
110	Rods in chains (P) (S)	M	OA	+	+	+	+
111	Cocci in clusters (p)	M	OA	+	+	+	+
112	Rods in chains (P) (S)	M	OA	+	+	+	+
Source of Isolation :Gluten water							
113	Thick rods (S)	M	FA	-	+	+	-
114	Pleomorphic forms)	T	FA	+	-	-	-
115	Rods in chains (S)	T	FA	+	-	-	-
116	Long rods	T	FA	+	-	-	-
117	Filamentous forms	T	FA	+	-	-	-
118	Cocci in clusters	M	FA	+	-	-	-
119	Cocci in tetrads	M	FA	+	-	-	-

Strain No.	Characteristics			Enzyme Activity			
	Morphological	Temp.	pH	C	A	P	L
Source of Isolation : Termite Mound soil							
120	Cocci in chains	M	OA	-	+	-	-
121	Filamentous forms	T	OA	+	+	+	-
122	Cocci in clusters	M	OA	-	+	-	-
123	Cocci in chains	M	OA	+	+	-	-
124	L - Forms (P)	M	FA	+	-	+	-
125	Pallisade rods (P) (S)	M	OA	+	-	-	-
126	Cocci in clusters	M	OA	-	-	-	-
Source of Isolation : Sambhar Salt lake							
127	Filamentous forms	T	OA	+	+	+	-
128	Filamentous forms	T	FA	+	+	+	-
129	Thick rods (S)	M	OA	+	+	+	+
130	Pallisade rods (P)	M	OA	+	+	-	-
131	Long rods (P)	T	OA	+	-	-	-
132	Filamentous forms	T	FA	+	+	+	-
133	Rods in singles (P)(S)	M	OA	-	+	-	-
134	Pallisade rods (P) (S)	M	OA	+	+	+	-
135	Pallisade rods (P)	T	FA	-	-	+	-
136	Filamentous forms (P)	T	OA	+	+	+	-
137	Rods in chains (S)	M	OA	+	+	-	-
138	**Halloalkaliphilic coccal forms (P)**	**T**	**OA**	**-**	**+**	**-**	**-**

Key:(P) Pigmented, (S) Spore formation, (M) Mesophilic, (T) Thermophilic, (FA) Facultative Alkaliphile, (OA) Obligate Alkaliphile, (C) Cellulase, (A) Amylase, (P) Protease (L) Lipase, (+) Positive, (-) Negative.

C.4. Solutions for determination of Buffering Capacity (130,217,409)

a. **200mM KCl:** Dissolved 14.912g of KCl in 1000mld/w.
(Mol.wt. of KCl =74.56)

b. **0.05MKOH:** Dissolved 2.811g of KOH in 1000mld/w.
(Mol wt. of KOH = 56.11)

c. **10% Triton X-100:** Dissolved 10ml of Triton X-100 in 90ml of 200mM KCl and mixed well before use.

Calculation of Buffering Capacity:

Buffering capacity is expressed in terms of nanomoles of OH ions consumed per mg of cell protein per unit change in pH.

Bo- Whole cell Buffering capacity.
Bt- Premeabilised cell Buffering capacity.
Bi- Cytoplasmic Buffering capacity.

$$\boxed{Bi = Bo - Bt}$$

Bo and Bt are determined experimentally for every small addition of base (10microlitres /50 nanomoles of OH^- ions) in which pH change is approximately 0.1pH unit or more. Average value encompassing change in 1pH unit is calculated. Bi is determined by calculating decrease in a buffering capacity after permeabilisation.

Eg: 10microlitres of 0.05 M KOH ⇨ 500 nanomoles of OH^-
1microlitre of 0.05 M KOH ⇨ 50 nanomoles of OH^-

Thus, if intact cells of *Bacillus subtilis* consume 100microlitres of 0.05 M KOH to change the pH from 6 - 11.

Buffering capacity of whole cells in the range of pH 6-11 is calculated as follows:
Nanomoles of OH^- consumed = 100x50= 5000
mg of protein used for titration =5
Number of pH units =5

Buffering capacity of whole cells in the range of pH 6-11 of *Bacillus subtilis* is :

$$\frac{\frac{5000}{5}}{5} = \frac{1000}{5} = 200$$

$$\frac{\text{nanomoles of } OH^-}{\text{mg protein}}$$
$$\text{pH unit}$$

Appendix D: Chemical estimations and standard graphs

D.1. Reagents and protocols for chemical estimation.

i) Phenol sulphuric acid method for total carbohydrate (312).

5 % Phenol: 50 g of redistilled phenol (reagent grade) dissolved in water and diluted to 1 L

Sulphuric Acid: 96 % reagent grade.

Standard glucose solution: 0.1 mg of glucose dissolved in 1 ml of d/w.

Procedure: To 1 mg of the sample, 1 ml of concentrated sulphuric acid was added followed by the adition of 1 ml of 2.5% aqueous phenol and 4 ml of concentrated $H_2 SO_4$. The tubes were rapidly kept in an ice bath and held for 10 minutes at room temperature. The yellow orange colour obtained was measured at 490 nm against a d/w blank. Standard curve was plotted using glucose (0-100 µgms/ml) as the standard, factor F calculated and the concentration of the total sugars in the samples determined.

ii) Glucose oxidase method for glucose (312).

Glucose oxidase Peroxidase reagent:
Dissolved 25 mg of *O*-dianisidine completely in one ml of methanol. Added 49 ml of 0.1 M phosphate buffer pH 6.5 (Appendix B). Then added 5 mg of peroxidase and 5 mg of glucose oxidase to the above prepared *O*-dianisidine solution.

Standard glucose solution: 0.1 mg of glucose dissolved in 1 ml of d/w.

Procedure: To 1 mg of the hydrolysed sample, 1 ml of d/w and 1 ml of glucose-peroxidase reagent was added. The tube was incubated at 35°C for 40 minutes. The reaction was terminated by addition of 2 ml of 6 N HCl. Colour intensity was read at 540 nm. A series of standard glucose (0-100 µgms/ml) were also treated in a similar manner.

iii) Dinitrosalicylic acid method for reducing sugars (312).

DNSA Reagent:

Dissolved 1.0 g of 3,5 - dinitrosalicylic acid, 30 g of sodium potassium tartarate and 1.6 g of sodium hydroxide in water and made volume upto 100 ml.

Standard maltose solution: 0.1 mg of maltose dissolved in one ml of d/w.

Procedure: To 1 ml of the sample, 1 ml of DNSA reagent was added. The tubes were heated at 100°C for 10 minutes. The reaction was stopped by addition of 1 N HCl. Samples were cooled and absorbance was recorded at 540 nm against reagent blank. The concentration of sugars was expressed as micrograms/ml using the standard graph prepared with maltose.

iv) Folin Lowry's method for Proteins (239):

Reagent A: 2% Na_2CO_3 in 0.1 N NaOH
Reagent B: 0.5% $CuSO_4$ in 1% potassium sodium tartrate.
Reagent C: Alkaline Copper solution-Mixed 50ml of A and 1ml of B prior to use.
Reagent D: Folin and Ciocalteau's phenol reagent
Commercially available reagent diluted with equal volume of d/w on the day of use. This reagent is a solution of sodium tungstate and sodium molybdate in phosphoric and hydrochloric acids.

Standard bovine serum albumin solution: 0.1mg of BSA dissolved in one ml of d/w.

Procedure: To 1ml of the sample, 5 ml of copper sulphate solution was added and kept at room temperature in the dark for 10 minutes. 0.5 ml of Folin and Ciocalteau's phenol reagent was then added and kept in the dark for 20 minutes. Absorbance was measured at 750 nm against reagent blank and the concentration of the samples determined from standard graph and Factor F calculated using Bovine serum albumin as the standard (0 - 100 µgms/ml).

v) Bicinchoninic acid method (BCA) for proteins (332):

Reagent A:	Commercially available BCA (Sigma) solution.
Reagent B:	Dissolved 4 g of $CuSO_4$ in 100 ml d/w.
BCA reagent:	Dissolved 0.2 ml of reagent B in 10 ml of reagent A just before use.

Standard used: 0.1 mg of BSA in 1 ml d/w.

Procedure: To 1 ml sample, 2 ml of BCA solution was added and incubated at 37°Cfor 30 minutes. The absorbance was measured at 562 nm and the concentration was calculated from the standard graph.

vi) Potassium dichromate method for lipids (284):

0.15% Acid dichromate solution:
Dissolved 0. 75 g of potassium dichromate in 10 ml of d/w with heating. Solution cooled and volume made up to 500 ml with concentrated sulphuric acid under cold condition. The reagent refrigerated in amber coloured bottle till further use.

Standard 0.1% Stearic acid:
Dissolved 0.1 g of stearic acid in 100 ml of distilled ethanol.

Procedure: 1 mg sample was dissolved in 1 ml of 0.5 N NaOH and placed in a boiling water bath for 30 mts. The sample was neutralised with 1 ml of 0.5 N HCl. The lipid content was estimated by the method outlined by Pande, Khan and Ventikasubramaniam using the principle of oxidation of lipids with acid dichromate.

Extraction of the sample was carried out with chloroform: Methanol: water (1:2:0.8). Further, 2 ml of chloroform and 2 ml of d/w was added to the extract. The contents were poured into a separating funnel and the lower chloroform layer was collected. It was evaporated to dryness under vaccum. To the stearic acid standard (3 µgms of 0.1% stearic acid), d/w blank and test solution, 2 ml of 0.15 % acid dichromate solution was added. The samples were vortexed, placed in a boiling water bath for 15 minutes and cooled under tap water. Centrifugation was carried out at 3000 rpm for 20 minutes and to this supernatant 4.5 ml of d/w was added. The solutions were mixed separately and the absorbance of the test and the standard was measured at 440 nm against d/w blank. The concentration was calculated as

$$\text{Concn. of lipids } \mu\text{gms/ml} = \frac{A}{B} \times \frac{b}{a} \times y$$

A = concentration of lipid standard in µgms.
B = Absorbance of lipid standard in nm.
b = Absorbance of the sample in nm
a = Volume of the sample for estimation in µl.
y = Volume of total lipid sample in µl.

vii) Estimation of Uronic acids (36):

a) Carbazole method

Sulphuric acid reagent: 0.025 M Na - tetraborate in concn. H_2SO_4. Dissolved 0.5485 g of Sodium tetraborate in 100 ml conc. H_2SO_4.

Carbozole reagent : 0.125% carbazole in ethanol/methanol. Dissolved 0.025 g of carbazole in 20 ml ethanol.

Standard galacturonic acid: 0.1 mg of galacturonic acid dissolved in 1 ml d/w.

Procedure: 5 ml of sulphuric acid reagent in a tube was cooled to 4°C using an ice bath. To this 1 mg of the test sample was added and the tube was closed with a stopper and shaken, at first gently and then vigorously on vortex mixer with constant cooling. Tube was heated for 10 minutes in a boiling water bath and

cooled at room temperature. 0.2 ml of carbzsole reagent was then added and after mixing, the samples were heated in a boiling water bath for 15 minutes, cooled to room temperature and the absorbance was measured against reagent blank at 530 nm. Concentration of uronic acid was determined using galacturonic acid (0 -100 µgms) as the standard.

b) Metahydroxy biphenyl method (92):

4 M Sulfamic Acid : Dissolved 3.8836 g of sulfamic acid in 5 ml of d/w. Added saturated KOH solution dropwise till sulfamic acid dissolved. The solution cooled and pH adjusted to 1.6 with KOH. The volume made to 10 ml with d/w.

H_2SO_4 containing 75 mM sodium tetraborate: Dissolved 2.8605 g sodium tetraborate in 100 ml of concentrated H_2SO_4.

0.15% *m*-hydroxybiphenyl : Dissolved 0.5 g NaOH in 100 ml of d/w to get 0.5 % NaOH. 0.15 g of *m*-hydroxybiphenyl dissolved in 100 ml of 0.5 % NaOH solution.

Standard galacturonic acid: 0.1mg of galacturonic acid dissolved in 1ml d/w.

Procedure: 40 µls of 4 M sulfamic acid was added to 1 mg of the sample and mixed thoroughly. 2.4 ml of concentrated sulphuric acid containing sodium tetraborate was then added to the tube and the solution was stirred vigorously by vortex mixing. The tubes were then heated in a boiling water bath at 100°C for 20 minutes with a stoppered knob. After cooling the tubes in an ice bath, 80 µls of metahydroxy biphenyl was added. The solution was then stirred vigorously and allowed to stand at room temperature for 10 minutes. Absorbance was measured at 525 nm and the concentration was calculated using glucoronic acid as the standard.

viii Cysteine hydrochloride method for methyl pentoses (81):

H_2SO_4: H_2O mixture: Added 1:6 (v/v) of H_2O and H_2SO_4.

3% Cysteine hydrochloride: Dissolved 3 g of cysteine hydrochloride in 100 ml d/w.

Standard arabinose solution : Dissolved 0.1 mg of arabinose in 1 ml d/w.

Procedure: To 1 mg of the sample, 4.5 ml of chilled mixture (1:6 water and concentrated sulphuric acid) was added. The mixture was then held at room temperature for a few minutes and then in a boiling water bath for 3-10 minutes and cooled under tap water. 0.1 ml of 3% aqueous cysteine hydrochloride was added and the absorbance of greenish yellow color was read at 396 nm and 430 nm.

Rhamnose (0 - 100 µgms/ml) was used as a standard and the readings of 430 nm were subtracted from 396 nm in order to calculate the factor.

xi) Dinitrophenylhydrazine method for pyruvates (331):

0.5 % 2,4 Dinitrophenylhydrazine : Dissolved 0.5 g of DNPH in 100 ml of 2 N HCl.

10 % Na_2CO_3: Dissolved 10 g of Na_2CO_3 in 100 ml of d/w.

Standard sodium pyruvate solution: Dissolved 5 mg of sodium pyruvate in 100 ml of 1 N HCl corresponding to 100 micrograms of pyruvate /ml.

Procedure: 1 mg of sample was taken in a thick walled glass ampule. 2 ml of 1 N HCl was added. The ampule was then flushed with nitrogen gas, sealed and hydrolysis was carried out at 100°C in an oven for 3 hours.

Content was cooled and transferred in a test tube to which 1 ml of dinitrophenyl hydrazine reagent was added and mixed for 5 minutes on a vortex mixer. The reaction mixture was extracted once with 5 ml of ethyl acetate. The lower aqueous layer was discarded using a separating funnel. To the reaction mixture, 5-7 ml of 10 % Na_2CO_3 was added and the content mixed thoroughly in a separating funnel. The lower aqueous layer was collected and the extraction was repeated thrice. The extracts were pooled and diluted to 25 ml with 10 % Na_2CO_3. Absorbance was measured at 375 nm and the concentration was calculated using factor F obtained from the standard graph of Sodium pyruvate prepared in 1 N HCl.

x) Barium Chloride gelatin method for sulphates (82):

Barium Chloride gelatin reagent: Dissolved 2 g of gelatin in 400 ml of hot d/w (60°C-70°C) and allowed to stand at 4°C overnight. Further dissolved 2 g of Barium chloride in the semigelatinous gelatin fluid and the resultant cloudy solution allowed to stand for 2-3 h before use.

1 N HCl

3% (w/v) Trichloroacetic acid: Dissolved 3 g of trichloroacetic acid (TCA) in 100 ml of d/w.

Standard Potassium Sulphate Solution: Dissolved 1.814 mg of Potassium sulphate in 10 ml of 1 N HCl corresponding to 100 µgms/ml of sulphate.

Procedure: To 1 mg sample, 1 ml of 1 N hydrochloric acid was added in a glass ampule. The ampule was flushed with nitrogen gas and sealed. The sample was hydrolysed in an oven at 105°C for 17 hours. The content of the tube was cooled and mixed before opening the tube and the content added to another tube containing

3.8 ml of 3% trichloroacetic acid. 0.1 ml of Barium Chloride gelatin reagent was then added; mixed thoroughly and kept at room temperature for 20 minutes. Absorbance of the white sulphate ppt was measured at 360 nm against a blank containing 1 ml 1 N HCl. Factor F was calculated using K_2SO_4 (0-100 µgms/ml) as the standard and the concentration of sulphates in the test samples determined. (All the glassware used for this estimation were washed with concentrated nitric acid.)

xi) Ammonium Molybdate method for Inorganic Phosphates (173,290):

Copper - acetate buffer pH 4.0: Dissolved 0.25 g of $CuSO_4$ and 4.6 g of sodium acetate in 100 ml of 2 M/L acetic acid.

Reducing agent: Dissolved 2 g of *p* - methylaminophenol sulphate in 10g/100ml solution of sodium sulphite and stored in dark bottle till required.

5% Ammonium molybdate: Dissolved 5g of ammonium molybdate in 100ml d/w.
Standard potassium dihydrogen phosphate solution: Dissolved 340 mg of KH_2PO_4 in 100 ml of water and volume made upto 250 ml using d/w. This solution has 1 µmole of phosphorous/ml.

Procuedure: To 1 mg of the sample was added 3 ml of copper acetate buffer, 0.5 ml of ammonium molybdate solution and 0.5 ml of reducing agent and incubated at room temperature for 10 minutes. Absorbance was read at 680 nm against stock phosphate solution containing KH_2PO_4.

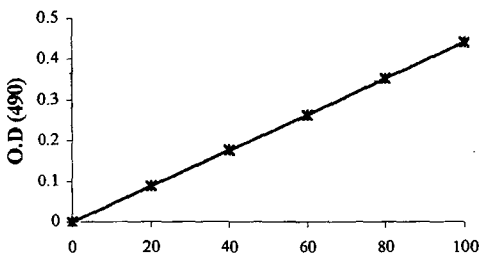
xiv Estimation of phenols (245):

80% Ethanol

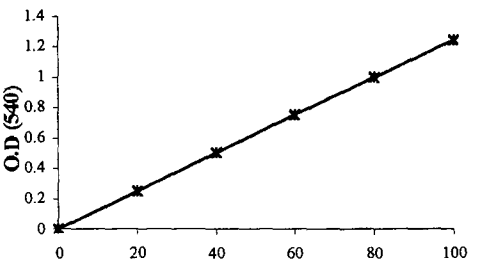
Folin-Ciocalteau reagent

Na_2CO_3 -20%

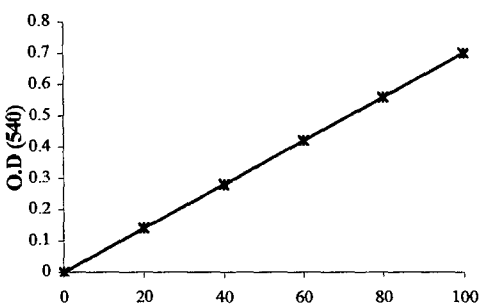
Procedure: 1 mg of the sample was dissolved in 1 ml hot water and 0.5 ml Folin coecalteau reagent was added. Content was mixed and the tube held at room temperature for 3 minutes. 2 ml of 20% Na_2CO_3 was then added and the tube heated in a boiling water bath for exactly 1 minute. Absorbance was read at 650 nm against a reagent blank and the concentration determined from the standard graph prepared for catechol (0-100 µgms/ml).



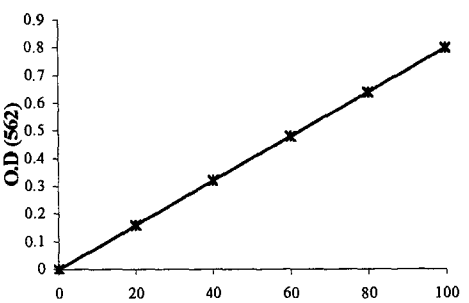
AD. 1. Standard graph for total sugars by phenol sulphuric acid method



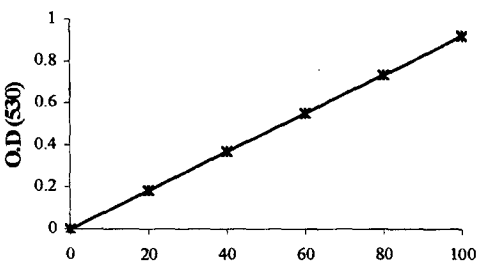
AD. 2. Standard graph for glucose by glucose oxidase method



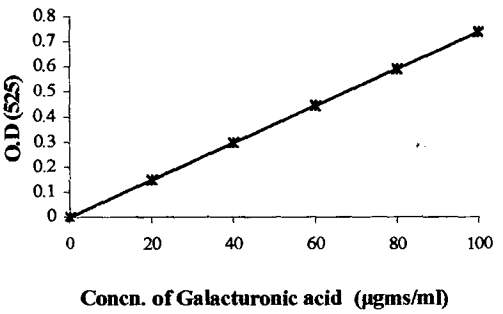
AD. 3. Standard graph for sugars by DNSA



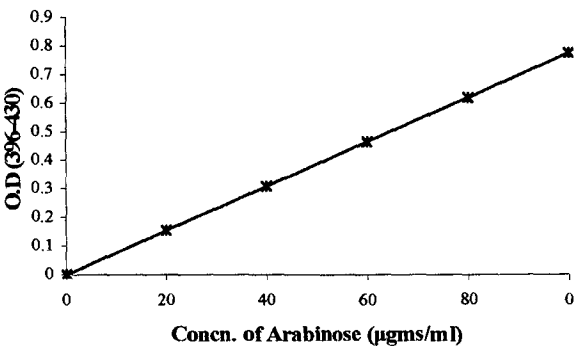
AD. 4. Standard graph for proteins by BCA method



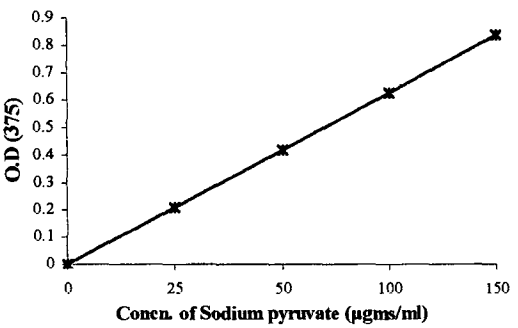
AD. 5. Standard graph for Uronic acids by Carbazole method



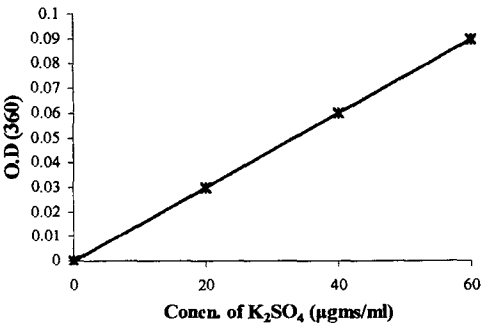
AD. 6. Standard graph for Uronic acids by Metahydroxybiphenyl method



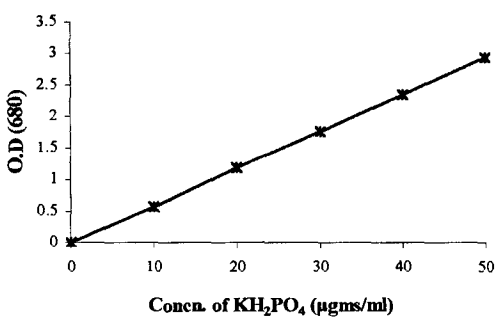
AD. 8. Standard graph for Methyl Pentoses by Cysteine hydrochloride method



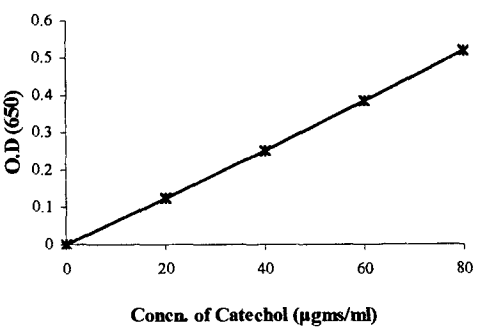
AD. 9. Standard graph for Pyruvates by Dinitrophenyl hydrazine method



AD. 10. Standard graph for Sulphates by Barium-chloride-gelatin method



AD. 11. Standard graph for Inorganic phosphates by ammonium molybdate method



AD. 12. Standard graph for phenols by Marambe & Ando's method

Appendix E-Techniques.

E.1. TLC

Dodecamolybdophosphoric acid spray reagent for mycolic acids: Dissolved 10 g of Dodecamolybdophosphoric acid in 100 ml ethanol.

Ninhydrin spray reagent for amino acids: **Dissolved 2 gm of ninhydrin in 25 ml of acetone. Add 25 ml of 0.2 M acetate buffer pH 5.5.**

Solvent systems with specific spray reagents used for identification of monosaccharides of hydrolysed polymers G1 and G2 by TLC.

Solvent System	Spray reagent
1. Butanol: Pyridine: water(3:1:1)	Aniline phthallate
2. Benzene:Glacial acetic acid:methanol(1:3:3)	0.2% naphthoresorcinol
3. Ethyl acetate: propanol (65:35)	2% aniline diphenyl amine reagent
4. Butanol:acetic acid:water (4:1:5)	Aniline phthallate
5. Isopropanol:pyridine:water:acetic acid(8:8:4:1)	Benzidine reagent

Aniline phthalate spray reagent for sugars: 2 ml of aniline added to 100 ml of *n*-butanol saturated with d/w. 3.25 of phthalic acid then dissolved in the aniline butanol solution.

Naphtho-resorcinol sulphuric acid reagent for sugars:

A. Naphtho-resorcinol Solution: Dissolved 0.2 g of naphtho-resorcinol in 100ml ethanol.

B. 20% H_2SO_4: 20 ml of concentrated sulphuric acid added to 80 ml of d/w.

Spray reagent: Mixed equal volumes of A and B before use.

Benzidine reagent for sugars: Dissolved 0.2 g of benzidine in 100 ml of acetic acid.

α-naphthol - Sulphuric acid spray reagent for glycolipids (180):

Reaent A: Dissolved 0.5 g of α-naphthol in 50 % methanol prepared in water.

Reagent B: 5 ml concentrated sulphuric acid added to 95 ml ethanol.
Plates sprayed first with reagent A, allowed to dry and then sprayed with reagent B. Heated at 110°C till the development of spots.

E.2. GC-Derivatisation of the hydrolysed samples to the alditol acetate forms: The hydrolysed sample was evaporated to dryness and washed in 1 ml d/w, thrice, evaporating the content to dryness everytime. The sample was dissolved in 1 ml d/w and to it 100 µls of inositol (1 mgm/ml) was added as the internal standard. Sample was vacuum dried in a perfit Rotavapor bath for 5 minutes. After adding 1 ml of d/w, the sample was passed through a dowex resin column, vacuum dried for 5 minutes and kept in a dessicator overnight. A drop of 10% triethylamine was added till the pH was 8.6-9.0. After 30 minutes, 0.5 ml of $NaBH_4$ (20 mg/ml) was added and incubated in the dark for 2-3 hours. The $NaBH_4$ was then destroyed by dropwise addition of glacial acetic acid (100 %) until no more effervescence was seen. Acid was removed by drying, followed by several rinses with 5 ml methanol. Sample was kept in the vacuum dessicator for atleast three hours. 1 ml of acetic anhydride (99 %) and 1 ml of pyridine (99 %) were added to the sample and kept overnight in the incubator at room temperature. It was evaporated to dryness and kept overnight under vacuum in a dessicator. Sample was washed with 1 ml of d/w and extracted thrice with 4 ml methylene chloride (99 %). The lower methylene chloride layer was collected in a test tube and filtered through Whatman paper, coated with anhydrous sodium sulphate powder. Sample was collected in a pear shaped flask and evaporated to dryness. A small amount of diethylether was added, transferred into a vial and the sample was dried by flushing with nitrogen gas. This step was repeated thrice. The sample was dissolved in 100 µls of methylene chloride before injection into GC. A capillary gas chromatography (GC equipped with CP sil-88 25 mm, id = 0.32 mm, df = 0.12, Chrompack, Model CP 9002 Middleburg, the Netherlands) and a flame ionisation detector (FID), was used to separate the alditol mixture. O.4µls of the sample was applied using a column injection when the oven temperature was 70 °C. Methylene chloride (0.4 µls) was injected into the GC as the blank and run for a period of 45 minutes. Standard sugars (100 µgms) and samples were also analysed for 45 minutes. The stationary phase used was a polar, highly substituted cyanopropyl siloxane phase, which was stabilised.

E.3. Stock Solution for SDS - PAGE

Acrylamide–bis–acrylamide solution [Monomer solution]: 29% acrylamide and 1% (w/v) N,N methylene *bis* acrylamide was dissolved in warm d/w. Checked pH to be 7.0 and stored in dark bottles at 4°C and used within 30 days.

Resolving gel buffer [1.5 M Tris, pH 8.8]: Prepared by dissolving 18.615g Tris, in 70ml d/w water and added 100 µls of 10% SDS in d/w. The pH of the solution was adjusted to 8.8 using concentrated HCl and the volume was build up to 100ml with d/w. The solution was stored at 4°C .

Stacking gel buffer [1.0 M Tris pH 6.8]: Prepared by dissolving 12.11g Tris, in 70ml d/w water and added 50 µls of 10% SDS in d/w. The pH of the solution was adjusted to 6.8 using concentrated HCl and the volume was build up to 100ml with d/w. The solution was stored at 4°C.

Ammonium per sulfate (APS, 10% w/v): Prepared by dissolving 0.1g of APS in 1.0ml d/w. The solution was prepared afresh each time.

Electrophoresis buffer:

Composition of 1X buffer is as follows:

Tris	3.0g
Glycine	14.4g
SDS(10%)	10ml
D/w	to make 1000ml
PH	8.4

Sample buffer:

Composition of 4X buffer is as follows:

Tris – HCl (1 M pH 6.8)	0.04ml
Glycine	0.04g
SDS	0.004g
β-Mercaptoethanol	0.004ml
d/w	to make 10ml

Tracking dye:

50% sucrose	10ml
Bromophenol blue	10mg

Staining Solution:
Coomassie Brilliant Blue G - 250 solution was prepared by dissolving 0.25g Coomassie Brilliant Blue G - 250 in 100ml of 25% methanol, 10% glacial acetic acid and 65% d/w.

Destaining Solution I

Methanol	40ml
Acetic acid	10ml
d/w	50ml

Destaining Solution II

Methanol	5ml
Acetic acid	7ml
d/w	88ml

Preparation of gel monomer

The composition of the resolving and stacking gels is as follows:

Solution	Resolving gel (10%) (ml)	Stacking gel(5%) (ml)
Monomer	2.5	0.33
1.5M Tris, pH 8.8	1.875	-
1.0M Tris, pH 6.8	-	0.625
10% SDS	0.075	0.025
10%APS	0.0375	0.025
d/w	0.003	1.525
TEMED	0.005	0.005

a. Preparation of sample: 100 µls of cell pellet (containing 100 mg of protein) was mixed with 10 µls of 25 % SDS and boiled for 2 minutes at 100 oC. 50 µls of sample buffer was then added and boiled for 5 minutes at 100 o C. After cooling, 20 µls of bromothymol blue was added and 50 µls of the samples were loaded in the gel with SDS PAGE molecular weight markers (sigma – St. Louis, MO USA).

b. Procedure: The SDS - PAGE was carried out in a Bangalore Genei apparatus. After a pre-run for 10 minutes, 30 µls of the samples containing 50 µgms of proteins along with the standard molecular weight markers were loaded in the gel. The electrophoresis was carried out at a constant voltage of 80 V for stacking gel and 120 V for resolving gel till tracking dye (Bromothymol blue) reached the bottom of the gel. At the end of the run, the gel was stained by Coomassie blue.

c. Staining and destaining procedure: Coomassie blue staining: The gel was stained in coomassie Brilliant Blue G-250 solution Staining was carried out overnight; followed by destaining under mild shaking using destaining solution I for 3-4 hours and destaining solution II for several hours till the protein bands became clearly visible with no background colour. The gels were dried and preserved between cellophane sheets .

E.4. SEM: Scanning Electron Micrographs of triton untreated and treated cells: Adopted from specimen preparation methods from SEM, JEOL application notes:

The cultures SB-D and SB-W grown in Horikoshi I medium were centrifuged at 10,000 rpm for 15 min and the supernatant was discarded. The cell pellet was dispensed in 200 mM KCl and smeared on the stub, further fixed in 2 ml of 2.5% glutaraldehyde fixative overnight at room temperature. The stub was further placed in 0.05 M Carbonate bicarbonate buffer (pH 10.0) and then in 30% acetone. It was allowed to stand for 10 min and the dehydration process was repeated likewise with 50%, 70% and 90% acetone for 10 min each, and finally in 100% aceton for 30 min. The stub was then put in the critical point drying device to replace acetone by liquid carbon dioxide to gaseous carbon dioxide. This process takes about 1 h. The stub was placed on the sputter coater (spi-module) Specimen holder, after drying. The position of the stage was set such that the specimen was approximately 50 mm from the bottom of the sputter head. After sputtering the specimen with 10-15 nm thin film of gold, the stub was placed onto the electron microscope sample chamber and observed with JEOL-5800 LV SEM. The similar procedure was repeated for the triton treated cells.

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CHAPTER IX

Bibliography.

BIBLIOGRAPHY.

1. **Abe, J.I., F.W.Bergmann, K.Obata** and **S.Hizukiri**. 1988. Production of the raw starch digesting amylase of *Aspergillus* sp K-27. Appl. Microbiol. Biotechnol. 27:447-450.

2. **Adhikary, S.P**. 1998. Polysaccharides from mucilaginous envelope layers of *Cyanobacteria* and their ecological significance. J. Scientific Ind. Res. 57: 454-466.

3. **Aguilar, A**. 1996. Extremophile research in the European union: from fundamental aspects to industrial expectations. FEMS. Microbiol. Rev. 18:89-92.

4. **Akiba, T**. and **K.Horikoshi**. 1976. Identification and growth characteristics of α-galatosidase producing microorganisms. Agri. Biol. Chem. 40:1845-1849.

5. **Annison, G**. and **I. Couperwhite**. 1986. Influence of calcium ion on alginate production and composition in continuous cultures of *Azotobacter vinelandii*. Appl. Microbiol. Biotechnol. 25: 55-61.

6. **Anton, J., I.Meseguer** and **F.R.Valera**. 1988. Production of an extracellular polysaccharide by *Haloferax mediterranei*. Appl. Environ. Microbiol. 54: 2381-2386.

7. **Aono, R** and **K. Horikoshi**. 1983. Chemical composition of cell walls of alkalophilic strains of *Bacillus*. J. Gen. Microbiol. 129:1083-1087.

8. **Aono, R** and **K. Horikoshi**. 1984. Composition of peptidoglycan of alkaliphile *Bacillus* sp. J. Bacteriol. 157:688-689.

9. **Aono, R**. 1985. Isolation and partial characterization of structural components of the walls of alkalophilic *Bacillus* strain, C-125. J. Gen. Microbiol. 131:105-111.

10. **Aono, R**. 1989. Characterization of cell wall components of the Alkalophilic *Bacillus* strain C-125. Identification of a polymer composed of polyglutamate and polyglucoronate. J. Gen. Microbiol. 135:265-271.

11. **Aono, R**. and **K. Horikoshi**. 1991. Carotenes produced by the alkalophilic yellow pigmented strains of *Bacillus*. Agric. Biol. Chem. 55:2643-2645.

12. **Aono, R., M. Ito** and **K. Horikoshi**. 1993. Occurrence of teichuronopeptide in cell walls of group 2 alkaliphilic *Bacillus* sp. J. Gen. Microbiol. 139, 2739-2744.

13. **Aono, R**. and **T. Sanada** 1994. Hyper autolysis of the facultative alkaliphile *Bacillus* sp C-125 cells grown at neutral pH: Culture pH dependent cross linking of the peptide moieties of the peptidoglycan. Biosci. Biotechnol. Biochem 58:2015- 2019.

14. **Aono, R**. 1995. Assignment of facultatively alkaliphilic *Bacillus* sp. C-125 to *Bacillus lentus* group 3. Int. J.Syst. Bacteriol. 45: 582-585.

15. **Aono, R., M. Ito, K. N. Joblin** and **K. Horikoshi**. 1995. A high cell wall negative charge is necessary for the growth of the alkaliphilic *Bacillus lentus*. C-125 at elevated pH. Microbiology. 141: 2955-2964.

16. **Aono, R., H.Kaneko** and **K. Horikoshi**. 1996. Alkaline growth pH dependent increase of respiratory and NADH Oxidation activities of facultatively alkaliphilic strain *Bacillus lentus* C-125. Biosc. Biotechnol. Biochem. 60: 1243-1248.

17. **Aono, R., M. Ito** and **K. Horikoshi**. 1997. Measurement of cytoplasmic pH of the alkalophilic *Bacillus lentus* C-125 with a fluorescent pH probe. Microbiology. 143: 2531 -2536.

18. **Aono R, M. Ito** and **T.Machida** 1999. Contribution of the cell wall component teichuronopeptide to pH homeostasis and alkaliphily in the alkaliphilic *Bacillus lentus* C-125. J.Bacteriol. 181: 6600-6606

19. **Atkinson, T.** 1996. Exploring and exploiting biodiversity-the conundrum. Trends in Biotechnol. 14: 181-182.

20. **Babu, K.R.** and **T. Satyanarayan**. 1993. Parametric optimisation of extracellular ∝- amylase production by Thermophilic *Bacillus coagulans*. Folia Microbiol. 38:77-80.

21. **Babu, K.R.** and **T. Satyanarayan**. 1993. Extracellular calcium inhibited ∝-amylase of *Bacillus coagulans* B 49. Enzyme Microbial Technology. 15: 1066-1069.

22. **Babu, K. R.** and **T. Satyanarayan**. 1995. ∝- Amylase production by Thermophilic *Bacillus coagulans* in solid state fermentation. Process Biochem. 30: 305-309.

23. **Bahm, Y.S., J. M. Park, D. H. Bai, S. Takase** and **J. H. Yu**. 1998. YUA001, a novel aldose reductase inhibitor isolated from alkaliphilic *Corynebacterium* sp. YUA 25. Taxonomy, fermentation, isolation and characterization. J. Antibiotics. 51: 902-907.

24. **Baig, S., M.A.Qadeer, M. Saeedakhtar** and **T.Ahmed** 1990. Utilisation of unhydrolysed cheese whey for the production of extracellular polysaccharide by *Xanthomonas cucurbitae* PCSIR-B-52 J. Ferment.Bioeng. 69: 345-349.

25. **Banerjee, S., S. Dattagupta** and **A.M.Chakrabarty**. 1983. Production of emulsifying agent during growth of *Pseudomonas cepacia* with2,4,5-trichlorophenoxyacetic acid. Arch. Microbiol. 135: 110-114.

26. **Bar-or, Y.** and **M.Shilo** 1987. Characterisation of macromolecular flocculants produced by *Phormidium* sp. Strain J-1 and by *Anabaenopsis circularis* PCC 6720. Appl. Environ. Microbiol. 53: 2226-2230.

27. **Beech I.B.** and **C.C.Gaylarde** 1991. Microbial polysaccharides and corrosion. International Biodeterioration 27: 95-107.

28. **Beech, I.B., L.Hanjagsit, M.Kalaji, A.L.Neal** and **V. Zinkewich.** 1999. Chemical and structural characterisation of exopolymers produced by *Pseudomonas* sp. NCIMB 2021 in continuous culture. Microbiology. 145: 1491-1497.

29. **Becker, K.** 1996. Exopolysaccharide production and attachment strength of bacteria and diatoms on substrates with different surface tensions. Microb. Ecol. 32: 23-33.

30. **Bhosle, N.B., S.S.Sawant, A. Garg** and **A.B.Wagh** 1995. Isolation and partial chemical analysis of Exopolysaccharides from the marine fouling diatom *Navicula subinflata*. Botanica Marina. 38: 103-110.

31. **Bhosle, N.B., S.S.Sawant, A. Garg, A.B.Wagh** and **L.V.Evans**. 1996. Chemical characterisation of exopolysaccharides from the marine fouling diatom *Amphora coffeaeformis*. Biofouling. 10: 301-307.

32. **Bhosle, N**, P.A.Suci, A.M.Baty, R.M. Weiner and G.G.Geesey 1998. Influence of divalent cations and pH on adsorption of a bacterial polysaccharide adhesin. J of colloid and Interface science. 205: 89-96.

33. **Bhushan, B., N. S. Dosanjh, K. Kumar** and **G. S. Hoondal**. 1994. Lipase production from alkalophilic yeasts sp. by solid state fermentation. Biotechnol. Lett. 16: 841-842.

34. **Bhushan, B.**, and **G. S. Hoondal**. 1998. Isolation, purification and properties of a thermostable chitinase from an alkalophilic *Bacillus* sp. strain BG-11. Biotechnol. Lett. 20:157-159.

35. **Bhushan, B**. 2000 Production and characterisation of a thermostable chitinase from a new alkalophilic *Bacillus* sp BG-11.J.Appl. Microbiol. 88:800-808.

36. **Bitter,** T. and **H.M.Muir**. 1962. A modified uronic acid carbazole reaction. Anal. Biochem. 4: 330-334.

37. **Blanco, A., T.Vidal., J.F.Colom** and **F.I.J.Pastor**. 1995. Purification and properties of xylanase from alkali-tolerant *Bacillus* sp. strain BP-23. Appl. Environ. Microbiol. 61:4468-4470.

38. **Bockle, B., B.Galunsky** and **R.Muller**. 1995. Characterisation of a keratinolytic serine proteinase from *Streptomyces pactum* Dsm 40530. Appl. Environ. Microbiol. 60:3705-3710.

39. **Bonet, R., M.D.S.Pujol** and **F.Congregado.** 1993. Effects of nutrients on exopolysaccharide production and surface properties of *Aeromonas salmonicida.* Appl. Environ. Microbiol. 59:2437-2441.

40. **Boyer, E.W.** and **M.B. Ingle**. 1972. Extracellular alkaline amylase from a *Bacillus* species. J. Bacteriol. 110:992-1000.

41. **Boyer, E. W., M. B. Ingle**, and **G. D. Mercer**. 1973. *Bacillus alkalophilus* sub sp. *halodurans* subsp. nov. :an alkaline β-amylase-producing alkalophilic organism. Int. J. Syst. Bacteriol. 23: 238-242.

42. **Boyle, C.D.**and **A.E. Reade.** 1983. Characterisation of two extracellular polysaccharides from marine bacteria. Appl. Enviro. Microbiol. 46: 392 –399.

43. **Breeuwer, P., J. C-Drocourt., F. M. Rombouts** and **T. Abee**. 1996. A novel method for continuous determination of the intracellular pH in bacteria with the internally conjugated fluorescent probe 5 (and 6-)- carboxyfluorescein succinimidyl ester. Appl. Environ. Microbiol. 62:178-183.

44. **Bretzel, W., W.Schurter, B.Ludevig, E. Kupfer, S. Doswald, M. Pfister** and **A.P.G.M.Vanloon**. 1999. Commercial riboflavin production by recombinant *Bacillus subtilis*: down stream processing and comparison of the composition of riboflavin produced by fermentation or chemical synthesis.J.Ind. Microbiol. Biotechnol. 22: 19-26.

45. **Brock, T.D.** 1986. Thermophiles In: General Molecular and Applied Microbiology. John Wiley and sons, New York.

46. **Brown, S., H. Constantino** and **R. Kelly**.1990. Characterisation of amylolytic enzymes associated with the hyperthermophilic archaebacterium *Pyrococcus furiosus.* Appl. Environ. Microbiol. 56:985-1991.

47. **Buck, D.P** and **G.D. Smith**.1995. Evidence for Na^+/ H^+ electrogenic antiporter in an alkalophilic *Cyanobacterium synechocystis*. FEMS Microbiol. Lett.128: 315-320.

48. **Bunthof, J., S.Braak., P.Breeuwer, F.M.Romobouts** and **T. Abee**. 1999. Rapid fluorescence assessment of viability of stressed *Lactococcus lactis*. Appl. Env. Microbiol. 65:3681-3689.

49. **Cadmus, M. C., Knutson, C. A., Lagoda, A. A., Pittsley, J. E.** and **Burton, K. A.** 1978. Synthetic media for the productin of quality xanthan gum in 20-liter fermentors. Biotechnol. Bioeng. 20: 1003-1014.

50. **Cao, J., L . Zheng** and **S. Chen**. 1992. Screening of pectinase producer from alkalophilic bacteria and study on its potential application in degumming of ramie. Enzyme Micro. Technol. 14:1013-1016.

51. **Catley, B. J.** 1971. Utilization of carbon sources by *Pullularia pullulans* for the elaboration of extracellular polysaccharide. Appl. Microbiol. 22: 641-649.

52. **Catley, B.J.** 1979. Pullulan synthesis by *Aureobasidium pullulans*. In: "Microbial polysaccharide and Polysaccharases" (R.C.W.Berkeley, G.W.Gooday and D.C.Ellwood, Eds.) Pg. 69-84. Academic Press, London, New York and San Franciso.

53. **Cerning, J., C. M. G. C. Renard, J. F. Thibault, C. Bouillanne, M. Landon, M. Desmazeaud** and **L. Topisirovic**. 1994. Carbon source requirements for exopolysaccharide production by *Lactobacillus casei* CG11 and partial structure analysis of the polymer. Appl. Environ. Microbiol. 60: 3914-3919.

54. **Chauthaiwale,J.** and **M.Rao.** 1994. Production and purification of extracellular *D*-xylose isomerase from an alkaliphilic, thermophilic *Bacillus* sp. Appl. Environ. Microbiol 60:4495-499.

55. **Cheng, S. W., H. M. Hu, S. W. Shen, H. Takagi, M. Asano** and **Y. C. Tsai**. 1995. Production and characterisation of keratinase of a feather – degrading *Bacillus licheniformis* PWD-1. Biosci. Biotechnol. Biochem. 59:2239-2243.

56. **Cheung, H.Y., S.Q.Sun, B.Sreedhar, W.M.Ching** and **P.A.Tanner.** 2000. Alterations in extracellular substances during the biofilm development of *Pseudomonas aeruginosa* on aluminium plates. J.Appl.Microbiol. 89:100-106.

57. ***Chislet, M.E.** and **D.J.Kushner**, 1961. A strain of *Bacillus circulans* capable of growing under highly alkaline conditions. J. Gen. Microbiol. 24:187-190 (cited in Ref. No. 157).

58. **Chitarra L.G, P.Breeuwer, R.W.V. Bulk** and **T.Abee**. 2000. Rapid fluorescence assessment of intracellular pH as a viability indicator of *Clavibacter michiganensis* subsp. *michiganensis*. J. Appl. Microbiol. 88: 809-816.

59. **Christensen B.E., J. Kjosbakken** and **O.Smidsrod.** 1985. Partial, chemical and physical characterisation of two extracellular polysaccharides produced by marine periphytic *Pseudomonas* sp. strain NCMB 2021. Appl. Environ.Microbiol. 50: 837 –845.

60. **Chua, N.H.** 1980. Electrophoretic analysis of chloroplast proteins. Methods Enzymol. 69:434-446.

61. **Clejan, S., T.A.Krulwich, K.R. Mondrus** and **D.Seto**. 1986. Membrane lipid composition of obligately and facultatively alkaliphilic strains of *Bacillus* sp. J. Bacteriol. 168, 334-340.

62. **Collins, M.D., B.M. Lund, J. A. E. Farrow** and **K. H. Schliefer**. 1983. Chemotaxonomic study of an alkaliphilic bacterium *Exiguobacterium aurantiacum* gen. nov. sp. nov. J. Gen Microbiol. 129:2037-2042.

63. **Cook, M. G., J. B. Russel, A.Reichert** and **J.Wiegel**. 1996. The intracellular pH of *Clostridium paradoxium*, an anaerobic alkalophilic and thermophilic bacterium. Appl. Environ. Microbiol. 62:4576-4579.

64. **Cooper, G. David** and **B.G.Goldenberg.** 1987. Surface active agents from two *Bacillus* sp. Appl. Environ. Microbiol. 53: 224 – 229.

65. **Costerton, J.W., K.J.Cheg, G.G.Geesey, T.I.Ladd, J.C.Nickle, M.Dasgupta** and **T.J. Marrie.** 1987. Bacterial biofilms in nature and disease. Ann. Rev. Microbiol. 41:435-464.

66. **Crabb, W.D.** and **C. Mitchinson**. 1997. Enzymes involved in the processing of starch to sugars. Trends in Biotechnol. 15:349-352.

67. **Danese, P.N., L.A.Pratt** and **R.Kolter.** 2000. Exopolysaccharide production is required for development of *Escherichia coli* K 12 Biofilm architecture. J.Bacteriol. 182 :3593-3596

68. **Dasilva, R., D. K. Yim, E. R. Asquieri** and **Y. K. Park**. 1993. Production of microbial alkaline cellulase and studies of their characteristics. Rev. Microbiol. 24:269-274.

69. **Davidson, M. W., K. A. Grayt, D. B. Knaff**. and **T. A. Krulwich**. 1988. Purification and characterization of two soluble cytochromes from the alkalophilic *Bacillus firmus* RAB. Biochim. Biophy. Acta. 933:470-477.

70. **Davis, C.M.** 1998. Making a living at the extremes. Trends in Biotechnol. 16: 102-104.

71. **De Philippis, R., M.C.Margheri, E. Pelosi** and **S.Ventura** 1993. Exopolysaccharide production by unicellular *Cyanobacterium* isolated from a hypersaline habitat. J. Appl Phycology. 5:387-394.

72. **De Philippis, R. R.** and **M. Vincenzini**. 1998. Exocellular polysaccharide from *Cyanobacteria* and their possible applications. FEMS Microbiol. Rev. 22: 151-175.

73. **De Vuyst, L.** and **B. Degeest**. 1999. Heteropolysaccharides from Lactic acid bacteria. FEMS Microbiol. Rev. 23: 153 – 177.

74. **Decho, W. A.** 1990. Microbial exopolymer secretions in ocean Environments: Their Roles in food webs and marine processes. Oceanog. Mar. Biol. Annu. Rev. 28: 73 – 153.

75. **Degeest, B.** and **L De Vuyst** 1999. Indication that the nitrogen source influences both amount and size of exopolysaccharides produced by *Streptococcus thermophilus* L Y O 3 and modelling of the bacterial growth and exopolysaccharide production in a complex medium. Appl. Environ. Microbiol. 65: 2863 – 2870.

76. **Desai, D.J.** and **I.M.Banat** 1997. Microbial production of surfactants and their commercial potential. Microbiol. Mol. Bio. Rev. 61: 47-64.

77. **Desmarais D.P., E. Jabalonski, N.S. Fedarko** and **M. F. Roberts**. 1997. 2- sulfatrehalose; a novel osmolyte in haloalkaliphilic archea. J. Bacteriol. 179:3146-3153.

78. **Desouza A.M.** and **I.W. Sutherland** 1994. Exopolysaccharide and storage polymer production in *Enterobacter aerogenes* type 8 strains. J. Appl. Bacteriol 76: 463 – 468.

79. **Deziel, E. , G. Paquette, R. Villemur, F. Lepine** and **J.G.Bisaillon**. 1996. Biosurfactant production by a soil *Pseudomonas* strain growting on polycyclic aromatic hydrocarbons. Appl. Environ. Microbiol. 62 : 1908 – 1912.

80. **Dimitrov, P.L., M.S.Kambourova, R.D. Mandeva** and **E. I. Emanuilova**. 1997.Isolation and characterization of xylan-degrading alkali-tolerant thermophiles. FEMS Microbiol. Lett. 157:27-30.

81. **Dische, Z.** and **L.B.Shettles**. 1948. A specific colour reaction of methyl pentoses and a spectrophotometric micro method for their determination. J. Biol.Chem.175: 595-603.

82. **Dodgson K.S.**and **R.C. Price**. 1962. A Note on the determinatiion of the ester sulphate content of Sulphate polysaccharides Biochem J. 84: 106-110.

83. ***Downie, A.W.**and **J. Cruickshank** 1928.The resistance of *Streptococcus faecalis* to acid and alkali media. Brit. J. Exp. Pathol. 9:171-173 (cited in Ref. No. 157).

84. **Duckworth, A. W., W. D. Grant, B. E. Jones** and **R. Vansteenbergen**. 1996. Phylogenetic diversity of soda lake alkaliphiles. FEMS Microbiol. Ecol. 19: 181-191.

85. **Dunkley, E.A., A. A. Guffanti, S. Clejan** and **T.A. Krulwich**. 1991. Facultative alkaliphiles lack fatty acid desaturase activity and loose the ability to grow at near neutral pH when supplemented with unsaturated fatty acid. J. Bacteriol. 173:1331-1334.

86. **Engle, M., Y.H. Li, C. Woese** and **J. Wiegel**. 1995. Isolation and characterization of a novel alkali tolerant thermophile, *Anaerobranca horikoshii* gen. nov. sp. nov. Int. J. Syst. Bacteriol. 45: 454-461.

87. **Escalante, A., C.W.Rodarte, M. G. Garibay** and **A. Farres**. 1998. Enzymes involved in carbohydrate metabolism and their role on exopolysaccharide production in *Streptococcus thermophilus*. J.Appl. Microbiol. 84 : 108 – 114.

88. **Egas, M.C.V., M.S.Da Costa** and **D. A. Cowan** 1998. extracellular α-amylase from *Thermus filiformis* ork A2: purification and biochemical characterization. Extremophiles. 2:23-32.

89. **Farres, J., G. Caminal** and **J. Lopez Santin**. 1997. Influence of Phosphate on rhamnose containing exopolysaccharide rheology and production by *Klebsiella* I-714. Appl. Microbiol. Biotechnol. 48: 522- 527.

90. **Ferris, F.J., S.Schultze, T.C.Witten, W.S.Fyee** and **T.J.Beveridge**. 1989 Metal Interactions with microbial Biofilms in acidic and neutral pH environments. Appl. Environ. Microbiol. 55:1249-1257.

91. **Fett, W.F., J.M.Wells, P.Cescutti** and **C.Wijey** 1995. Identificatiion of exopolysaccharides produced by fluorescent *Pseudomonads* associated with commercial Mushroom (*Agaricus bisporous*) production. Appl. Environ. Microbiol. 61: 513 – 517.

92. **Filisetti, M.C.C.** and **N.C.Carpita** 1991. Measurement of uronic acids without interference from neutral sugars. Biochemistry 197: 157 – 162.

93. **Florenzano, G., C.Sili, E.Pelosi** and **M. Vincenzine.** 1985. *Cyanospira rippkae* and *Cyanospira capsulata* (gen. nov. and sp. nov.): New filamentous heterocystous cyanobacteria from Magadi lake (Kenya). Arch. Microbiol. 140: 301-306.

94. **Fogarty, M.W., P.J. Griffith** and **A.M. Joyce**. 1974. Enzymes of *Bacillus* species-Part 1. Process Biochem. 9:11-24.

95. **Fry, K.N., J.K.Fredrickson, S.Fishbain, M. Wagner** and **D.A.Stahl.** 1997. Population structure of microbial communities associated with two deep, anaerobic alkaline aquifers. Appl. Environ. Microbiol. 63: 1498-1504.

96. **Fujiwara, N., K. Yamamoto** and **A. Masui.** 1991.Utilization of thermostable alkaline protease from an alkalophilic thermophile for the recovery of silver from used X-ray film. J. Ferment. Bioeng. 72: 306-308.

97. **Fujiwara, N., A. Masui** and **T. Imanaka.** 1993. Purification and properties of the highly thermostable alkaline protease from an alkaliphilic and thermophilic *Bacillus* sp. J. Biotechnol. 30: 245-256.

98. **Fukumori, F., T.Kudo** and **K. Horikoshi.** 1985. Purification and properties of a cellulase from an alkalophilic *Bacillus* sp. no. 1139. J. Gen. Microbiol. 131: 3339 – 3345.

99. **Fukumori, F., T.Kudo** and **K. Horikoshi.** 1987. Truncation analysis of an alkaline cellulase from an alkaliphilic *Bacillus* sp. FEMS.Microbiol. Lett. 40: 311 – 314.

100. **Gamar,L., K.Blondeau** and **J.M.Simonet.** 1997. Physiological approach to extracellular polysaccharide production by *Lactobacillus rhamnosus* strain C83. J. Appl. Microbiol. 83: 281 – 287.

101. **Garcia, M.L, A.A. Guffanti** and **T.A. Krulwich.** 1983. Characterisation of the Na^+/H^+ antiporter of alkalophilic *Bacilli* in vivo; $\Delta \psi$ dependent $^{22}Na^+$ efflux from whole cells. J. Bactriol. 156:1151-1157.

102. **Garg, A. P., J. C. Roberts** and **A. J. McCarthy.** 1998. Bleach boosting effect of cellulase free xylanase of *Streptomyces thermoviolaceus* xylanase preparations on birchwood kraft pulp. Enzyme Microbiol. Technol. 18:261-267.

103. **Gas, Coyne, D. J., J. A. Connor** and **A. T. Bull.** 1991. Isolation of bacteria producing siderophores under alkaline conditions. Appl. Microbiol. Biotechnol. 36: 130-135.

104. **Gas, Coyne, D. J., J. A. Connor** and **A. T. Bull.** 1991. Capacity of siderophore-producing alkalophilic bacteria to accumulate iron, gallium and aluminium. Appl. Microbiol. Biotechnol. 36: 136-141.

105. **Geddie, L.J.**, and **I.W.Sutherland** 1993. Uptake of metals by bacterial polysaccharides. J.Appl. Bacteriol 74: 467 – 472.

106. **Gee. J. M., B. M. Lund, G. Metcalf** and **J. L. Peel.** 1980. Properties of a new group of alkalophilic bacteria. J. Gen. Microb. 117: 9-17.

107. **Georganta, G., T. Kaneko, N. Nakamura, T. Kudo** and **K. Horikoshi.** 1993. Isolation and partial properties of cyclomaltodextrin glucanotransferase producing alkaliphilic *Bacillus* sp. from a deep-sea mud sample starch. 45: 95-99.

108. **Gerasimenko, L. M., A. V. Dubinin** and **G. A. Zavarzin.** 1996. Alkaliphilic *Cyanobacteria* from soda lakes of Tuva and their ecophysiology. Microbiology. 65: 736-740.

109. **Gessesse, A.** 1998. Purification and properties of two thermostable alkaline xylanases from an alkaliphilic *Bacillus* sps. Appl. Environ. Microbiol. 9: 3533 – 3535.

110. ***Gibson, T.** 1934. An investigation of *Bacillus pasteurii* gp. II, Special physiology of organisms. J. Bacteriol. 28:313-322 (cited in Ref. No. 157).

111. **Giesecke, U.E., G.Bierbaum, H.Rudde, U.Spohn** and **C.Wandrey** 1991. Production of alkaline protease with *Bacillus licheniformis* in a controlled fed batch process Appl. Micriobiol. Biotechnol. 35: 720 – 724.

112. **Gilmour, R.** and **T.A. Krulwich**. 1996. Purification and characterization of the succinate dehydrogenase complex and co-reactive b-type cytochromes from the facultative alkalophile *Bacillus firmus* OF4. Biochem. Biophy. Acta. 1276: 57-63.

113. **Gilmour, R., P.messner, A.A.Guffanti, R.Kent, A.Schebrel, N.Kerdrick** and **T.Krulwich**. 2000. Two-dimensional gel electrophoresis analyses of pH dependent protein expression in facultatively alkaliphilic *Bacillus pseudofermus* OF4 lead to characterisation of an S – Layer Protein with a role in alkaliphily. J.Bacteriol 182: 5969 – 5981.

114. **Giraud, E., L. Gosselin, B. Marin, J.L. Parada** and **M. Raimbault**. 1993. Purification and characterisation of an extracellular amylase from *Lactobacillus plantarium* strain A 6. J. Appl. Bacteriol. 75:276-282.

115. **Glymph, J.L.** and **F.J. Stutzenberger.** 1977. Production, purification and characterisation of ∝- amylase from *Thermomonospora curvata.* Appl Environ. Microbiol.34: 391-397.

116. **Gordon, R.E.** 1980. 107 years of the genus *Bacillus*. In: the aerobic endospore forming bacteria: classification and identification. Edited by R.C.W. Berkeley and M.Goodfellow. London: Academic Press Pg.1 – 15.

117. **Gordon, R.E.** and **J.L.Hyde**. 1982. The *Bacillus firmus – Bacillus lentus* complex and pH 7.0 variants of some alkalophilic strains. J.Gen.Microbiol. 128: 1109 – 1116.

118. **Goodfellow, M.** and **E. Minikin**. 1985. Introduction to chemosystematics. Chemical methods in bacterial systematics. Academic Press Ltd., London.

119. **Grant, D.W., A. A. Mills** and **A.K.Schofield**.1979 .An alkalophilic sp of *Ectothiorhodospira* from a Kenyan soda lake. J. Gen. Microbiol. 110, 137-142.

120. **Grant, D.W** and **K.Horikoshi**. 1989. Alkaliphiles In: Microbiology of Extreme environments and its potential for biotechnology. Elseiver Science Publishers. Pg 346-366.

121. **Grant, W. D., W. E. Mwatha** and **B. E. Jones**.1990. Alkaliphiles: Ecology diversity and applications FEMS. Microbio. Rev. 75: 255-270.

122. **Grant, S., W.D.Grant, B.E.Jones,** and **C.K.L.Li.** 1999. Novel archaeal phylotypes from an East African alkaline saltern. Extremophile 3: 139 – 145.

123. **Grobben, G.J., I.Chin Joe, V.A. Kitzen** *et al.* 1998. Enhancement of exopolysaccharide production by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772 with a simplfied defined medium. Appl. Environ. Microbiol. 64: 1333 – 1337.

124. **Guerra-Santos, L. H., O. kappeli** and **A. Flechter**. 1986. Dependence of *Pseudomonas aeruginosa* continuous culture biosurfactant production on nutritional and environmental factors. Appl. Microbiol. Biotech. 24: 443-448.

125. **Guffanti, A. A., P. Susman., R. Blanco** and **T.A. Krulwich**. 1978. The proton motive force and ∝-amino iso-butyric acid transport in an obligately alkaliphilic bacterium. J. Biol. Chem. 253:708-715.

126. **Guffanti, A. A., R. Blanco; R. A. Benenson** and **T. A. Krulwich.** 1980. Bioenergetic properties of alkaline tolerant and alkalophilic strains of *Bacillus firmus*. J. Gen. Microbiol. 119:79-86.

127. **Guffanti, A.A., D.E.Cohn, H.R.Kaback** and **T.A.Krulwich**. 1981. Relationship between the Na $^{+}$/H^{+} antiporter and Na^{+}/Substrate symport in *Bacillus alcalophilus*. Proc. Natl. Acad. Sci. USA 78: 1481-1484.

128. **Guffanti, A.A.** and **H.C.Eisenstein** 1983. Purification and characterisation of flagella from the alkalophilic *Bacillus firmus* RAB. J.Gen. Microbiol. 129: 3239 – 3242.

129. **Guffanti, A.A., O. Finkelthal., D.B. Hicks., L. Falk., A. Sidhu., A. Garro** and **T.A. Krulwich.** 1986. Isolation and characterization of New facultatively alkalophilic strains of *Bacillus* sp. J. Bacteriol. 167:766-733.

130. **Guffanti, A. A** and **E. Hou.** 1987. The effect of pH on the passive proton conductance of *Bacillus acidocaldarius*. FEMS Microb. Lett. 41: 275-278.

131. **Guffanti, A.A** and **D.B.Hicks.** 1991. Molar Growth yields and bioenergetic parameters of extremely alkalophilic *Bacillus* sps in batch cultures & growth in a chemostat at pH 10.5. J. Gen. Microbiol.137: 2375-2379.

132. **Gupta, S., B.Bhushan** and **G.S.Hoondal.** 2000. Isolation, purification and characterisation of xylanase from *Staphylococcus* sps SG 13 and its appplication in biobleaching of Kraft pulp. J.Appl. Microbiol. 88: 325 – 334.

133. **Hakamada, Y., K. K. Koike, T. Yoshimatsu, H. Mori, T. Kobayashi** and **S. Ito.** 1997. Thermostable alkaline cellulase from an alkaliphilic isolate, *Bacillus* sp KSM-S 237. Extremophiles. 1:151-156.

134. **Hamamoto, T. M. Hashimoto, M. Hino, M. Kitada, Y. Seto, T. Kudo** and **K. Horikoshi.** 1994. Characterisation of a gene for Na $^{+}$ /H^{+} antiporter system of alkalophilic *Bacillus* sps. strain C-125 Mol. Microbiol. 14: 939 – 946.

135. **Hamana, K., T. Akiba., F. Uchino** and **S. Matsuzaki.** 1989. Distrubution of spermine in Bacilli and Lactic acid bacteria. Can. J. Microbiol. 35:450-455.

136. **Hamasaki, N., S. Shirai, M.Niitsu, K. Kakinuma** and **T. Oshima.** 1993. An alkalophilic *Bacillus* sp. produces 2-phenylethylamine. Appl. Environ. Microbiol. 59: 2720-2722.

137. **Hancock,I.C.** and **I.R.Poxton.** 1988. Bacterial cell surface techniques. John Wiley and Sons, New York.

138. **Hansen, S.Jacob** and **B.K.Ahring.** 1997. Anaerobic microbiology of an alkaline Icelandic hot spring. FEMS Microbiol. Ecol. 23:31-38.

139. **Hartman, P.A., R.Willerson** and **P.A.Tetrault.** 1995. *Bacillus stearothermophilus* 1: Thermal and pH stability of the amylase. Appl. Microbiol. 3:7-10.

140. **Hashimoto, M., T.Hamamoto, M.Kitada, M. Hino, T.Kudo** and **K. Horikoshi.** 1994. Characterisation of alkali sensitive mutants of alkaliphilic *Bacillus* sps strain C-125 that show cellular morphological abnormalities. Biosci. Biotechnol. Biochem. 58:2090-2092.

141. **Hayashi, T,. T.Akiba** and **K.Horikoshi.** 1988. Production and purification of new maltohexose forming amylase from alkalophilic *Bacillus* sp. H-467. Agri. Biol. Chem. 52:443-448.

142. **Hayashi, T,. T. Akiba** and **K. Horikoshi**. 1988. Properties of new alkaline maltohexaose – forming amylases. Appl. Microbiol. Biotechnol. 28:281-285.

143. **Helm, R.F., Z.Huang, D.Edwards, H.Leeson, W.Peery** and **M.Potts**. 2000. Structural characterisation of the released polysaccharide of Desiccation – tolerant *Nostoc commune* DRH–1, J.Bacteriol 182: 974-982.

144. **Hicks, D. B.** and **T. A. Kurlwich**. 1986. The membrane ATPase of alkaliphilic *Bacillus firmus* RAB is an F_1- type ATPase. J. Biol. Chem. 261:12896-12902.

145. **Hirota, N.** and **Y. Imae**. 1983. Na^+ driven flagellar motors of an alkalophilic *Bacillus* strain YN-1. J. Biol. Chem. 258:10577-10581.

146. **Hisatsuka, K.I., T. Nakahara, N. Sano** and **K. Yamada**. 1971. Formation of Rhamnolipid by *Pseudomonas aeruginosa* and its function in hydrocarbon fermentation. Agric. Biol. Chem. 35:686 - 692.

147. **Holdom, R.S.** and **A.G.Turner**. 1969. Growth of *Mycobacterium rhodochrous* on *n*-Decane: a new growth factor and emulsifying agent. J. Appl. Bacteriol. 34: 448 – 456.

148. **Honda, H., T. Kudo, Y. Ikura** and **K. Horikoshi**. 1985. Two types of xylanases of alkalophilic *Bacillus* sps no. C-125. Can. J. Microbiol. 31: 538 – 542.

149. **Honda, H., T, Kudo** and **K. Horikoshi**. 1985. Purification and partial characterization of alkaline xylanase from *E.coli* carrying pCX311. Agic. Biol. Chem. 49: 3165-3169.

150. **Horan N.J., T.R.Jarman** and **E.A.dawes**. 1981. Effects of carbon source and inorganic phosphate concentration on the production of algenic acid by a mutant of *Azotobacter vinelandii* and on the enzymes involved in the synthesis. J.Gen Microbiol. 127: 185-191.

151. **Horikoshi, K**. 1971. Production of alkaline enzymes by alkaliphilic microorganisms part I. Alkaline protease produced by *Bacillus* No. 221. Agric. Biol. Chem. 36:1407-1414.

152. **Horikoshi, K**. 1971. Production of alkaline enzymes by alkaliphilic microorganisms II. Alkaline amylase produced by alkaline *Bacillus* no.A-40-2. Agric. Bio. Chem. 35:1783-1791.

153. **Horikoshi, K**. 1972. Production of alkaline enzymes by alkaliphilic microorganisms part III. Alkaline pectinase of *Bacillus* No. P-4-N. Agric. Bio. Chem. 36:285-293.

154. **Horikoshi, K**. and **Y. Atsukawa.** 1973b. Xylanase produced by alkaliphilic *Bacillus* No. C-59-2. Agric. Bio. Chem. 37:2097-2103.

155. **Horikoshi, K**. and **T. Akiba**. 1982. In: Alkaliphilic microorganisms: A new microbial world. Springer- Verlag KG; Heidelberg; Germany.

156. **Horikoshi, K., M. Nakao, Y.Kurono** and **N.Sashihara**. 1984. Cellulases of an alkalophilic *Bacillus* strain isolated from soil. J. Microbiol. 30: 774-779.

157. **Horikoshi, K**. 1991. In: Microorganisms in Alkaline environments. Kodansha. VCH, Japan.

158. **Horikoshi, K**. 1996.Alkaliphiles- from an industrial point of view. FEMS. Microb. Rev. 18:259-270.

159. **Horikoshi, K**.1999.Alkaliphiles: Some applications of their products for Biotechnology. Microbiol. and Mol. Biol. Rev. 63: 735-750.

160. **Hussain, M.**, **M.H.Wilox** and **P.J.White**. 1993. The slime of coagulase – negative *staphylococci*: Biochemistry and relation to adherence FEMS Microb. Rev. 104: 191-208.

161. **Igarashi, k., Y. Hatada, H.Hagihara, K.saeki, M.Takaiwa, T.Uemura, K.Ara, K.Ozaki, S.Kawai, T.Kobayashi** and **S.Ito**. 1998. Enzymatic properties of novel liquefying α-amylase from an alkalophilic *Bacillus* isolate and entire nucleotide and amino acid sequences. Appl. Environ.Microbiol. 64: 3282-3289.

162. **Ikura Y.** and **K.Horikoshi**. 1977. Isolation and some properties of alkalophilic bacteria utilizing rayon waste. Agri.Biol. Chem. 41:1373-1377.

163. **Ingle, M.B.** and **R.J.Erikson**. 1978. Bacterial ∝-amylases. Adv. Appl. Microbiol. 24:257-279.

164. **Ito, S., S.Shikata, K.Ozaki, S.Kawai K.I. Okamoto, A.takei, y. Ohta** and **T.Satoh**. 1989. Alkaline cellulase for laundry detergents: production by *Bacillus* sp. KSM-635 and enzymatic properties. Agri. Biol. Chem. 53:1275-1281.

165. **Ito, S.** 1997. Alkaline cellulases from alkalophilic *Bacillus*: Enzymatic properties, genetics, and application to detergents. Extremophiles. 1:61-66.

166. **Ito, M., A. A. Guffanti., J. Zemsky., D. Mackivey** and **T.A.Krulwich**. 1997. Role of the nha. C encoded Na^+/H^+ antiporter of the alkaliphilic *Bacillus firmus* OF4.J. Bacteriol. 179:3851-3857.

167. **Itoh, S., H. Honda, F. Tomita** and **T. Suzuki.** 1971. Rhamnolipids produced by *Pseudomonas aeruginosa* grown on *n* – paraffin. J.Antibiot. 12: 855-860.

168. **Jain, D. K., Thompson, D. L .C., Lee, H.** and **Trevors, J .T.** 1991. A drop collapsing test for screening surfactant producing microorganism. J. Microbiol. Methods. 13: 271-279.

169. **Janda, L., T. Pavelka, P. Tichy, J. Spizek** and **M. Petricek**. 1997. Production and properties of ∝-glucosidases from the thermotolerant bacterium *Thermomonospora curvata.* J. Appl. Microbiol. 83:470-476.

170. **Jansson, P.E., L. Kenne** and **B. Lindberg**. 1975. Structure of the extracellular polysaccharide from *Xanthomonas campestris*. Carbohydrate Research 45: 275 – 282.

171. **Jarman, T.R., L.Deavin, S.Slocombe** and **R.C.Righelato**. 1978. Investigation of the effects of environmental conditions on the rate of exopolysaccharide synthesis in *Azotobacter vinelandii.* J. Gen. Microbiol. 107 : 59-64

172. **Jarman, T.R.**and **G.W.Pace.** 1984. Energy requirements for microbial exopolysaccharide synthesis Arch.Microbiol. 137: 231-235.

173. **Jayaraman, J.** 1996. In: Laboratory manual in Biochemistry. New Age International Ltd. Pub. New Delhi. India.

174. **Jones, B. E., W. D. Grant, A. W. Duckworth** and **G. G. Owenson**. 1998. Microbilal diversity of Soda Lakes. Extremophiles. 2:191-200.

175. **Kanekar, P.P., S.S.Sarnaik** and **A.S.Kelkar**. 1999. Bioremediation of phenol by alkaliphilic bacteria isolated from alkaline lake of Lonar, India. J. Appl. Microbiol. Symposium Supplement. 85:128 S – 133 S.

176. **Kano, M.** 1986. A *Bacillus acidocaldarius* α-amylase that is highly stable to heat under acidic conditions. Agric. Biol. Chem. 50: 23-31.

177. **Kaplan, D., D.Christiaen** and **S.Arad**. 1987. Chelating properties of extracellular polysaccharide from *Chlorella* sp. Appl. Environ. Microbiol. 53: 2953-2956.

178. **Kaplan, N. Z. Zosim** and **E.Rosenberg**. 1987. Reconstitution of emulsifying activity of *Acinetobacter calcoaceticus* BD4 Emulsan by using pure polysaccharide and protein. Appl. Environ. Microbiol. 53: 440 – 446.

179. **Karlsson, S. Z., G.Banhide** and **A.C.Alberssen**.1988.Identification and characterization of alkali-tolerant *Clostridia* isolated from biodeteriorated casein-containing building material. Appl. Microbiol. Biotechnol. 28: 305-310.

180. **Kates, M**. 1986. Techniques of Lipidology. Isolation, analysis and identification of lipids. Elseiver, Amsterdam, New York, Oxford.

181. **Kato, C., A. Inoue** and **K.Horikoshi**. 1996. Isolating and characterizing deep-sea marine microorganisms. Trends in Biotechnol. 14: 6-12.

182. **Kazamias, M.T**. and **Jay F.Sperry**. 1995. Enhanced fermentation of mannitol and release of cytotoxin by *Clostridium difficile* in alkaline culture media. Appl. Environ. Microbiol. 61:2425-2427.

183. **Keller, M., F. J. Braun., R. Dirmeir., D. Hafenbradl., S. Burggraf., R. Rachel** and **K. U. Stetter**. 1995. *Thermophilus alcaliphilus* sp. nov. , a new hyperthermophilic archaeum growing in polysulfide at alkaline pH. Arch. Microbiol. 164:390-395.

184. **Kelly, C.T**. and **M.W.Fogarty**. 1978. Production and properties of polygalacturonate lyase by an alkalophilic microorganism, *Bacillus* sps. R.K.9. Can. J. Microbiol. 24: 1164-1172.

185. **Kelly, C.T., F.O'Reilly** and **W.M. Fogarty**. 1983. Extracellular ∝-glucosidase of an alkalophilic microorganism *Bacillus* sp ATCC 21591. FEMS Microbiol. Lett. 20:55-59.

186. **Kelly, C.T., M. A. Mctigue, E. M. Doyle** and **W.M. Fogarty**. 1995. The raw starch degrading alkaline amylase of *Bacillus* sp IMD 370. J. Ind. Microbiol. 15:446-4448.

187. **Kevbrin, V.V., T.N.zhilina, F.A.Rainey** and **G.A.Zavarzin**. 1998. *Tindallia magadii* gen.nov.,sp.nov, an alkalophilic anaerobic ammonifier from soda lake deposits.Curr.Microbiol.37: 94-100.

188. **Khmelenina, V.N., M.G.Starostina, N.E.Suzina** and **Y.A.Trotsenko**. 1997.Isolation and characterization of halotolerant alkalophilic methanotrophic bacteria from Tuva soda lakes.Curr.Microbiol.35: 257-261.

189. **Kim S.H., S. Ramaswamy** and **J.Downward**. 1999. Regulated exopolysaccharide production in *Myxococcus xanthus*. J. Bacteriol 181: 1496- 1507.

190. **Kim, T.U., B.G.G.U, J.Y. Joeng, S.M. Byun** and **Y.C.Shin**. 1995. Purification and characterisation of maltotetrose forming alkaline ∝-amylase from an alkalophilic *Bacillus* strain GM 8901. Appl. Environ. Microbiol 61:3105-3112.

191. **Kimura, T**. and **K.Horokoshi**. 1988.Isolation of bacteria which can grow at both high pH and low temperature. Appl. Env. Microb .54:1066-1067.

192. **Kimura, K.A. Tsukamoto, Y. Ishii, T.Takano** and **K. Yamane**. 1988. Cloning of a gene for maltohexose producing amylase of an alkalophilic *Bacillus* and hyperproduction of the enzyme in *Bacillus subtilis* cells. Appl. Microbiol. Biotechnol. 27:372-377.

193. **Kimura, T.,** and **K. Horikoshi**. 1989. Carbohydrate use by alkalopsychrotrophic bacteria. Agri. Biol. Chem. 53: 2019-2020.

194. **Kimura, T.,** and **K. Horikoshi**. 1989. Production of almylase and pullulanase by an alkalopsychrotrophic *Micrococcus* sp. Agric. Biol. Chem. 53:2963-2968.

195. **Kimura, T.** and **K. Horikoshi**. 1990. Characterisation of Pullulan – hydrolysing enzyme from an alkalo-psychrotrophic *Micrococcus* sp. Appl. Microbiol. Biotechnol. 34: 52-56.

196. **Kimura, H., Okamura A** and **H. Kawaide**. 1994. Oxidation of 3,7 and 12 hydroxyl groups of cholic acid by an alkaliphilic *Bacillus* sp. Bioc. Biotechnol. Biochem. 58:1002-1006.

197. **Kitada, M.** and **K.Horikoshi**. 1977. Na^+ ion stimulated α-(14c) amino isobutyric acid uptake in alkaliphilic *Bacillus* sp. J. Bacteriol. 131(3), 784-788.

198. **Kitada, M., A.A.Guffanti** and **T.A.Krulwich** 1982. Bioenergetic properties and viability of alkalophilic *Bacillus firmus* RAB as a function of pH and Na^+ contents of the incubation medium.J.Bacteriol. 152: 1096-1104.

199. **Kitada, M.** and **T.A.Krulwich**. 1984. Purification and characterization of the cytochrome oxidase from alkaliphilic *Bacillus firmus* RAB. J. Bacteriol. 158:963-966.

200. **Kitada, M., I. Wijayanti** and **K.Horikoshi**. 1987. Biochemical properties of a thermophillic alkalophile. Agri.Biol.Chem. 51:2429-2435.

201. **Kitada, M., K. Onda** and **K. Horikoshi** 1989. The different Na^+ /H^+ antiport system in a newly isolated alkalophilic *Bacillus* sp. J. Bacteriol. 171:1879-1884.

202. **Kitada, M., M.Hashimoto, T.kudo** and **K.Horikoshi**. 1994. Properties of two different Na^+/H^+ antiport systems in Alkalophilic *Bacillus* sp. strain. C-125. J.Bacteriol. 176:6464-6469.

203. **Kitada, M., S. Morotomi, K. Horokoshi** and **T.Kudo**. 1997. K^+/ H^+ antiporter in alkalophilic *Bacillus* sp. no. 66 (JCM 9763). Extremophiles 1:135-141.

204. **Kobayashi, Y.** and **K.Horikoshi**. 1980. Identification and growth characterization of alkaliphilic *Coreynebacterium* sp. which produces NAD (P)- dependent maltose dehydrogenase and glucose dehydrogenase. Agri. Biol. Chem. 44(1): 41-47.

205. **Kobayashi, T., H. Kanai, T. Hayashi, T. Akiba, R. Akaboshi** and **K. Horikoshi**. 1992. Haloalkalophilic maltotriose forming ∝-amylase from the archebacterium *Natronococcus* sp. strain Ah-36. J.Bacteriol. 174:3439-3444.

206. **Kobayashi, T., H.Kanal, R.Aono, K.Horikoshi** and **T. Kuo**. 1994. Cloning, expression and nucleotide sequence of the amylase gene from the haloalkalophilic archaeon *Natronococcus* sp.strain Ah-36. J.Bacteriol 176: 5131-5134.

207. **Kobayashi, T., Y. Hakamada, S. Adachi, J. Hitomi, T. Yoshimatsu, K. Koike, S. Kawai** and **S. Ito**. 1995. Purification and properties of an alkaline protease from alkalophilic *Bacillus* sp. KSM-K16. Appl. Microbiol. Biotechnol.43:473-481.

208. **Koch, R.A., P Zablowski, A. Spreinat** and **G. Antranikian**. 1990. Extremely thermostable amylolytic enzymes from the archebacterium *Pyrococcus furiosus*. FEMS.Microbiol Lett. 71:21-26.

209. **Koch, R.A., K. Lemka** and **G.Antranikian**. 1991. Purification and properties of a hyperthermophilic ∝- amylase from the archebacterium *Pyrococcus woesei.* Microbiol. 572-578.

210. **Kojic, M., M.Vujcic, A.Banina, P.Cocconcelli, J.Cepning** and **L.Topisirovic.** 1992. Analysis of exopolysaccharide production by *Lactobacillus casei* CG11 isolated from cheese. Appl. Environ. Microbiol. 58: 4086 – 4088.

211. **Kosono, S., Y.Ohashi, F.Kawamura, M.kitada** and **T.Kudo**. 2000. Function of a principal Na^+/ H^+ antiporter, Sha A is required for initiation of sporulation in *Bacillus subtilis*. J. Bactriol .182:898-904.

212. **Kouker, G.** and **K.E.Jaeger**. 1987. Specific and sensitive plate assay for bacterial lipases. Appl. Environ. Microbiol. 53: 2111 – 2113.

213. **Koyama, N., H. Takinishi** and **Y. Nosoh.** 1983. A Possible relation of membrane proteins to the alkalostability of a facultatively alkalophilic *Bacillus* FEMS Microbiol. Lett. 16:213-216.

214. **Koyama, N.** and **Y. Nosoh** 1995. Effect of potassium and sodium ions on the cytoplasmic pH of a *Bacillus*, Biochim. Biophy. Acta. 812: 206 – 212.

215. **Krahe, M.G.Antranikian** and **H.Markl.** 1996. Fermentation of extremophilic microorganisms. FEMS. Microbiol.Rev.18: 271-285.

216. **Kroll, R.G.** 1990. Alkalophiles. In: Microbiology of Extreme Environments, Edward C. (Ed) McGraw Hill Publ company, New York Pg 55-92.

217. **Krulwich, A.T., R.Agus, M.Schneier** and **A. A. Guffanti**. 1985. Buffering capacity of *Bacilli* that grow at different pH ranges. J. Bacteriol. 162: 768-772.

218. **Krulwich, T.A.** 1986 Bioenergetics of alkalophilic bacteria J.Memb. Biol. 89: 113-125.

219. **Krulwich, T.A, A.A.Guffanti, M.Y.Fong, L.Falk** and **D.B.Hicks**. 1986 Alkalophilic *Bacillus firmus* RAB generates variants, which can grow at lower Na^+ concentration than the parental strain. J.Bacteriol. 185: 884-889.

220. **Krulwich, T.A., D.B.Hicks., D. Seto Young** and **A.A.Guffanti**. 1988. The bioenergetics of alkalophilic bacilli. Crit. Rev. Microbiol. 16:15-36.

221. **Krulwich, T.A** and **A.A.Guffanti**. 1989. Alkalophilic Bacteria. Annu.rev.Microbiol. 43:435-463.

222. **Krulwich, T.A**. 1995. Alkaliphiles: 'basic' molecular problems of pH tolerance and bioenergetics. Mol. Microbiol. 15: 403 – 410.

223. **Krulwich, T.A, M.Ito** and **R. Gilmour**. 1997. Mechanism of cytoplasmic pH regulation in alkalophilic strains of *Bacillus*. Extremophiles 1:163-169.

224. **Krulwich, T.A., I.Masahiro, G.Gilmour, D.B.Hicks** and **A.A.Guffanti**. 1998. Energetics of alkaliphilic *Bacillus* sp. Physiology and Molecules. Advances in Microbial Physiology 40: 401-436.

225. **Krulwich, T. A., M. Ito, D, B. Hicks, R. Gilmour** and **A. A. Guffanti**. 1998. pH homeostasis and ATP synthesis: Studies of two processes that necessitate inward proton translocation in extremely alkaliphilic *Bacillus* species. Extremophiles 2: 217-222.

226. **Kudo, T.**, and **K. Horikoshi**. 1979. The environmental factors affecting sporulation of an alkaliphilic *Bacillus* sp. Agri. Biol. Chem. 43, 2613-2614.

227. **Kudo, T. M. Hino, M. Kitada** and **K.Horikoshi**. 1990. DNA Sequences required for the alkalophily of *Bacillus* sp. Strain C-125 are located close together on its Chromosomal DNA. J.Bacteriol. 172: 7282 – 7283.

228. **Kurono, Y.** and **K. Horikoshi**. 1973. Alkaline catalase produced by *Bacillus* No. Ku-1. Agri. Biol Chem. 37, 2565-2570.

229. ***Kushner, D. J.** and **T. A. Lisson**. 1959. Alkali resistance in *Bacillus cereus*. J. Gen. Microbiol. 21:96-108 (cited in Ref. No. 157).

230. **Labare, M.P, K.Guthrie** and **R.M.Weiner**. 1989. Polysaccharide exopolymer adhesives from periphytic marine bacteria. Adhesion Sci. Technol. 3: 213 – 223.

231. **Lemoine, J., F. Chirat, J. M. Wieruszeski, G.Strecker, N. Favre** and **J.R.Nesser.** 1997. Structural characterisation of the Exocellular polysaccharides produced by *Streptococcus thermophilus* SFi39 and SFi12. Appl. Env. Microbiol. 63: 3512 – 3518.

232. **Lesuisse, E., Schanck, K** and **C. Colson**. 1993. Purification and preliminary characterisation of an extracellular lipase of *Bacillus subtilis* 168, an extremely alkaline pH-tolerant enzyme. Eur. J. 155-160.

233. **Lewis, R., J. E. kaback** and **T.A.Krulwich**. 1982. Pleotrophic properties of mutation to non-alkalophily in *Bacillus alcalophilus*. J. Gen Microbiol. 128: 427-430.

234. **Li, Y. H., M. Engle, N. Weiss, L. Mandelco** and **J. Wiegel**. 1994. *Clostridium thermoalcaliphilum* sp. nov, an anaerobic and thermotolerant facultative alkaliphile. Int. J. Syst. Bacteriol. 44:111-118.

235. **Lin, F.C., R.M.Brown, J.B.Cooper** and **D.P.Delmer**. 1985. Synthesis of Fibrils in vitro by a solubilised synthase from *Acetobacter xylinum*. Science 230: 822-825.

236. **Looijestein, P.J, I.C.Boels, M.Kleerebezem** and **J.Hugenholtz**. 1999. b. Regulation of exopolysaccharide production by *Lactococcus lactis* subsp. *cremoris* by the source of sugar. Appl. Environ. Microbiol. 65: 5003-5008.

237. **Looijestein, P.J., W.H.M.Van casteren, R. Tuinier, C.H.L. Doeswijk – Voragen** and **J.Hugenholtz**. 2000. Influence of different substrate limitations on the yield, composition and molecular mass of exopolysaccharides produced by *Lactococcus lactis* subsp. *cremoris* in continuous cultures. J. Appl. Microbiol 89: 116-122.

238. **Lowe, et al**. 1993. Anaerobic bacteria adapted to environmental stress. Microb. Rev. 57:483-492.

239. **Lowry, O.H., N.J.Rosebrough, A.Farr** and **R.J. Randell** 1953. Protein measurement with the Folin Phenol reagent. J of Biological Chem. 193: 265-275.

240. **Mac Cormick, C.A., J.E.Harris, A.J.Jay, M.J.Ridout, I.J.Colquhoun** and **V.J.Morris**. 1996. Isolation and characterisation of a new extracellular polysaccharide from an *Acetobacter* sp. J.Appl. Bacteriol. 81: 419 – 424.

241. **Madigan, T., T. Michael** and **B.L. Marrs**. 1997. Extermophiles. Scientific American, April 1997, 66-71.

242. **Malhotra, R. S.M. Noorwez** and **T.Satyanarayan**. 2000. Production and partial characterisation of thermostable and calcium independent ∝- amylase of an exterme thermophilic *Bacillus thermooleovorans*, NP 54. Lett. Appl. Microbiol.31: 378-384.

243. **Mamo, G.** and **A.Gessesse**. 1999. Effect of cultivation conditions on growth and ∝-amylase production by a thermophilic *Bacillus* sp. Lett. in Appl. Microbiol.29: 61-65.

244. **Manaca, C.M., L. Lama, R. Improta, E.Esposito, A. Gambacorta** and **B. Nicolaus.** 1996. Chemical composition of two exopolysaccharides from *Bacillus thermoantarcticus*. Appl. Environ. Microbiol. 62: 3265 – 3269.

245. **Marambe, M.** and **T. Ando**. 1992. Phenolic acids as potential seed germination inhibitors in animal waste composts. Soil science and plant nutrition. 38: 727-733.

246. **Mattimore, V.** and **J.R. Battista**. 1996. Radio resistance of *Deinococcus radiodurans* : Functions necessary to survive ionizing radiation are also necessary to survive prolonged desiccation. J.Bacteriol. 178 : 633 – 637.

247. **Mc Genity, T. J.** and **W. D. Grant**. 1993. The haloalkaliphilic archea *Natronococcus occultus* represents a distinct lineage within the halobacteria closely related to the other haloalkaliphilic lineage (*Natronobacterium*). Syst. Appl. Microbiol. 16:239-243.

248. **Mc Klane, L.** and **J. Kandel**. 1996. In: Microbiology: Essentials and applications. Mc Graw Hill. 2nd edition.

249. **Mc lean, R.J.C., D. Beauchemin, L. Clapham** and **T.J. Beveridge**. 1990. Metal binding characteristics of the Gamma – glutamyl capsular polymer of *Bacillus licheniformis* ATCC 9945. Appl. Environ. Microbiol. 56: 3671 – 3677.

250. **Mcneely, W.H**. Biosynthetic polysaccharides In: Microbial technology edited by Henry J. Peppler 381-403.

251. **McTigue, M.A, C.T. Kelly, W.M. Fogarty** and **E.M. Doyle**. 1994. Production studies on the alkaline amylases of three alkalophilic *Bacillus* sp. Biotechnol. Lett. 16: 569-574.

252. **Medda, S.** and **A.K. Chandra**. 1980. New strains of *Bacillus licheniformis* and *Bacillus coagulans* producing thermostable ∝- amylase active at alkaline pH. J. Appl. Bactieriol. 48:47-58.

253. ***Meek, C. S** and **C. B. Lipman**. 1922. The relation of the reactions of the salt concentrations of the medium to nitrifying bacteria. J. Gen. Physiol. 5, 195-204 (cited in Ref. No. 157).

254. **Mengistu, Y., C.Edwards** and **J.R.Saunders**. 1994. Continuous culture studies on the synthesis of capsular polysaccharide by *Klebsiella pneumoniae* K1. J. Appl. Bacteriol. 75: 424-430.

255. **Mittelman, M.W. and G.G.Geesey.** 1985. Copper binding Characteristics of Exopolymers from a freshwater sediment bacterium. Appl. Environ. Microbiol. 49: 846-851.

256. **Morales, P., A. Madarro, J.A.Perez – Gonzalez, J.M.Sendra, F.Pinaga** and **A. Flors**. 1993. Purification and Characterisation of alkaline xylanases from *Bacillus polymyxa.* Appl. Environ. Microbiol. 59: 1376 – 1382.

257. **Moreno, J., C. Vargas – Garcia, M.J. Lopez** and **G. Sanchez Serrano.** 1999. Growth and exopolysaccharide production by *Azotobacter vinelandii* in media containing phenolic acids. J. Appl. Microbiol 86: 439 – 445.

258. **Mountfort, D.O.** and **R.A.Asher**.1988. Production of ∝- amylase by the ruminal anaerobic fungus *Neocallimastix frontalis*. Appl. Environ. Microbiol.54: 2293-2299.

259. **Mulligan, C. N., D. G. Cooper** and **R. J. Neufield.** 1984. Selection of microbes producing biosurfactants in media without hydrocarbons. J. Ferm. Technol. 62:311-314.

260. **Myers, F. S.** and **A. Anderson**. 1992. Microbes from 20,000 feet under the sea. Science. 255:28-29.

261. **Nakajima S., H. Funahashi** and **T. Yoshida**. 1990. Xanthan gum production in a Fermentor with twin impellers. J. Ferment. Bioeng. 70: 392 – 397.

262. **Nakamura, N., K. Watanabe** and **K. Horikoshi**. 1975. Purification and some properties of alkaline pullulanase from a strain of *Bacillus* No. 202-1 an alkaliphilic microorganism. Biochem. Biophy. Acta. 397:188-193.

263. **Nakamura, S. K.** and **K. Horikoshi**. 1976d. Purification and properties of neutral cyclomaltodextrin glycosyl transferase of an alkaliphilic *Bacillus* sp. Agri. Biol. Chem. 40:1785-1796.

264. **Nakamura, S. K. Wakabayashi, R. Nakai, R. Aono** and **K. Horikoshi**. 1993. Purification and some properties of an alkaline xylanase from alkaliphilic *Bacillus* sp. Strain 41 M-1. Appl. Environ. Microbiol 59:2311-2316.

265. **Neu, R.T.** and **K. Poralla.** 1988. An amphiphilic polysaccharide from an adhesive *Rhodococcus* strain. FEMS Microbiol. Lett. 49: 389-392.

266. **Neu, R.T.** and **K.C.Marshal**. 1991. Microbiol "Footprints": a new approach to adhesive polymers. Bio fouling. 3: 101-112.

267. **Neu, R.T., T. Dengler, B. Jann** and **K. Poralla**. 1992. Structural studies of an emulsion stabilizing exopolysaccharide produced by adhesive, hydrophobic *Rhodococcus* strain J. Gen. Microbiol. 138:2531-2537.

268. **Neu, R.T.** 1996. Significance of bacterial surface active comopounds in interaction of bacteria with interfaces. Microbiol. Rev. 60 : 151-156.

269. **Nicolaus, B., M.C.Manca, I. Romano** and **L. Lama.** 1993. Production of an exopolysaccharide from two thermophilic archaea belonging to the genus *Sulfolobus.* FEMS Microbiol. Lett. 109: 203 – 206.

270. **Niehaus, F., C.Bertoldo, M. Kahler** and **G.Antranikian**.1999. Extermophiles as a source of novel enzymes for the industrial application. Appl. Microbiol. Biotechnol. 51:711-729.

271. **Nihalani, D.** and **T. Satyanarayan.** 1992. Isolation and characterisation of extracellular alkaline enzyme producing bacteria. Ind. J. Microbiol. 32: 443 – 449.

272. **Nimura, Y., F. Yanangida, T. Uchimura, N. Ohara, K. Suzuki** and **M. Kozaki**. 1987. A new facultative anaerobic xylan using alkalophile lacking cytochrome, quinone and catalase. Agric. Biol. Chem. 51:2271-2275.

273. **Nishihara, M., H. Mori** and **Y. Koga**. 1982. Bis- (monoacylglycerol) phosphate in alkaliphilic bacteria. Agri. Biol. Chem. 52:1643-1647.

274. **Nomoto, M., T. -C. Lee, C. -W. Liao, T.-M. Yen** and **C.-P. Yang**. 1984. Alkaline proteinases from alkalophilic bacteria of Taiwan. Agric.Biol. Chem. 48: 1627-1628.

275. **Nomoto, M., M. Oshawa, H. L. Wang, C.C. Chen** and **K. W. Yeh**. 1988. Purification and characterization of extracellular alkaline phosphatase from an alkaliphilic bacterium. Agri. Biol. Chem. 52:1643-1647.

276. **Novak, S.J., S.W.Tanebaum** and **J.P.Nakas**. 1992. Heteropolysaccharide formation by *Arthrobacter viscosus* grown on xylose and xylose oligosaccharides. Appl. Env. Microbiol. 58: 3501-3507.

277. **Obi, S.K.C.** and **F.J.C. Odibo**. 1984.Some properties of highly thermostable ∝- amylase from a *Thermoactinomyces* sp. Can. J. Microbiol.30:780-785.

278. **Ohta, K. A. Kiyomiya, N. Koyama** and **Y. Nosoh**. 1975. The basis of the alkalophilic property of a species of *Bacillus*. J. Gen. Microb. 86: 259 – 266.

279. **Okazaki, W., T. Akiba, K. Horikoshi** and **R. Akahoshi**. 1985. Purification and characterization of xylanases from alkalophilic thermophilic *Bacillus* sp. Agric. Biol. Chem. 49: 2033-2039.

280. **Ophir, T.** and **D.L.Gutnick**. 1994. A role for exopolysaccharide in the protection of microorganisms from Desiccation. Appl. Environ. Microbiol. 60 : 740 – 745.

281. **Oren, Aharon**. 1994. The ecology of the extremely halophilic archaea. FEMS Microbiol. Rev. 13: 415- 440.

282. **Oto, S., R. Aono** and **K. Horikoshi**. 1981. *Exophiala alcaliphila*, new black yeast like hypomycete with an accompanying *Phaeococcomyces alkaliphilus*, morphology and its physiological characteristics. Trans. Mycol. Soc. Japan. 22, 429-439.

283. **Paavilainen, S., P. Helisto** and **T. Korpela**. 1994. Conversion of carbohydrates to organic acids by alkaliphilic *Bacilli*. J. Ferm. Bioeng. 78:217-222.

284. **Pande, Khan** and **Ventikasubramaniam**. 1963. Anal. Biochem. 6:Pg 415.

285. **Park, J.S., S.Horinouchi** and **T. Beppu**. 1991. Characterisation of the leader peptide of an endo type cellulase produced by Alkalophilic *Streptomyces* strain. Agric. Biol. Chem. 55: 1745-1750.

286. **Pennisi, E**. 1997. In Industry Extremophiles begin to make their mark. Science, 276: 705-706.

287. **Persidis, A**. 1998. Extremophiles. Unusual and useful molecules are found in life on the edge of environmental tolerance. Nature Biotech. 16: 593-594.

288. **Petry, S., S. Furlan, M.J.Crepeau, J.Cerning** and **M.Desmazeaus**. 2000. Factors affecting exocellular polysaccharide production by *Lactobacillus delbrueckii* subsp. *bulgaricus* grown in a chemically defined medium. Apppl. Environ. Microbiol. 66: 3427 – 3431.

289. **Pfiffner, S.M., J. Michael, M.C.Inerney, E. Gary, Jenneman** and **M.K.Roy**. 1986. Isolation of halotolerant, thermotolerant, facultative polymer producing bacteria and characterisation of the polymer. Appl. Env. Microbiol. 51: 1224-1229.

290. **Plummer, D.T**. 1980. In:introduction to practical Biochemistry. Tata Mc graw Hill.

291. **Podkovyrov, S.** and **J. G. Zeikus**. 1992. Structure of the gene encoding cyclomaltodextrinase from *Clostridium thermohydrosulfuricum* – 39E and characterization of the enzyme purified from *E. coli*. J. Bacteriol. 174:5400-5405.

292. **Pollock, J.T.** 1993. Gellan related polysaccharides and the genus *Sphingomonas.* Microbiol 139: 1939-1945.

293. **Powell, D.A.** 1979. Structure, solution properties and biological inter actions of some microbial extracellular polysaccharides. In Microbial polysaccharides and polysaccharases (R.C.W.Berkeley, G.W.Goodday, D.C.Ellwood ed) Pg. 117 – 160. Academic press, London, New York, San Francisco.

294. **Prasad, P.,** and **A. K. Kashyap.** 1991. Ammonium transport in the alkalophilic diazotropic cyanobacterium *Nostoc calcicola-* influence of phosphate limitation and metabolic inhibitors. J. Plant Physiol. 138: 244-247.

295. **Prasad, R.** and **S. Sarkar.** 1997. Evolutionary extremophilic Archaeal domain of life. Curr. Sc. 73: 842-853.

296. **Prescott, M. L., J.P. Harley** and **D.A. Klein.**1999. In: Microbiology:Mc Graw Hill companies. 4th edition.

297. **Pringle, J.H.** and **M. Fletcher** 1986. Adsorption of bacterial surface polymers to attachment substrata. J. Gen. Microbiol. 132: 743- 749.

298. **Promma, K., M.Matsuda** and **K.Okutani.** 1997. Structural analysis of a malic acid-containing polysaccharide from an *Aureobasidium pullulans* of marine origin. J.Mar.Biotechnol. 5:219-24.

299. **Qureshi, M.H., T. Fujiwara** and **Y. Fukumori.** 1996. Succinate:Quinone oxidoreductase (Complex II) containing a single Heme *b* in facultative alkaliphilic *Bacillus* sp. Strain YN-2000. J. Bacteriol. 178: 3031 – 3036.

300. **Raguenes, G.H.S., A. Peres, R. Ruimy, P.Pignet, R.Christen, M.Loaec, H.Rougeaux, G.Barbier** and **J.G.Guennec.** 1997. *Alteromonas infernus* sp. nov. a new polysaccharide producing bacterium isolated from a deep sea hydrothermal vent. J. Appl. Microbiol. 82:389-398.

301. **Rajaram, S.** and **A.Varma** 1990. Production and characterisation of xylanase from *Bacillus thermoalkalophilus* grown on agricultural wastes. Appl. Microbiol. Biotechnol. 34:141-144.

302. **Ray, R.R.** and **G.Nanda.** 1996. Microbial β-amylases: Biosynthesis, characteristics and Industrial applications. Critical Rev. in Microbiol.22: 181-199.

303. **Read, R.R.** and **W.Costerton.** 1987. Purification and characterisation of adhesive exopolysaccharides from *Pseudomonas putida* and *Pseudomonas fluorescens.* Can. J. Microbiol. 33:1080-1090.

304. **Richard, F. H., Huang, Z., Edwards, D., Leeson, H., Perry, W.** and **Potts, M.** 2000: Structural characterization of released polysaccharide of dessication tolerant *Nostoc commune* DRH1. J. Bacteriol. 182(4): 974-982.

305. **Roberts, I.S.** 1995. Bacterial polysaccharides in sickness and in health. Microbiol. 141:2023-2031.

306. **Robyt, J.** and **R.J. Ackerman.** 1971. Isolation, purification and characterisation of a maltotetrose producing amylase from *Pseudomonas stutzeri.* Arch. Biochem. Biophys. 145:105-114.

307. **Rosa, M.D.A.Gambacorta, B. Nicolaus, H.N.M.Ross, W.D.Grant** and J.D.Bu'lock. 1982. An asymmetric archaebacterial diether lipid from alkaliphilic halophiles. J.Gen. Microbiol. 128: 343-348.

308. **Rosenberg, E.A. Zuckerberg, C. Rubinovitz** and **D.L.Gutnick**. 1979. Emulsifier of *Arthrobacter* RAG – I: Isolation and Emulsifying Properties. Appl. Environ. Microbiol. 37: 402 – 408.

309. **Royan, S. C. Parulekar** and **S. Mavinkurve**. 1999. Exopolysaccharides of *Pseudomonas mendocina* P_2D. Lett. Appl. Microbiol. 29: 342-346.

310. **Rubinovitz, C., D.L.Gutnick** and **E. Rosenberg**. 1982. Emulsan production by *Acientobacter calcoaceticus* in the presence of chloramphenicol. J. Bacteriol 152 : 126-1

311. **Ruijssenaars, H.J., F. Stingele** and **S. Hartmans**. 2000. Biodegradability of food associated extracellular polysaccharide. Current Microbiol 40: 194-199.

312. **Sadasivam, S.** and **A. Manickam** 1996. In: Biochemical methods. New Age International (P) Ltd. Publishers, New Delhi, India.

313. **Salva, T. D. G., V. B. Delima** and **A. P. Pagana**. 1997. Screening of alkaliphilic bacteria for Cyclodextrin glycosyl transferase production. Rev. Microbiol. 28:157 – 164.

314. **Samad, M.Y.A., C.N.Razak, A.B.Salleh, W.M.Zin Wan Yunus, K.Ampon** and **M. Basri.** 1989. A Plate assay for primary screening of lipase activity. J.Microb. Methods 9:51-56.

315. **Sancheztorres, J., P. Perez** and **R. I. Santamaria**. 1996. A cellulase gene from a new alkoliphlic *Bacillus* sp. (strain N186-1). Its cloning, nucleotide sequence and expression in *Escherichia coli.* Appl. Microbiol. Biotechnol. 46:149-155.

316. **Sanderson, G.R.** and **R.C.Clark.** 1983. Gellan Gum. Food Technology 37: 63-70.

317. **Satoh, E., T.Uchimura, T. Kudo** and **K. Komagata**. 1997. Purification, characterization, and nucleotide sequence of an intracellular maltotriose – producing α-amylase from *Streptococcus bovis* 148. Appl. Environ. Microbiol. 63: 4941 – 4944.

318. **Savidge, R.A.** and **J.R.Colvin.** 1985. Production of Cellulose and soluble polysaccharides by *Acetobacter xylinum*. Can J.Microbiol. 31: 1019-1025.

319. **Schleper, C., G. Puhler; I. Holz, A. Gambacorta, D. Janekoric, U. Santarium, H. P. Klerk** and **W. Zillig**. 1995, Picrophilus genus nov. fam. nov., a novel aerobic heterotrophic thermoacidophilic genus and family comprising archaea capable of growth around pH O. J. Bact., 177:7050 – 7059.

320. **Schnaitman, C.A.** 1970. Examination of the protein composition of the cell envelope of *Escherichia coli* by polyacrylamide gel electrophoresis. J. Bacteriol. 104: 882 – 889.

321. **Schnaitman, C.A.** 1971 Solubilisation of the cytoplasmic membrane of *Escherichia coli* by Triton X -100 J.Bacteriol. 108: 542 – 552.

322. **Schutten, G., E.J.Faber, E.Smit, K. Bonting, M.R.Smith, B.T.Bink, J.P.Kamerling, J.F.G. Vliegenthart** and **L.Dijkhuizen**. 1999. Biochemical and structural characterisation of the glucan and fructan exopolysaccharides synthesised by the *Lactobacillus reuteri* wild type strain and by mutant strains. Appl. Environ. Microbiol. 65: 3008 – 3014.

323. **Scott, J. A.** and **palmer, S. J.** 1988. Cadmium biosorption by bacterial exopolysaccharide. Biotechnol. Lett. 10:21-24.

324. **Shafi, R.** and **S. Khanna**. 1995. Biosurfactants. I.J.Microbiol. 35: 165 – 184.

325. **Shah, A.K.** and **A.A.Ashtaputre.** 1999. Evaluation of rheological properties of the exopolysaccharide of *Sphingomonas paucimobilis* GS – 1 for application in oil exploration. J.Ind. Microb. Biotechnol. 23: 442 – 445.

326. **Shikata, S., K. Saeki, H. Okoshi, T. Yoshimatsu, K. Ozaki, S. Kawai** and **S. Ito**. 1990. Alkaline cellulase for laundry detergents: Production by alkalophilic strains of *Bacillus* and some properties of the crude enzymes. Agric. Biol. Chem. 54: 91-96.

327. **Shimao, M., S.Onishi, S.Mizumori, N.Kato** and **C.Sakazawa**. 1989. Degradation of 4-chlorobenzoate by facultatively alkalophilic *Arthrobacter* sp. strain SB-8. Appl. Env. Microb. 55:478-482.

328. **Silman, R.W., W.L.Bryan** and **T.D.Leathers**. 1990. A comparison of polysaccharides from strains of *Aureobasidium pullulans*. FEMS Microbiol. Lett. 71: 65- 70.

329. **Singh, S.** 1995. Partial purification and some properties of urease from the alkaliphilic cyanobacterium *Nostoc calcicola.* Folia Microbial. Prague. 40:529-533.

330. **Slodki, M.E.** and **M.C.Cadmus**. 1978. Production of Microbial polysaccharides. In: Adv. In Appl. Microbiol. Academic press, New York, San Francisco, London. 23: 19-54.

331. **Sloneker, J.H.** and **D .G. Orentas**. 1962. Pyruvic acid, a unique component of an exocellular bacterial polysaccharide. Nature 194: 478 – 479.

332. **Smith, P.K., R.I. Krohn, G.T. Hermanson** and **A.K.Mallia**. 1985. Measurement of protein using Bicinchoninic acid. Analy. Biochem. 150 V : 76-85

333. **Sneath, P.H. A, Nicholas S. Mair, M. Elisabeth, J.Sharpe** and **G. Holt**. 1986. In: Bergeys manual of systematic bacteriology. Williams and Wilkins; Baltimore.

334. **Sorokin, D.Y., A.M.Lysenko** and **L.L.Mityushina**. 1996.Isolation and characterisation of alkalophilic chemoorganoheterotrophic bacteria oxidizing reduced inorganic sulfur compounds to tetrathionate. Microbiology 65: 326-338.

335. **Sorokin, D.Y., G. Muyzer, T.Brinkhoff, J.G.Kuenen** and **M.S.M. Jetten**. 1998. Isolation and characterisation of a novel facultatively alkalophilic *Nitrobacter* sps, *N.alkalicus* sp. nov. Arch. Microbiol .170. :345-352

336. **Sorokin, D.Y.**, and **L.L.Mityushina**. 1998. Ultra structure of alkalophilic heterotrophic bacteria oxidising sulfur compounds to tetrathionate. Microbiology 67: 78-85.

337. **Souw, P.** and **A.L.Demain**. 1979. Nutritional studies on Xanthan production by *Xanthomonas campestris* NRRL B 1459. Appl. Environ. Microbiol 37: 1186 - 1192.

338. **Souza, K.A, P.H.Deal, H.M.Mack** and **C.E.Turnbill**. 1974.Growth and reproduction of microorganisms under extremely alkaline conditions. Appl. Microbiol. 28:1066-1068.

339. **Souza, A.K** and **P.H Deal**. 1977. Characterisation of a novel extremely alkalophilic bacterium .J. Gen. Microbiol. 101:103-109.

340. **Sowers, K.R.** and **R.P.Gunsalus.** 1988. Adaptation for growth at various saline concentrations by the archaebacterium *Methanosarcina thermophila.* J. Bacteriol 170: 998 – 1002.

341. **Speelmans, G. B.Poolman** and **W.N. Konings**. 1993. Amino acid transport in the thermophilic anaerobe *Clostridium fervidus* is driven by an electrochemical sodium gradient. J. Bacteriol. 175: 2060-2066.

342. **Stack, R.J.** 1988. Neutral sugar composition of extracellular polysaccharides produced by strains of *Butyrovibrio fibrisolvens.* Appl. Environ. Microbiol. 54: 878- 883.

343. **Stingele, F., J.R.Nesser** and **B. Mollet**. 1996. Identification and characterisation of the eps (exopolysaccharide) gene cluster from *Streptococcus thermophilus* sfi6. J.Bacteriol. 178: 1680 – 1690.

344. **Studdert, C.A., R.E. Decastro, K.H. Seitz** and **J.J.Sanchez**. 1997. Detection and preliminary characterization of extracellular proteolytic activities of the haloalkalophilic archeaon *Natronococcus occultus*. Arch. Microbiol. 168, 532-535.

345. **Sturr, M.G., A.A.Guffanti** and **T.A.Krulwich**. 1994. Growth and bioenergetics of alkalophilic *Bacillus firmus* OF4 in continuous culture at high pH. J.Bacteriol. 176:3111- 3116.

346. **Sunaga, T., T. Akiba** and **K. Horikoshi**. 1976. Production of penicillinase by an alkalophilic *Bacillus*. Agric. Biol.Chem. 40: 1363- 1367.

347. **Sutherland, I.W.** 1979. Microbial exopolysaccharide: Control of synthesis and acylation. In, Microbia polysaccharides and Polysaccharases. Edited by R.C.W. Berkeley *et al.* Academics press New York Pg.-1-34.

348. **Sutherland, I. W.** 1982. Biosynthesis of microbial exopolysaccharides. In: Advances in Physiology Vol. 23, pg. 79-150 edited by A. H. Rose and J. Gareth Morris. Academic Press. New York, San Francisco, London.

349. **Sutherland, I.W.** 1985. Biosynthesis and composition of Gram negative bacterial extracellular and wall polysaccharides. Annu. Rev. Microbiol. 39: 243-270.

350. **Sutherland, I.W.** 1998. Novel and established applications of microbial polysaccharides. Trends in Biotechnol. 16: 41-46.

351. **Swamy, M.V., M.Sai Ram** and **G.Seenayya**.1994. β-Amylases from *Clostridium thermocellum* SS 8- a thermophilic, anaerobic, cellulolytic bacterium. Lett. In Appl. Microbiol. 18:310-304.

352. **Tait, M.I., I.W.Sutherland** and **J.C.Sturman**. 1986. Efect of growth conditions on the production, composition and viscocity of *Xanthomonas campestris* exopolysaccharide. J. Gen. Microbiol 132: 1483-1492.

353. ***Takahara, Y.** and O. Tanabe. 1960. Studies on the reduction of Indigo in industrial fermentation vat (VII). J. Ferm. Technol. 38, 329-331 (cited in Ref. No. 157).

354. **Takami, H., T. Akiba** and **K.Horikoshi**. 1989. Production of extremely thermostable alkaline protease from *Bacillus* sps. no. AH-101.Appl. Microbl. Biotechnol. 30: 120-124.

355. **Takami, H., T. Akiba** and K.Horikoshi. 1989. Characterisation of an alkaline protease from *Bacillus* sp.no. AH-101.Appl. Microbl. Biotechnol. 33: 519-523.

356. **Takami, H., A.Inoue, F.Fuji** and **K.Horikoshi**. 1997. Microbiol flora in the deepest sea mud of the Mariana Trench. FEMS Microbiol.Lett .152: 279-285.

357. **Tallgren, A.H., V.Airaksinen, R.V.Weissenberg, H.Ojamo, J.Kuusisto** and **M.Leisola**. 1999. Exopolysaccharide producing bacteria from sugar beets. Appl. Environ. Microbiol. 65: 862-864.

358. **Tanabe, H., K.Yoshihara, K.Tamura, Y. Kobayashi, I.Akamatsu, N.Niyomwan** and **P. Footrakul**. 1987. Pretreatment of Pectic wastewater from orange canning process by an alkalophilic *Bacillus* sp. J.Ferment. Technol. 65: 243-246.

359. **Tanabe, H., Y. Kobayashi** and **I.Akamatsu**. 1988. Pretreatment of Pectic wastewater with pectate lyase from an alkalophilic *Bacillus* sp. Agric. Biol.Chem. 52:1855-1856.

360. **Tayama, K, H. Minakami, E. Entani, S. Fujiyama** and **H.Masai** 1985. Structure of an acidic polysaccharide from *Acetobacter Sp.* NBI 1022. Agri Bioch. 49: 959-966.

361. **Teri** - Biotechnological exploitation of extremophiles for industrial applications. 2000. Proceedings of the Brainstorming session sponsored by DBT, India.

362. **Thomas, R.N.** 1996. Significance of bacterial surface active compounds in interaction of bacteria with interfaces. Microbiol. Rev. 60: 151 – 166.

363. **Tindall, B.J, A. Allan, A. Mills** and **W.D.Grant**. 1980. An alkalophilic red halophilic Bacterium with low magnesium requirement from a Kenyan soda lake. J.Gen Microbiol. 116: 257-260.

364. **Tindall, B.J., H.N.M. Ross** and **W.D.Grant**. 1984. *Natronobacterium* gen. nov. and *Natronococcus* gen. nov. two new genera of haloalkaliphilic archaebacteria. Syst. Appl. Microbiol. 5: 41-57.

365. **Tsai, Y., S. Lin, Y. Li, M. Yamasaki** and **G. Tamura**. 1986. Characterisation of an alkaline elastase from alkalophilic *Bacillus* YA-B. Biochim. Biophys. Acta. 883: 439-447.

366. **Tsuchida, O., Y. Yamagata, T. Ishizuka, T. Arai, J. Yamada, M. Takeuchi** and **E. Ichishime**. 1986. An alkaline proteinase of an alkalophilic *Bacillus* sp. Curr. Micrbiol. 14: 7-12.

367. **Tsuchiya, K., H.Sakashita, Y.Nakamura** and **T.Kimura**. 1991. Production of thermostable alkaline protease by alkalophilic *Thermoactinomyces* sp. HS682. Agric. Biol. Chem. 55:3125-3127.

368. **Tsujibo, H., T.Sato, M.Inui, h. Yamamoto** and **Y.Inamori**.1988. Intra-cellular accumulation of phenazine antibobiotics production by an alkalophilic *actinomycete*. Agric.Biol.Chem. 52:301-306.

369. **Tsujibo, H., Yoshida. Y., Miyamato. K., T. Hasegawa** and **Y. Inamori**. 1992. Purification and properties of two types of chitinases produced by an alkaliphilic actinomycete. Biosci. Biotechnol. Biochem. 56:1304-1305.

370. **Tsvetkov, V.T.** and **E.I. Emanuilova**. 1989. Purification and properties of heat stable ∝- amylase from *Bacillus brevis*.Appl.Microbiol. Biotechnol. 31:246-248.

371. **Uchino, F.** 1982. A thermophilic and unusually acidophilic amylase produced by a thermophilic acidophilic Bacillus sp. Agric. Biol. Chem. 46:7-13.

372. **Upsani, V.** and **S. Desai.** 1990. Sambhar Salt Lake-chemical composition of the brines and studies on haloalkaliphilic archaebacteria. Arch. Microbiol. 154: 589 – 593.

373. **Upton, M.E.** and **W.M.Fogarty**.1977. Production and purification of the thermostable amylase and protease of *Thermomonospora viridis*. Appl. Environ. Microbiol. 33:59-64.

374. **Van den Berg, D.J.C., G.W.Robjin** and **A.C.Janssen et al**. 1995. Production of a novel extracellular polysaccharide by *Lactobacillus sake* O-1 and characterisation of the polysaccharide. Appl. Environ. Microbiol. 61: 2840- 2844.

375. **Van Kranenbug, R., J.D.Marugg, I.I.Van Swam, N.J.L. Willem** and W.M.de vos 1997. Molecular characterisations of the plasmid encode eps gene cluster essential for exopolysaccharide biosynthesis in *Lactococcus lactis*. Mol. Microbiol. 24: 387-397.

376. **Vandevivere, P.** and **D.L.Kirchman.** 1993. Attachment stimulates exopolysaccharide synthesis by a bacterium. Appl. Environ, Microbiol. 59: 3280-3286.

377. ***Vedder, A.** 1934. *Bacillus alcalophilus* n. sp. benevens enkle ervaringen met sterk alcalische voedingsbodems. Anton. Van. Leeu. I, 141-147 (cited in Ref. No. 157).

378. **Vermani, M.V., S.M.Kelkar** and **M.Y.Kamat**. 1997. Studies on polysaccharide production and growth of *Azotobacter vinelandii* MTCC 2459, a plant rhizosphere isolate. Lett. Appl. Microbiol. 24: 379-383.

379. **Vielle, C.** and **J.G. Zeikus**. 1996. Thermozymes: Identifying molecular determinants of protein structural and functional stability. Trends in Biotechnol. 114: 183-190.

380. **Vincenzini, M., R.D.Phillips, C.Sili** and **R.Materassi**. 1990. Studies on exopolysaccharide release by diazotrophic batch cultures of *Cyanospira capsulata*. Appl. Microbiol. Biotechnol. 34:392-396.

381. **Wang, Y.X., K.C.Srivastava, G.J.Shen** and **H.Y.Wang**. 1995. Thermostable alkaline lipase from a newly isolated thermophilic *Bacillus*, strain A30-1 (ATCC 53841). J. Ferment. Bioeng. 79:433-438

382. **Watanabe, N., Y.Ota, Y.Minoda** and **K.Yamada**. 1977. Isolation & identification of alkaline lipase-producing microorganisms, culture conditions and some properties of crude enzymes. Agric. Biol. Chem. 41: 1353-1358.

383. **Weiner, M.R., R.R.Colwell, R.N.Jarman, D.C. Stein, C.C.Sommerville** and **D.B.Bonar**. 1985. Applications of biotechnology to the production, recovery and use of marine polysaccharides Biotechnology 3: 899-902.

384. **Weiner, M.R.** 1997. Biopolymers from marine prokaryotes. Trends in Biotech. 15: 390-394.

385. **Whitehead, T.R.** and **M.A. Cotta**. 1995. Identification of intracellular amylase activity in *Streptococcus bovis* and *Streptococcus salivarius*. Curr. Microbiol. 30:143-148.

386. **Whitfield, C.** 1988. Bacterial extracellular polysaccharides Microbiol. 34: 415-420.

387. **Whittaker, D.K.** and **D.B.Drucker.** 1970. Scanning electron microscopy of intact colonies of microorganisms. J.Bacteriol. 104: 902 – 909.

388. **Wicken, A.J.** 1985. Bacterial cell wall and surfaces. In Bacterial adhesion edited by D.C.Savage and M.Fletcher Plenum Press, New York Pg 45-70.

389. **Wiegel, J.** 1998. Anaerobic alkalithermophiles, a novel group of extremophiles. Extremophiles. 2: 257-267.

390. **Wijbenga, D.J., G.Beldman, A.Veen** and **D.J.Binnema**. 1991. Production of native starch degrading enzymes by a *Bacillus firmus/lentus* strain. Appl. Microb. Biotechnol. 35:180-184.

391. **Williams, A.G.** and **J.W.T. Wimpenny**. 1980. Extracellular polysaccharide biosynthesis by *Pseudomonas* NCIB11264. Studies on precursor forming enzymes and factors affecting exopolysaccharide production by washed suspensions. J.Gen. Microbiol 116: 133-141.

392. **Windish, W.W.** and **M.S.Mhatre**. 1965. Microbial amylases. In: Adv. Appl. Microbiol 7: 273 – 297 AC Press. New York, London.

393. **Wrangstadh, M., P.L.Conway** and **S. Kjelleberg** 1986. The production and release of extracellular polysaccharide during starvation of a marine *Pseudomonas* sp. And the effect thereof on adhesion. Arch. Microbiol. 145:220-227.

394. **Xu, Y., P.J.Zhou** and **X.Y.Tian**. 1999. Characterisation of two novel haloalkalophilic archaea *Natronorubrum bangense* gen. Nov., sp. Nov., and *Natronorubrum tibetense* gen. nov., sp. nov. Int.J.Syst. Bacteriol. 49: 261-266.

395. **Yamamoto, M., Y. Tanaka** and **K. Horikoshi**. 1972. Alkaline amylases of alkalophilic bacteria. Agri. Biol. Chem. 36:1819-1823.

396. **Yankov, D., E. Dobreva, V. Beschkov** and **E. Emanilova**. 1986. Study of optimum conditions and kinetics of starch hydrolysis by means of thermostable ∝- amylase. Enzyme Microbiol. Technol. 8: 665-

397. **Yonezawa, Y.** and **K. Horikoshi**. 1978. Polyamines in alkaliphilic *Bacillus* Y-25. Agri. Biol. Chem. 42:1955-1956.

398. **Yoshihara, K.** and **Y.Kobayashi**. 1982. Retting of Mitsumata bast by alakophilic *Bacillus* in papermaking. Agri. Biol.Chem. 46:109-117.

399. **Yoshimatsu, T., K. Okazaki, S. Shikata, Y. Obta, K. Koike, S. Kawai** and **S. Ito**. 1990. Purification and characterisation of alkaline endo 1,4, β-glucanases from alkalophilic *Bacillus* sp KSM-635. J. Gen. Microbiol. 136: 1973-1979.

400. **Yu, T. X**. 1991. Protease of haloalkalophiles 1991 In Superbugs-1991. ed. K. Horikoshi and W. D. Grant. Springer Verelag, New York.

401. **Yum, D. Y., H. C. Chung, D. H. Bai, D.H. Oh** and **J. H. Yu**. 1994. Purification and characterisation of alkaline serine protease from an alkalophilic *Streptomyces* sp. Biosci. Biotechnol. Bioche. 58: 470 – 474.

402. **Yumoto, I. Y. Fukumori** and **T. Yamanaka**. 1991. Purification and characterisation of two membrane bound c type cytochromes from a facultatively alkalophilic *Bacillus*. J. Biochem. 110: 267 – 273.

403. **Yuuki, T., T. Nomura, H. Tezuka, A. Tsuboi, H. Yamagata, N. Tsukagoshi** and **S. Udaka**. 1985. Complete nucleotide sequence of a gene for heat and pH stable ∝- amylase of *Bacillus licheniformis*: Comparison of the amino acid sequence of three bacterial liquefying ∝- amylases deduced from the DNA sequences. J. Biochem. 98: 1147-1156.

404. **Zaghloul, T.I., M.Albahra** and **H.Alazneh**. 1998. Isolation, identification and keratinolytic activity of feather-degrading bacterial isolates. Appl. Biochem. Biotechnol. 70:207-213.

405. **Zhang, Y.** and **R.M.Miller**. 1992. Enhanced octadecane dispersion and biodegradation by a *Pseudomonas* rhamnolipid surfactant (Biosurfactant). Appl. Environ. Microbiol. 58:3276-3282.

406. **Zhilina, T.N.** and **G.A. Zavarzin.** 1994. Alkalophilic anaerobic community at pH 10.0. Curr.Microbiol. 29: 109-112.

407. **Zhilina, T.N., G.A. Zavarzin, F.Rainey, V.V.Kevbrin, N.A.Kostrikina** and **A.M.Lysenko**. 1996. *Spirochaeta alkalica* sp.nov. *Spirochaeta africana* sp. Nov., and *Spirochaeta asiatica* sp.nov. alkalophilic anaerobes from the continental soda lakes in Central Asia and the East African Rift. Int.J. syst .Bacteriol. 46: 305-312.

408. **Zhilina, T.N., G.A. Zavarzin, F.Rainey, E.N. Pikuta, G.A.Osipov** and **N.A.Kostrikina**. 1997 *Desulfonatronovibrio hydyogenovorans* gen. nov., sp.nov., an alkalophilic, sulfate-reducing bacterium .Int.J.Syst .Bacteriol. 47: 144-149.

409. **Zychlinsky, E.** and **A. Matin**. 1983. Cytoplasmic pH homeostasis in an acidophilic bacterium Thiobacillus *acidophilus*. J. Bact. 156:1352-1355.

*** - References not seen in original.**

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