SCREENING OF MICRO-ORGANISMS FROM COASTAL MARINE AND ESTUARINE HABITATS: STUDY OF THEIR BIO-ACTIVE METABOLITES

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By

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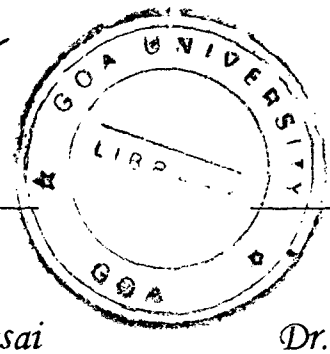
CERTIFICATE

*This is to certify that Ms. Yogita N. Sardessai has worked on the thesis entitled "**Screening of Micro-organisms from Coastal Marine and Estuarine Habitats : Study of their Bio-active Metabolites**" under my guidance and supervision.*

This thesis, being submitted to the Goa University, Taleigao Plateau, Goa, for the award of the degree of Doctor Of Philosophy in Microbiology, is an original record of the work carried out by the candidate herself and has not been submitted for the award of any other degree or diploma of this or any other university in India or abroad.



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Biodiversity is the earth`s most important but least utilised resource.

-----Edward Wilson (1988)

Introduction

Bio-active metabolites produced by microbes and other living organisms exert biological activity by virtue of their structure on other living cells such as micro-organisms, plants, higher animals and humans. Traditionally, microbes are known for production of antibiotics, which is the largest group of bio-active compounds. However, bacteria, fungi and even micro-algae are reported to produce a diverse range of pharmacologically and immunologically active molecules such as enzymes, enzyme inhibitors, anti-parasitic agents, insecticides, phyto-hormones etc. In the recent years, various advances have been made in virtually every field of drug discovery, and the total number of compounds with clearly defined biological activity isolated from natural sources is close to 100,000 out of which about 10,000 compounds are of microbial origin [37]. Besides this, the potential of micro-organisms to convert and transform natural products has been widely exploited for the production of bio-active compounds, particularly steroid molecules.

The present thesis is therefore focussed to screen the ability of micro-organisms from marine and estuarine sources for steroid transformation and L-asparaginase production. The work was diverted towards studies on:

1) L-asparaginase producing bacteria

And

2) Bacteria capable of steroid transformation in an organic-aqueous biphasic system (Cholesterol was selected as a model steroid compound). Since Goa has a tropical climate and extensive coastline known to harbour rich bio-diversity, all the bacterial cultures used in this study were isolated

from sediment samples collected from the Arabian Sea and Mandovi estuary.

1) **L-asparaginase (L-asparagine aminohydrolase E.C. 3.5.1)**

This enzyme cleaves the amino acid L-asparagine to L-aspartic acid and ammonia is of extreme importance in the treatment of certain cancers and tumors particularly acute lymphoblastic leukemia (A.L.L.) [320]. Leukemic cells have a lower asparaginase synthetase activity and a higher requirement for asparagine as compared to normal cells. The amino acid starvation leads to apoptosis or cell suicide. L-asparaginase selectively suppresses the synthesis of ribosomal proteins at the level of mRNA translation. The medical use of the enzyme was initiated in the early 1960s when its significance in destroying cancer cells was reported by Broome [58]. Currently, the industrial production of L-asparaginase is by *E.coli* and *Erwinia chyrasenthemi*. These enzymes are highly effective in cancer treatment, however there are drawbacks such as toxicity effects induced by immunological reactions and the need for extensive purification protocols to ensure complete removal of endotoxins and related impurities. Hence the search for a source of asparaginase continues. For clinical activity, the enzyme besides having a high affinity for asparagine should be stable and active at 37 degrees C and pH 7.4 [352]. Studies were therefore diverted towards:

a. Isolation of L-asparaginase producing bacteria from marine and estuarine habitats.

b. Determination of L-asparaginase activity of the bacterial isolates at 37 degrees C and pH 7.

c. Optimisation of enzyme activity.

d. Identification of selected isolates.

2) **Bio-transformation of steroids** is a multi-million dollar industry having numerous pharmaceutical uses. The major limiting factor in this process is the extremely poor solubility of steroids in the aqueous medium (< 10^{-2} to 10^{-3} g/l), which lowers the transformation rates and increases monetary implications. It is therefore desirable that solubility conditions be improved during bio-transformation. It has been established that cholesterol dissolved in organic solvents at a high concentration is converted at a much higher rate with cells or enzymes suspended in the water- phase [269]. However, organic solvents are toxic to all ordinary bacteria even in low concentrations. Therefore, to have adequate production of chemicals by micro-organisms in such a two-phase system, it is necessary to develop micro-organisms which contain the relevant enzymes in sufficient amounts and with high specific activities, even in the presence of the generally destructive organic phase. This problem can be overcome by using organic solvent tolerant bacteria (OSTB) which can carry out the desired bio-transformations in an organic solvent saturated system. OSTB are a relatively novel group of extremophilic microbes which have developed various adaptations to withstand solvent toxicity. They have tremendous potential in industrial processes involving non-aqueous bio-catalysis and transformation in presence of an organic phase [91], as the cells and enzymes of OSTB are found to be active in the

presence of organic solvents [297, 298]. It has been reported that the number of OSTB in marine habitats is much higher than in soil [201]. Hence, the present work was undertaken to isolate organic solvent tolerant cholesterol transforming bacteria from this ecosystem. An attempt was therefore made to study the following aspects:

a. Isolation of bacteria capable of cholesterol transformation in an organic – aqueous biphasic system from marine/ estuarine habitats.

b. Identification of selected bacterial isolates.

c. Development of a suitable organic-aqueous biphasic fermentation system for cholesterol transformation

d. Identification of the intermediate obtained from cholesterol.

e. Study of the solvent tolerance of these isolates.

Chapter 1

Literature Survey

Section 1)

Microbes as a source of novel bio-active metabolites

The role of micro-organisms in the production of biologically active molecules is well-recognised. In addition to antibiotics, bacteria and fungi are now known to produce a diverse range of bioactive compounds such as enzyme inhibitors, pharmacologically and immunologically active compounds, insecticides, anti-parasitic agents, phyto-hormones and others during secondary metabolism. Screening of the microbial bio-diversity from extreme and unexplored habitats, isolation of rare/ novel taxa, high-throughput, super-sensitive and selective screening techniques, computer-assisted structural analysis, combinatorial libraries and advances in bio-technology and bio-engineering have all contributed to increase the possibility of obtaining new bio-active metabolites of microbial origin. This section discusses the factors responsible for the recent thrust in bio-active microbial metabolites research, with special emphasis on the role of marine micro-organisms in yielding novel drugs.

1.1.1 Introduction:

Bio-active metabolites are biologically active molecules produced by living organisms. Microbes have traditionally served as a rich source of several potent bio-active metabolites. The discovery of Penicillin and its subsequent clinical success ushered in the golden era of chemotherapy (1938-1945), which resulted in intensive research in the field of antibiotics from terrestrial micro-organisms. Bio-active metabolites generally represent a structurally diverse group of secondary metabolites of microbes and other organisms which can exert various biological activities in other microbes, plants and higher animals. The total number of compounds with clearly defined biological activity isolated from natural sources is close to 100,000 including about 10,000 compounds of microbial origin. A large proportion of these (over 8,000) are antibiotics, but in recent times the number of bio-active metabolites isolated having other activities is slowly increasing **[table 1.1]**. Non-antibiotic bio-active metabolites include enzyme inhibitors, pharmacologically and immunologically active agents phytotoxins, herbicides, anti-parasitic compounds, insecticides and other. Two examples of commonly used drugs include the immuno-suppressant cyclosporin and the cholesterol lowering agent mevinolin, both of which are fungal metabolites [37].

Table 1.1: Publications dealing with biologically active natural substances that appeared in 1996 in international journals: [76]

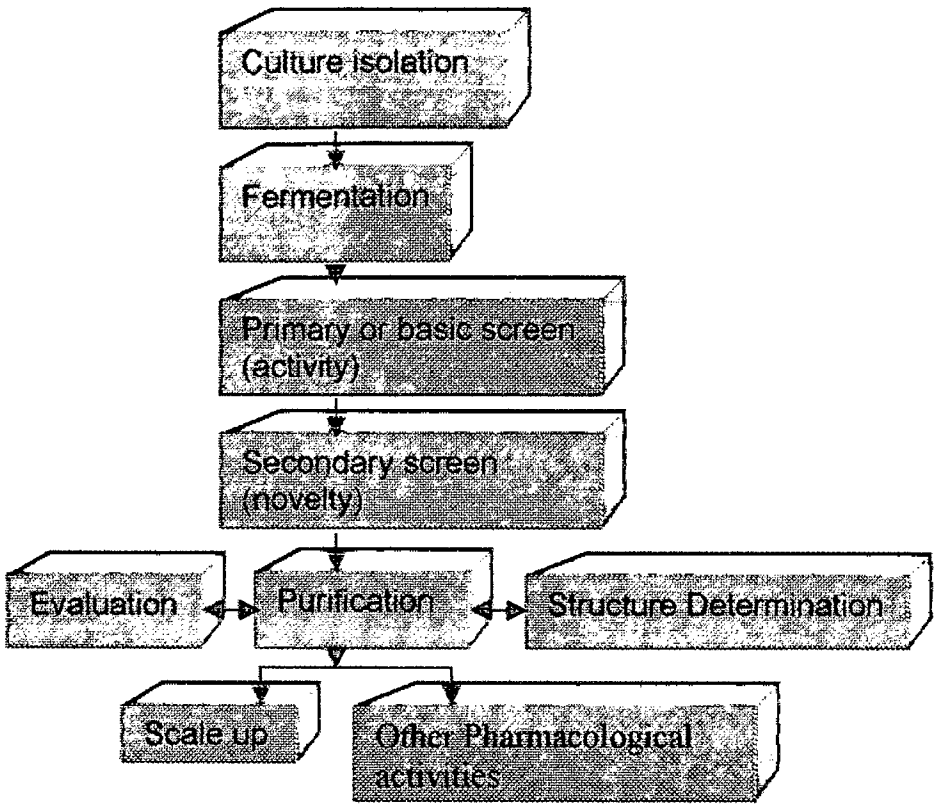
Biological activity	Number of publications
Analgesics	13
Antihelminthics	13
Antibacterial substances	176
Antidiabetic substances	7
Antifeedants	19
Antifungals	91
Antiinflammatory	22
Antimalarial	13
Antimitotic	5
Antioxidants	5
Antitumor	79
Antiviral	36
Cardiovascular	25
Cytotoxic substances	25
Enzyme inhibitors	115
Hepatoprotective agents	9
Immunomodulators	29
Insecticides	10
Molluscicides	7
Muscle relaxants	8
Neuropharmacological agents	10
Pheromones	52
Plant growth modulators	17
Platelet aggregation inhibitors	18
Toxic substances	71
Miscellaneous	134

1.1.2 The recent thrust in research on microbial bio-active metabolites:

Several factors are responsible for the recent thrust in research on microbial bio-active products research. These include a) new rapid, superselective and sensitive screening techniques, b) technical advances in biotechnology and bioengineering, c) emergence of drug-resistant strains and new pathogens, d) The constant need for further specific agents against infections, neoplastic cells, viral diseases, e) Isolation of unique bio-active metabolites from novel microbial species isolated from less explored habitats and f) the discovery of applications of microbial metabolites in new areas of human therapy [37].

Drug discovery is a multi-step multi-disciplinary procedure, involving the screening process and isolation of the producing species from its natural habitat followed by cultivation and detection of biological activity **[flowcharts 1.1, 1.2 and 1.3].** Microbes are known to produce an inexhaustible variety of low molecular weight compounds, many with desirable bio-activity, yet to pinpoint the truly useful ones, a well-devised precise, efficient and sensitive assay procedure is required. The recognition of a new microbial metabolite with promising bio-activity leads to a detailed clinical evaluation which includes animal tests, toxicology, pharmaco-kinetics, scale-up, structure determination and modification, investigation of side-effects, mode of action, which is eventually followed by a clinical trial.

Flowchart 1.1:Screening process for a new bioactive microbial metabolite:- [37]



Flowchart 1.2: Evaluation of a new bioactive metabolite (Drug Development Process): [126]

Culture→Screening → new active compound→.....structure elucidation, chemical modifications, licensing , patenting

preclinical investigation (animal tests ,toxicology testing, Pharmacokinetics: absorption, distribution, elimination, resistance) →

Clinical trials (interactions , combinations etc)→...Regulatory agencies→

Production→...scale up, fermentation improvement, genetic manipulation, isolation technology →large scale fermentation (quality control, biosynthesis, biotechnology)→ formulation

Market

Flowchart 1.3: Strategies in a microbial screening programme [126]

Strain Isolation :

-------Conventional microflora (***Streptomyces, Bacillus*** , Imperfect fungi)

-------Neglected microflora(***Actinoplanes, Nocardia, Micromonospora, Myxobacteria, Basidiomycetes, Yeasts , marine bacteria)***

Secondary metabolite profile:

-------Conventional testing methods

-------Novel testing methods

Exhaustive <-> Intensive

Genetic Manipulation (Mutation, Recombinant DNA strategies

Biochemical Manipulation (Controlled fermentation, Mixed culture fermentation)

Therefore, the development of a new microbial drug from culture isolation to the market place requires at least 8 to 10 years and millions of dollars. Scientists well-versed in novel drug discovery unanimously agree that all screening procedures should be based on faith in the genetic versatility and biochemical potential of micro-organisms. Indeed, it is believed that virtually every type of bio-activity can be expected from microbes provided that the culture conditions allow the expression of the desired genes and the screening method used is effective [61, 126, 302]. Classical screening methods were time-consuming, labour-intensive and produced a low hit rate. However, the new target-oriented pharmacological screens involve highly specific detection methods based on detailed understanding of biological systems, e.g. screens based on receptor-ligand binding and enzyme inhibition.

In high –throughput screening (HTS), a large number of compounds can be tested in an automated fashion, for activity as inhibitors or activators of a biological target. Combinatorial chemistry has made it possible to create chemical libraries of millions of compounds for testing. A combinatorial library is built around a structural motif of known bio-active natural products and the library is then subjected to HTS in the hope of identifying substances with improved properties as compared to the original natural product. The whole HTS technology has been miniaturised in order to reduce the time, material and cost spent on each product. Ultra-HTS using nano-technology has been developed. Conventional screening procedures generally involve a basic screen which is a simple front-line process such as the brine shrimp lethality test which predicts cyto-toxicity

and the crown gall tumor bioassay which monitors the inhibition of crown gall tumors on potato discs and is fairly accurate in predicting 3PS in vivo murine anti-leukemic activity. This is followed by more specialised and sophisticated secondary assays such as screening for anti-viral activity [82]. An interesting observation is that most microbial bioactive metabolites having novel pharmacological or immunological activities exhibit some sort of antimicrobial action eg. the immuno-suppressant cyclosporin was first noticed for its antibiotic action, a feature which can be exploited in discovering new drugs. Various novel strategies are being devised to design new drugs [63, 64, 76, 95, 141]. For instance, the new antimicrobial drugs target novel and non-traditional sites of microbial cells such as efflux pumps. Advances in chromatographic techniques and extraction methods have simplified product isolation and computer assisted identification techniques based on N.M.R., I.R, U.V. and mass spectroscopy facilitate rapid and economic identification of bio-active compounds. The Bio-active Natural Products Database has been developed which collects the important physical, chemical, biological and pharmacological properties, origin, isolation methods and literature citations of more than 15,000 natural compounds including over 10,000 metabolites. It covers anti-microbial, antitumor, anti-viral compounds isolated from microbes, higher plants, animals and all non-antibiotic bioactive metabolites isolated from micro-organisms and marine species including algae and marine invertebrates. To identify an unknown compound from the fermentation broth, selected information such as molecular weight of the unknown substance should be entered. The

computer program compares the entered information with properties of compounds covered in the database and selects those best matching the entered characteristics [37] **[table 1.2].**

1.1.3 Significance of marine micro-organisms in novel drug discovery:

Although numerous drugs have been discovered from terrestrial microbes, the ocean remained as a vast untapped habitat for several years. This scenario has changed in the recent years due to technical advances such as the development of deep-sea submersibles which have made it possible to collect speminens under visual observation in the remote ocean floors at 300 to 400 metres depth. Another reason for the increased interest in marine microbes is the realisation of the immense genetic potential of the tremendous bio-diversity harboured in the marine habitat. The ocean comprises 70% of the earth`s surface and has a diverse range of zones and micro-niches within the open sea, submarine vents, shallow seeps and coastal ecosystems such as coral reefs and mangroves. Marine bacteria are broadly of two types; free-living and symbiotic. The latter are very significant because they have evolved various complex associations with marine plants and animals in whom they produce bio-films. Since nutrients are scarce in sea, competition is intense, associational specificity with nutrient rich plants and animals is high and microbes.

Table 1.2 : Screening for bioactive metabolites is a multidisciplinary exercise [37]

Biologists	***Isolation, identification, maintenance of organisms***
Biochemists	Extraction and purification, characterisation of metabolites, assay design
Chemists	Molecular determination, synthesis
Molecular biologists	Improvement of titres, gene cloning, development of probes, sequencing methods for detection and recognition
Information technologists	Databases, computer search protocols
Engineers	Automated screening procedures and instruments

produce a large number of compounds for their defense and survival. It is these compounds which constitute a major drug source. Several drugs which were believed to originate from marine plants and animls are now known to originate from their bacterial symbionts.This is a fortunate occurrence since microbes are easier to manipulate genetically and large-scale cultivation for industrial scale production is much more feasible for microbial products than any other living source. Bryostatin 1, the anti-cancer agent was initially thought to be produced by the bryozoan *Bugula neritina.* However, the compound is now believed to be produced by its bacterial symbionts. Bacterial origin of metabolites is indicated by; a) low yields, b) isolation of the same metabolite from diverse taxa, c) consistent microbial associations in the producer, d) similarity of the molecule obtained to other known microbial products. Marine organisms are known to produce many bio-active compounds having antibiotic, anti-viral, anti-cancer, insecticidal, analgesic, anti-spasmodic, anti-depressant, cardiovascular, neuroactive effects. These compounds belong to various chemical groups such as glycosidases, nucleosides, saponins, peptides, depsipeptides, diterpenes, macrolides and alkaloids. The actual source of these is obscure. However, considering the fact that bacterial symbionts occupy about 40% of the total volume of these organisms, it is interesting to speculate that these compounds could be of bacterial origin.

It has been proved that the marine habitat contains many novel, unidentified taxa which have a high probability of yielding novel products. 16 S rRNA cataloguing has revealed the presence of bacterial assemblies of several undescribed phylogenetic entities attached to

marine flora and fauna. Many micro-organisms may have devised novel metabolic pathways to yield new metabolites.Under the unique conditions prevalent in the marine ecosystem such as presence of salt, marine nutrients, increased hydrostatic pressure, structurally unique compounds belonging to novel chemical classes have been obtained. For instance, a structurally novel group of antibiotics called istamycins has been isolated from a novel streptomycete when grown in sea-water. A novel class of cytotoxic and anti-viral macrolides called macrolactins have been obtained from a deep-sea bacterium. The anti-inflammatory salinamides and the bromine containing antibiotic marinone are other examples of the structural novelty of marine products

However, certain factors are critical in obtaining novel drugs from marine microbes. These include collection of the correct groups of bacteria from the proper location, duplication of culture conditions as existing in the region of isolation and use of sensitive screens. It has been found that production of the bio-active metabolite is strongly affected by availability of nutrients and temperature of incubation. To illustrate, an actinomycete strain from sea-mud produced an anti-plasmodial substance only in presence of salt and diluted yeast extract. A streptomycete strain was found to produce different antibiotics at different incubation temperatures. Most marine bacteria are non-culturable on normal nutrient media lacking salt and marine nutrients. Since bioactive compounds are known to be produced through secondary metabolism, simultaneous production of small quantities of related substances is common. It is therefore crucial to

increase the sensitivity of screens used to detect the activity in fermentation broths.

Marine natural products are now considered as a significant drug resource and an integral component of natural products chemistry. *Bacillus* and *Alteromonas haloplanktis* strains isolated from deep-sea sediment have been known to produce anti-cancer agents. An actinomycete isolated from jelly fish is known to produce an anti-inflammatory agent. [24, 114, 138, 299, 328,329, 335,336,432]. **[table 1.3]**

1.1.4 Current Status of International Research:

It is not surprising that most of the significant drug discovery programmes world-wide are centred around marine organisms.

Major programmes now exist in the U.S. and Japan and there are significant research programmes worldwide. Many marine organism derived agents are in various stages of preclinical development. Japan`s ministry of International Trade and Industry has sanctioned a 10-year project to develop new industries for extraction of fine chemicals from the sea. Over half of NCI (U.S. National Cancer Institute) natural product programmes involve exploration of marine sources and the efforts are being richly rewarded. At least 6 marine drugs are scheduled for clinical evaluation under NCI [82,114].

Table 1.3 : Novel Organic Molecules Reported From Marine Bacteria:

[114]

Producing strain	Source	Bioactivity
Actinomycete		
Chainia purpurea	Sediment	Antibiotic, anticancer
Streptomyces sioyaensis	Sediment	Anticancer
Streptomyces griseus	Sediment	Antibiotic
Actinomycete	Gorgonian soft coral	Anticancer, antiblotic
Streptomyces sp.	Jellyfish	Antibiotic, anti –inflammatory
Streptomyces sp.	Sponge	Antibiotic
Non-actinomycete		
Alteromonas rubra	Not reported	Bronchodilator
Alteromonas haloplanktis	Deep sea sediment	Anticancer
Alteromonas	Sea water	Enzyme inhibitor
Pseudomonas fluorescens	Ascidian	Antimicrobial
Thermococcus	Hydrothermal vents	Antifungal
Bacillus	Deep sea sediments	Anticancer
Unidentified gram +ve species	Deep sea sediments	Anticancer, antiviral

1.5 The Indian Scene:

India is surrounded by the sea on 3 sides and is bestowed with a very long coastline of 7500 km. The new ocean regime has enabled India to control about 2.01 million square km of the sea as its exclusive economic zone.

A systematic effort towards pharmacologic exploration of the marine wealth of our country began in 1980, with the collaboration of National Institute of Oceanography, Goa, Central Drug Research Institute, Lucknow and Bose Institute, Calcutta. Their study involved broad-based pharmacological screening of approximately 500 marine samples in which activities like anti-fertility, anti-viral, hypotensive, CNS stimulation etc were detected. The leads were promising, therefore it was decided to have a broad-based project and thus a National Multicentric Project on the Development of Potential Drugs from the Ocean funded by Department of Ocean Development, Government of India, started in 1991 [70].

Section 2)

Bacterial L-asparaginase: An Important Drug in Cancer Therapy

The enzyme L-asparaginase (L-asparagine aminohydrolase EC. 3.5.1), which cleaves the amino acid L-asparagine to L-aspartic acid and ammonia, is of extreme importance in the synergistic therapy used in the treatment of certain cancers and tumors, particularly acute lymphoblastic leukemia. The medical use of the enzyme was initiated after the early 1960s when its significance in destroying cancer cells was reported by Broome. Neoplastic cells were found to be destroyed due to the lack of availability of free exogenous L-asparagine, an essential growth requirement, resulting in inhibition of protein synthesis and death. The industrial production of this enzyme is from bacterial cells of *E.coli* and *Erwinia*. This section traces the evolution of the bacterial enzyme L-asparaginase into a powerful anti-cancer drug and discusses modern developments in the use of L-asparaginase in cancer therapy in addition to its microbiological, pharmaco-kinetic and pharmacological aspects.

1.2.1 Introduction:

The enzyme L-asparaginase (L-asparagine amino-hydrolase E.C.3.5.1.) is an important element in the therapy of acute lymphoblastic leukemia (ALL), which is the most common pediatric malignancy accounting for 75% of all newly diagnosed leukemias and 25% of all the cancers of childhood. Statistics reveal that there has been a 20% cumulative increase in the

incidence of childhood ALL from 1973-1991 in the U.S. Except B-cell ALL, which can be treated with short term (< 6 months) but very intensive chemotherapy, including high dose cyclophosphamide, doxorubicin, vincristine, methotrexate and cytarabine, subtypes of ALL require prolonged treatment for 2 to 2.5 years. The contemporary protocols include : 1) Remission Induction, 2) Intensification and Consolidation, 3) Prevention of CNS disease, 4) Continuation of remission. Induction of remission includes administration of a glucocorticoid such as prednisone or dexamethasone along with vincristine and L-asparaginase. The belief that cancer therapy would be curative if early treatment were sufficient to eradicate malignant cells before they acquire drug resistance led to the aggressive multi-drug regimen, of which L-asparaginase is a crucial component. The addition of L-asparaginase is found to improve the remission induction rate to 95%. This therapy is particularly advantageous for children with B cell precursor ALL, higher risk cases and those with T cell ALL [320].

L-asparaginase is an amino-hydrolase enzyme which catalyses the hydrolysis of L-asparagine to aspartic acid and ammonia and depletes asparagine in serum and cells. Leukemic cells have a lower asparagine synthetase activity and higher requirement for asparagine than normal cells, therefore the amino acid starvation leads to apoptosis or cell suicide. It thus suppresses the growth of malignant cells that are more dependent on an exogenous source of asparagine [352]. Asparaginase is the only effective drug against ALL which results in inhibition of protein synthesis. It has been demonstrated that L-asparaginase selectively suppresses the synthesis of ribosomal proteins at the level of mRNA translation [189, 283]. Kidd (1953) reported that certain transplanted murine leukemias were suppressed by treatment with guinea pig serum and Broome in 1961 identified L-asparaginase as the anti-lymphoma factor of Kidd [58, 59, 60]. However, the revelation that 60 guinea pigs would be required for a single therapeutic dose of 100 units/kg body weight for an adult human made its commercial application impractical. The finding that bacterial L-asparaginase from *E.coli* (E.C. 3.5.1.1.) had anti-tumor activity gave a new impetus to this field and many screening programmes were conducted to assess asparaginase activities of different micro-organisms. The advantage of a microbial source is obvious, since microbial production of the enzyme could be scaled up to meet the increasing demand of pharmaceutical industries [352].

1.2.2 Production of L-asparaginase:

L-asparaginase is found to be present in many animal tissues, bacteria and plants and in the serum of certain rodents, but not that of man. A wide range of micro-organisms (bacteria and fungi) from different sources such as soil, sewage, plant, animal and human systems as well as coastal marine and estuarine habitats have been screened which has yielded promising and potent strains with high asparaginase activity [178, 224, 411, 412, 352, 361]. Although, most of the studies have been centred around bacterial L-asparaginase, this enzyme also occurs in some yeast *(Saccharomyces cerevisiae)* [301] and micro-algae *(Chlamydomonas)* [309]. Traditionally, screening for L-asparaginase producers has been carried out by nesslerisation of culture supernatants of individually isolated cultures from samples, which is a lengthy and time-consuming procedure [411,412,178]. One international unit of the enzyme is defined as the amount of enzyme required to release one micromole of ammonia under standard assay conditions [433].

Recently, novel assays have been developed which simplify this process. For instance, Gulati et al's semi-quantitative plate assay incorporates the pH indicator phenol red which contains asparagine as the sole nitrogen source. Asparaginase activity leads to evolution of ammonia, which makes the medium alkaline producing pink zones around the culture [144].

Studies conducted on parameters affecting L-asparaginase production by different bacteria have identified factors such as medium composition, asparagine concentration, presence of certain amino acids, carbon-

nitrogen ratio, oxygen content, pH and temperature of incubation as important in asparaginase production by different cultures [271,285]. In some bacteria, the enzyme is extracellular whereas in some it is intracellular [107,46]. For industrial production, extracellular enzymes are preferred as they are easier to extract and have a higher affinity for the substrate.

In the procedure of J.D. Teller (U.S. patent 3, 440, 142, April 22, 1969) assigned to the Worthington Biochemical Corporation), cells of *E.coli* strain B are grown in a fermentation medium in which peptone serves as a source of carbon The cells are harvested and then extracted with an aqueous medium. The aqueous extract is purified by a combination of precipitation and chromatography to obtain a fraction rich in L-asparaginase activity [147].

1.2.3 Factors affecting enzyme activity:

Although asparaginase can be isolated from numerous sources, not all enzymes possess the anti-lymphoma activity. Of the two asparaginases produced by *E.coli* (E.C-1 and E.C-2), only E.C-2 possesses anti-lymphoma activity. It is the most extensively studied asparaginase and differs from E.C-1 by its broad pH activity profile and higher substrate affinity. The effectiveness of asparaginase in therapy is based on certain characteristics of the enzyme such as ; a) high affinity for L-asparaginase (low Km value) which will enable enzyme activity even at very low asparagine concentrations and b) activity at physiological pH and temperature, and c) stability which means the sub-units should not

undergo rapid dissociation. L-asparaginase activity is measured either by determination of ammonia or L-aspartic acid [433,434].

1.2.4 Use of L-asparaginase as an anti-cancer drug:

Numerous recent clinical studies corroborate the significance of L-asparaginase in ALL therapy [73,112,151,255,273,294,352,433,434] With asparaginase as an element of therapy, complete remission was achieved in a patient suffering from acute monocytic leukemia in the secondary stage. L-asparaginase was found to induce complete remission in Epstein Barr virus positive, multidrug resistant, cutaneous T-cell lymphoma. The ten year event -free survival of patients with asparaginase treatment was found to be superior to those without the treatment [256]. Intensive high-dose asparagine consolidation has been consistently found to increase the survival of pediatric patients in T-cell ALL and advanced stage lympoblastic leukemia [6]. In fact, the lack of response to asparaginase in vitro predicts a higher risk for clinical relapse regardless of risk assignment. Hence, resistance to asparaginase is of great prognostic significance in treatment of ALL [22,311] .

1.2.5 L-asparaginase induced toxicity:

Despite its unique mode of action which shows relative selectivity with regard to malignant cells, multiple toxic effects are also reported to occur in some patients.

L-asparaginase, being a foreign protein in the human body, is found to induce immunological reactions which include enzyme inactivation

without any clinical manifestations as well as with anaphylactic shock. L-asparginase is now frequently being administered intra-muscularly to decrease the incidence of severe sensitivity reactions leading to a reduction in the occurrence of immediate hypersensitivity and anaphylaxis. However, sometimes delayed allergic reactions occur [375,413]. An enzyme-linked immuno-sorbent assay was used to measure anti-asparaginase antibodies in plasma samples, using *E.coli* asparaginase as the antigen [430]. Antibody levels were inversely related to the time elapsed between the reaction and sampling. It was found that patients with hypersensitivity reactions had higher antibody levels than identically treated control patients at comparable time points in therapy. Anti-asparaginase antibodies play a key role in immuno-clearance of the enzyme. Severe functional disorders of organ systems result from impaired homeostasis of the amino acids asparagine and glutamine. Both the malignant process and the drugs used in combined therapy cause a decrease in natural inhibitors and in procoagulant activity and endothelial injury. These hemostatic changes may contribute to a thrombotic tendency in patients with ALL [5, 303]. Changes affecting proteins of the coagulation system have considerable clinical impact as they may induce bleeding as well as thrombo-embolic events and cause serious complications, such as intra-cranial haemmorhage when the C.N.S. is involved. L-asparaginase may induce subclinical pancreatitis and estimation of pancreatic serum trypsin and elastase may be useful in early diagnosis of L-asparaginase induced acute pancreatitis [221,243,292,360].

Unlike other anti-cancer agents, L-asparaginase does not suppress production of blood cells by the bone marrow.

1.2.6 Pharmaco-kinetics & pharmacological parameters:

Currently, the L-asparaginase used in therapy is either the *E.coli* or *Erwinia chyrsanthemi* enzyme or the *E.coli* enzyme linked to polyethylene glycol called pegaspargase [137,283,373]. There is no difference between the acute toxicity of all the three preparations. Therapeutic efficacy is however found to be affected when different asparaginase preparations are given by identical therapy schedules. A mere 2500 u/m^2 of the *E.coli* preparation asparaginase (Medac) resulted in trough levels comparable to 10,000 u/m^2 of crasnitin (*E.coli* preparation by Bayer company). The *Erwinia* preparation Erwinase did not maintain measurable trough levels at the protocol schedule, hence, different L-asparaginase preparations are not readily interchangeable and changes in preparation design or schedule require careful observation and pharmaco-kinetic monitoring. Another independent study showed that an increased dose of 9 x 20000 iu / m^2 of *Erwinia* asparaginase within 3 weeks resulted in a pharmacokinetic dose intensity comparable to 4 x 10000 iu / m^2 of the *E.coli* product Crasnitin which is no longer marketed. High inter-individual variability and the phenomenon of silent inactivation necessitates monitoring, whenever different preparations are used .

Several modifications have been tried to decrease the hypersensitivity reactions and increase the bio -availability of asparaginase. Due to intense immunological reactions generated by it, the enzyme has a short

serum half-life. Immobilisation of asparaginase into a biocompatible matrix greatly decreases immunogenicity and increases half-life in vivo and therapeutic index. Hence, the poly-ethylene glycol conjugated form of the *E.coli* enzyme (PEG-L-asparaginase) called pegaspargase was developed. The majority of patients who have had clinical hypersensitivity reactions to both *E.coli* and *Erwinia* native enzyme preparations tolerate pegaspargase without any further hypersensitivity reactions. The recommended dose of pegaspargase is 2500 iu/m2 administered by intramuscular or intravenous reactions every 2 weeks in combination with other chemotherapeutic agents Studies reveal that PEG-L-asparaginase is generally well-tolerated in patients with advanced solid tumors, and a dosage of 2000 iu/ m2 by intramuscular injection every 2 weeks results in significant depletion of serum L-asparagine. Various pharmacologic evaluation studies on pegaspargase are being carried out [173,283,382,439]. A bio-conjuate of L-asparaginase obtained by linkage of palmitic acid chains to the native enzyme in the presence of the substrate as a protective molecule was prepared. The activity of the bio-conjugate was found to be dependent on the production of miscelles [253]. It has been found that the use of RBCs as carriers of L-asparaginase greatly improves the pharmacodynamic parameters of the drug. Sustained elimination of plasma L-asparaginase occurred, the duration of which depended on the injected dose [225,226,347]. Liposomal preparations of L-asparaginase have also been prepared. Pharmaco-kinetic studies showed that encapsulation in large liposomes (median diameter, 1249 nm) decreased the circulation time of the

enzyme, whereas encapsulation in small liposomes (median diameter, 158-180 nm) prolonged it by a factor of 10. Liposome encapsulation of L-asparaginase prevents induction of anti-asparaginase antibodies and mitigates the anaphylactic reaction, as no death occurred in animals presensitised and challenged with liposomal formulation, in contrast to animals treated with free enzyme. The survival of animals bearing P1534 tumors was prolonged by a factor of 2 after treatment with selected liposomal formulations as compared with the free enzyme [127].

1.2.7 Asparaginase resistance

Lack of sufficient cellular activity of asparaginase synthetase in blast cells compared to normal tissues is the basis of the anti-leukemic effect of asparaginase. However, complete amino acid deprivation in mammalian cells causes a significant enhancement in gene expression for a number of cellular activities, among which is asparaginase synthetase. This may play a role in resistance to asparaginase therapy. Hence, further advances in asparaginase therapy require additional knowledge of amino acid dependent regulation of asparaginase synthetase genes [176].

1.2.8 Recent trends in L-asparaginase research

A large number of investigations have been performed in order to clarify the molecular structure , the mechanism of catalysis and the genetic determinants involved in *E.coli* L-asparaginase II bio-synthesis and regulation. [48,75,119,171,197,353,354,355,356,357,358,384,422,423] Researchers are also studying the effect of mutations on the enzyme

structure, specificity and function [86,94,303,415]. More recently, design of recombinant strains of *E.coli* and *Erwinia* over-producing L-asparaginase with high enzymatic activity and anti-tumor activity has been undertaken. This is because even the best strains currently available *Erwinia carotovora* SCR 193 and *Erwinia carotovora* 268M and *Erwinia chrysanthemi* NCPB 1066 yield quantities of L-asparaginase which are lower than would be desirable.

Cornea et al describe experimental studies observed on spheroplast fusion, performed in order to over-produce L-asparaginase II in two local isolated *E.coli* strains. For this purpose, bacterial strains were marked with antibiotic resistance by transformation with chromosomal DNA. Seven of the transformants (Kmr or Smr), having 48.6 –78 % higher level of L-asparaginase production were selected for spheroplast fusion. Some of the selected recombinants were found to have an enzyme activity 2-3 fold higher than parental strains and some showed a constant high level of asparaginase II biosynthesis during 3 months. Construction of stable and efficient recombinant strains over-producing L-asparaginase by standard methods of molecular biology and genetic engineering can considered an important step for the creation of a novel recombinant medical preparation of L-asparaginase [86].

Section 3)

Steroid bio-transformation in biphasic (organic –aqueous) systems by organic solvent tolerant bacteria (OSTB)

A) Microbial transformation of steroids

The steroids are a diversified class of oxygenated tetracyclic isoprenoid derivatives bearing the ring system consisting of four fused rings, that are vital in many ways to the life of a eukaryotic organism. The sterols are essential for membrane stability and cell growth and proliferation and are precursors of other steroids of importance. Bile salts are essential in lipid digestion and absorption. Vitamin D is required for calcium absorption and metabolism. Cardiotonic steroids are widely used in medical management of congestive heart failure. Commercial biotechnology operations are centred on the use of microbial agents in specific transformation of individual steroid substrates into useful intermediates or final products. The microbial hydrolysis of plant saponins to sapogenins (dioscin to diosgenin) and microbial degradation of side chain features of sterols are examples of commercially operated processes.

1.3A.1 History:

The earliest interest in microbiological transformation of steroids developed out of 19th century interests in the metabolic origins of the fecal sterol coprosterol from cholesterol and from interest in bacterial decomposition of bile acids in putrid bile. At the same time, capacity of Mycobacterium species to utilise cholesterol as a carbon source was recognised. The earliest systematic investigation of the use of microbes for alteration of steroids was the work of Mamoli and Vercellone.

The announcement by Hench in 1949 , at the Mayo clinic, that cortisone had a dramatic beneficial effect on bedridden patients suffering from the rheumatoid arthritis ushered in the cortisone era. The clinical results reported in 1949, combined with the complexity of the partial synthesis, stimulated highly innovative research to discover new routes to cortisone and cortisol, the active hormone. The significance of microbial steroid transformation became apparent only with the important discovery by the Upjohn Company in 1950 in which a hydroxyl group was introduced in the 11 alpha position in progesterone by means of *Rhizopus.* Through this reaction a new shorter path to cortisone was opened up. This led to description of numerous other transformations using bacteria and fungi Prednisolone, discovered at the Schering Corporation, was the first compound that combined a high level of anti-inflammatory activity with reduced salt retention. Landmarks in steroid bioconversion

research include 11 beta hydroxylation of substance S by *Curvularia lunata* NRRL 2380 which became the basis for commercial hydrocortisone production and transformation of hydrocortisone to prednisolone by *Arthrobacter simplex* ATCC 6946. The cortisone era had a profound impact on drug discovery also, since it led to the logical application of steric and electronic concepts to medicinal chemistry. Last but not the least the cortisone era taught drug-receptor interactions.

With the discovery that the side chain of naturally occuring steroids such as cholesterol, beta- sitosterol or campesterol can be degraded selectively by micro-organisms, this bio-transformation methodology has attracted much attention leading to the development of several useful processes. Today, an increasing number of pharmacologically active steroids are annually produced in the industry through the initial microbial transformation of sterols [370].

1.3A.2 Applications:

The world market for finished steroids appears to be ever increasing, both in terms of products and money value and sales are known to exceed billions of dollars. Sales of anti-inflammatory steroids, male and female sex hormones, oral contraceptive agents, anabolic agents, diuretics, anti-cancer agents, anti-lipemic agents, anesthetics, anti-androgenic agents, tumor localising agents continue to grow. One of the areas with an early interest in the directed application of the micro-

organisms for synthetic purposes was the steroid hormones manufacturing industry. This industry is essentially based on the production of stereospecific compounds with many chiral centres. Although it is possible to produce nearly all molecular weight compounds by organic synthesis, production routes of stereospecific compounds are always very complicated and expensive, bioconversion has, therefore, attracted a great deal of attention. Steroids are of vital importance for biotechnology based pharmaceutical industry because of their life saving nature. All the steroid hormones of the body, with the exception of retinoic acid are synthesised through cholesterol.

Cholesterol oxidase is an enzyme which was commonly used in clinical laboratories to determine serum cholesterol levels. Recent studies prove that the enzyme is a new class of insectidal protein which is very effective against cotton boll weevil [85,74].

1.3A.3 General processes used for steroid production:

World steroid producers use three general processes : 1) direct isolation from natural sources as the recovery of conjugated estrogens from horse urine and cardiotonic steroids from Digitalis and related plants, 2) partial synthesis from relatively inexpensive steroid raw material of animal (deoxycholic acid) and plant origin (diosgenin) 3) total synthesis from non-steroidal materials. The strains chosen for steroid transformation are generally maintained as spores, lyophilised cultures, stored in liquid nitrogen media, or cultures on agar and must

be inoculated into liquid nutrient media for use in aerated fermenters. Media must contain suitable sources of nitrogen, carbohydrate, mineral salts and trace nutrients and buffering components. Commercial transformations are generally carried out in stainless steel, glass lined or other fermenters under sterile conditions. After scale up, contents are transferred into production stage fermenter 50,000 to 75,000 litre size. After suitable period of growth, the substrate is added under sterile conditions in a suitable solvent like acetone, ethanol, dimethyl formamide, propylene glycol, dimethyl sulfoxide etc. Final solvent level is generally kept at a minimum of about 1% total volume, in order to limit toxicity of solvent to the organism. Critical operating parameters include temperature, stirring and aeration rates, pH and foam control. The high costs of substrate and operations demand that microbial fermentations of steroids be under close monitoring by suitable analytical means. Product recovery for analysis is accomplished by solvent extractions. Ultraviolet light absorption, colorimetric methods and chromatographic methods are useful. Despite the commercial success of using flourishing vegetative cell cultures for steroid hydroxylation, several alternative processes have been evaluated with a view to increase yields, control side reactions, simplify the process and improve economics. Among such alternatives are processes using resting vegetative cells, spores, immobilised spores, cell free hydroxylases and immobilised enzymes [370].

There is a vast database available on microbial steroid transformation. Some of the references are cited here [17,23,28,40,41,44,62,83,84 ,117,132,196,231,233,234,275,276,277, 343,344,371,389,436]. The subject has been extensively reviewed [3,250,251,252,370,398].

Cleavage of modified substrates:

The traditionally used natural sterol substrates for steroid drug synthesis are deoxycholic acid, hecogenin, diosgenin, solasodine, ergosterol, stigmasterol, sitosterol and cholesterol. The microbial conversion of chemically modified substrates has also been carried out. Likewise, the fermentation products can also be modified such that they can not serve as substrates for enzymes which subsequently attack the ring structure. The various type reactions of steroids are listed in **table 1.4**.

1.3A.4 Microbial Cleavage of sterol side chains:

With the first discovery by Sohngen that micro-organisms can degrade cholesterol and phytosterols, bacterial genera represented by *Arthrobacter, Coynebacterium, Nocardia, Pseudomonas, Mycobacterium, Rhodococcus* shot into prominence due to their capability to utilise sterols as a sole source of carbon and energy. It was found that the sterol ring structure and side chain were metabolised by different mechanisms **[fig 1.4]**.

Table 1.4 : Type reactions with steroids

Oxidations

1. hydroxylation

2.alcohol oxidation

3. epoxidation

4. carbon-carbon bond scission

5. double bond introduction

6. peroxidation

7. heteroatom oxidation

Reductions

1. double bond saturation

2. ketone to alcohol

3. aldehyde to alcohol

4. hydroperoxide to alcohol

5. enol to primary alcohol

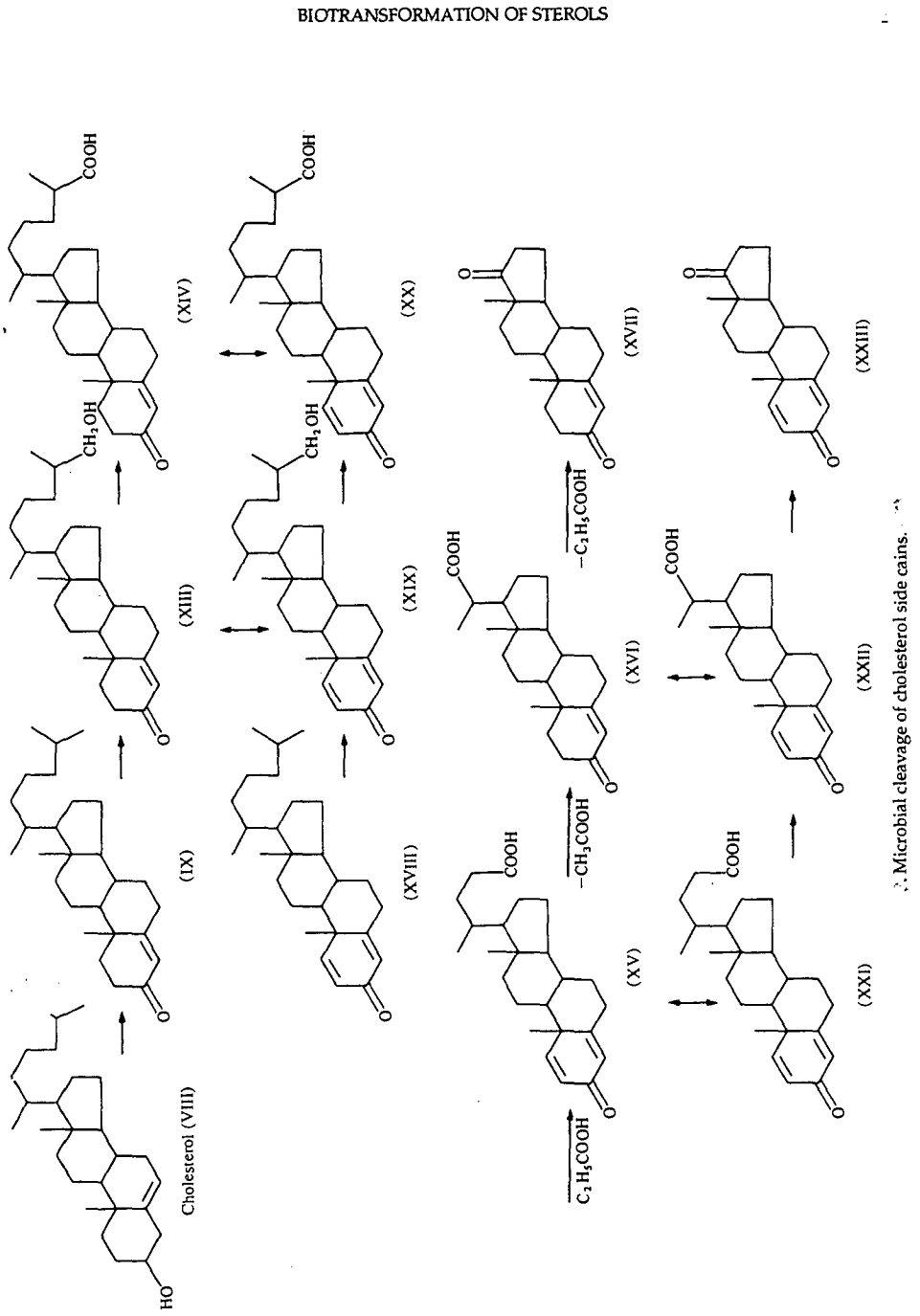
Isomerisation

Conjugation

Hydrolyses

Introduction of hetero-atoms.

Fig 1.1: Degradation of steroid nucleus by microbes.



The enzymatic reactions did not follow a given order but occurred simultaneously and independently. Thus if the ring structure of the substrate was modified, the enzyme normally involved in the degradation of the ring system was unable to catalyse ring fission. If, however, the side chain was modified, the enzyme responsible for its degradation was unable to cleave the side chain of the substrate. The pathway of side chain cleavage of sterols was completely elucidated by Sih and co-workers. Contrary to the mammalian system where C19 steroids are formed via cleavage of C17-C20 bond, micro-organisms shorten the side chain of cholesterol by mechanism similar to beta oxidation of fatty acids. Thus cholesterol is stepwise converted from a C27 steroid via C22 and C24 carboxylic acid intermediates to a C 19 steroid with the production of 2 molecules of propionic acid and one molecule of acetic acid. These end products have been observed for various genera including *Arthrobacter, Bacillus, Brevibacterium, Mycobacterium, Nocardia, Pseudomonas, Protaminobacter, Rhodococcus and Streptomyces.*

The first reaction of microbial side chain cleavage is hydroxylation of C26 and its subsequent oxidation to a steroid carboxylic acid. A C26 steroid acyl CoA is formed next by the action of a specific acyl CoA synthetase. This enzyme was found to have a molecular weight of 66 Kda and a pH optima of 7.6 in Mycobacterium sp. NRRL B-3683 and NRRL-3805, a multi-enzyme complex completes the degradation of C 26 steroid acyl CoA to 4 cholenic acid CoA and proplonic acid. This enzyme complex contains an FMN dependent steroid acyl CoA oxidase, a hydratase, a beta hydroxy CoA dehydrogenase and thiolase. The multi-enzyme

complex from sp. NRRL B-3805 grown with beta-sitosterol has a molecular weight of 250 Kda and contains at least 6 sub-units of 46, 41, 35, 24, 16.5 and 11 Kda. Since the complex is highly unstable during purification, it has led to the assumption that the units are only loosely associated by hydrophobic forces.

Selective Cleavage of steroid side chain:

It is evident that numerous species of bacteria possess the ability to utilise cholesterol and phytosterols as the sole source of carbon and energy. The complete degradation of these compounds is however of no commercial interest. An important goal of microbial side chain clevage therefore is to produce C19 or C22 steroids with intact steroid nucleus. For a long time 4-androsten-3,17-dione (AD) has been the main starting material for preparation of androgens and anabolic steroids. It has gained increasing importance as the base for production of spironolactone. New and commercially effective methods for chemical construction of the pregane side chains from AD(D) have opened up newer routes for synthesis of progesterone and corticoids. On the other hand 1,4 andro-sta-diene 3,17 -dione, can be aromatised by pyrolytic action estrone, which can be susequently reduced to C19 - nor-steroids.Selectlve removal of side chain requires blocking of enzymes responsible for ring cleavage. C-1(2) dehydrogenase and or 9 alpha hydroxylase. To this end, three different approaches have been employed: structural modification of the substrate, inhibition of 9 alpha hydroxylase by chemical means and mutation of the bacterium.

Enzyme inhibitors for selective cleavage:

The key enzyme in sterol ring fission, 9-alpha –hydroxylase is a monooxygenase consisting of several proteins forming an electron transfer chain. Some of these proteins contain Fe^{+2} as an essential metal ion. Removal or replacement of these ions results in complete inactivation of enzyme activity. Employing enzyme inhibitors, numerous processes for selective side chain cleavage of sterols has been developed. 2,2'-dipyridyl, 1,10 –phenanthroline and 8-hydroxy quinoline have been most frequently used. Since most of such compounds are toxic or inhibitory to growth as well, they are usually added to pregrown bacterial cultures. In the presence of enzyme inhibitors, cholesterol as well as beta sitosterol, campesterol, stigmasterol and their mixtures can be effectively converted to C 19, C22 or C27 steroids. C19 steroids are generally the major products accompanied by smaller amounts 1-dehydrotestosterone and testosterone. The ratio of 4-ene—3-oxosteroids to 1,4 diene –3-oxosteroids is enhanced if aerobic degradation phase is followed immediately by anaerobic conditions. In addition to enzyme inhibitors, other chemicals such as oils or adsorbents can increase the yields of C19 steroids. Adjustment of optimal concentration of the chelating agent in the fermentation broth is very essential for high productivity but usually difficult to achieve. Iron or other metal ions in the culture medium react with these compounds and neutralise their inhibitory activity on the enzyme activity as they are no longer available for complexing with the intracellular protein bound Fe^{+2}. Therefore, the composition of the culture

medium has to be controlled exactly especially if complex ingredients such as cornsteep liqour or molasses are to be used.

Low levels of the inhibitor in the medium leads to complete degradation of the substrates to a large extent. This is the result of incomplete inhibition of the enzyme 9-alpha hydroxylase. On the other hand, inhibitor concentration higher than optimal leads to inhibition of C 26-hydroxylase, which results in incomplete substrate conversion and thus low yields of C 19 steroids. At times, chelating agents exhibit adverse effects on cell growth even if added to a pre-grown culture. To overcome these problems, methods have been devised to either trap the accumulating C19 steroids to prevent further degradation or to add the chelating agent in the optimal concentration range. Toxic effect of the inhibitors is found to be partly diminished by adsorption onto styrene divinyl benzene co-polymers. Such as amberlite XAD2, ability to trap iron ions however was not affected. Further these resins are capable of adsorbing C19 and C22 steroids selectively. Other sterols form micelles in aqueous suspension and thus are not adsorbed. The addition of such polymers has been reported to result in 2-3 fold increase in yields of C 19 steroids. Presumably, stimulation of conversion rates by addition of active carbon is based on a similar reaction. A substantial increase in C19 steroids yield was also reported after the addition of oils or fats to the fermentation broth together with the chelating agents.

1.3A.5 Future trends:

a) Side chain cleavage with mutants:

Mutagenic treatment has been used for improving the efficiency of sterol side chain cleaving bacteria. Such mutants are biochemically blocked at 9-alpha hydroxylase and or C-1-(2) dehydrogenase and / or C1-(2) dehydrogenase expression level, unable to produce either both or at least 9 alpha hydroxylase. Such mutants can degrade sterol side chain selectively without the necessity of modification of the substrate or addition of enzyme inhibitors. A process for microbial degradation of sterols to C19 sterols was described for two newly isolated bacteria designated as *Mycobacterium* NRRL B3683 and *Mycobacterium* species NRRL B –3805. The former was produced by u.v. irradiaion of a soil isolate capable of selective degradation of side chains, no inhibitors of ring degradation were necessary.

b) Immobilisation :

Since the first application of an immobilised microbial catalyst, steroid biotransformation in 1970 by Mosbach and Larson, many such reports have appeared. Immobilised cells of *Mycobacterium phlei* were able to degrade side chains of 4-cholesten-3-(0-carboxymethyl)oxime to 4-androstene –17-one-3(o-carboxymethyl)oxime in a continuous system for a period of more than 40 days. The half life of immobilised cells is found to be several fold higher than free cells. Proper immobilisation on or in

suitable supports seems to be most promising. It has been demonstrated that bioconversion efficiency of the microbial cell depends largely on the immobilisation method used, for instance, hydrophobicity of the matrix and organic solvent used.

c) Protoplasts and Genetic Recombination: The steroid hydroxylation frequency of *Cunninghamella elegans* protoplasts was found to be four times higher than mycelia. Recently, spheroplasts of *Mycobacterium* sp. NCAIM 00349 and *Mycobacterium phlei* NCAIM were fused to achieve recombinant mycobacterial strains. The mycobacterial recombinants produce several novel metabolites including 1,4 –andro sta diene –3,17 dione.

d) Bi-phasic systems:

1) **Aqueous 2-phase systems:**

An aqueous 2-phase system is obtained in a water solution of 2 chemically different polymers, when the concentration of the polymers exceeds a critical value. A 2-phase aqueous system is also obtained in a solution containing one water soluble polymer (Poly- ethylene gycol) and an inorganic salt at high concentration. Aqueous 2-phase systems have been applied in different bioconversions including the use of micro-organisms, cell organelles, enzymes and the combination of cells and enzymes. Flygare and Larsson have exploited this system for side chain cleavage of cholesterol.

1) **Organic –aqueous biphasic systems:**

The possibility of side chain cleavage of sterols in organic media has been recently highlighted, to overcome solubility problems. [3]

1.3A.6 Steroid bio-transformations: Advantages of using a biphasic organic –aqueous bio-conversion system

Bio-transformations have been used over a considerable period of time in the chemical synthesis of pharmaceutical products of various classes. The advantages of microbial conversions as opposed to the chemical processes lie in the regio and stereo- specificity of their enzymes and their mild reaction conditions. The objective may be a specific reaction with minimum side- reactions or a sequence of reactions as part of the bio-transformation process.

Biocatalysts have been mainly used in the aqueous reaction systems. However, water serves as a poor solvent for nearly all reactions in industrial chemistry. There are tremendous limitations imposed on steroid transformations in an aqueous system because steroids and their intermediates being highly hydrophobic have extremely poor water-solubility ($< 10^{-2}$ to 10^{-3} grams per l) which lowers conversion rates and increases costs. If excessive quantities of water are used to dissolve small quantities of steroids, magnitude of waste-water generated increases. Steroids can be dissolved in solvents and supplied to cells present in the medium [14,269,201]. However, organic solvents being highly toxic destroy most microbial cells and inactivate/ denature their

enzymes. This problem was resolved to an extent by using immobilised cells and enzymes.

Enzymatic catalysis in non-aqueous media has undergone rapid expansion in recent times. Furthermore, it has now been realised that many enzymes or multi-enzyme complexes including lipases, esterases, dehydrogenase function in natural environments and in some cases it is essential to reduce the water ratio in the system to achieve the desired product.

Numerous steroid conversions have been carried out in presence of organic solvents. An ideal organic solvent should be

a) practically immiscible in water

b) exhibit high solubility for the product

c) it should not exert an inhibitory effect on the biocatalyst

d) non-flammable solvents are preferred for reasons of safety.

Organic solvents like n-alkanes, cyclohexane, toluene, benzene, carbon tetrachloride, chloroform, methylene chloride, ethyl or butyl acetate, diethyl or dibutyl ether have been used with intact cells and enzymes.

A biphasic bio-conversion system would consist of microbial cells suspended in appropriate medium (aqueous phase) in contact with the organic phase containing dissolved substrate. It has been stated that for the adequate production of chemicals by micro-organisms in an 2-phase system, it will be necessary to develop micro-organisms which contain the relevant enzymes in sufficient quantities and high specific activities, even in the presence of the generally destructive apolar phase [348].

If side-chain cleavage becomes economically feasible in an organic solvent containing monophasic or biphasic system, it would be a highly attractive proposition. This can be made possible by employing organic solvent tolerant bacteria (OSTB) for the same. OSTB are a novel group of extremophiles which are capable of functioning in media saturated with organic solvent.

The general and applied aspects of OSTB are discussed in the following sections.

B) Tolerance of bacteria to organic solvents

Organic solvent tolerant bacteria are a relatively novel group of extremophilic micro-organisms. These overcome the toxic and destructive effects of organic solvents due to the presence of various adaptive mechanisms. Extensive studies done on the toluene tolerance of certain *Pseudomonas* strains have led to an understanding of the mechanisms of organic solvent tolerance involving novel adaptations such as the toluene efflux pumps, cis-trans isomerisation of membrane fatty acids, rapid membrane repair mechanisms, etc. Organic solvent tolerant mutants of *E.coli* have been constructed and genes enhancing such tolerance characterised. However, there is practically no information available on the tolerance mechanisms of the reported gram - positive organic solvent tolerant bacterial strains like *Bacillus, Rhodococcus* and *Arthrobacter.*

This section discusses the general aspects of organic solvent tolerant bacteria, their history, biodiversity, mechanisms of tolerance and proposes certain probable adaptations of gram - positive bacteria in tolerance to organic solvents.

1.3B.1 Introduction:

Organic solvents are known to be extremely toxic to microbial cells, even at very low concentrations of 0.1% (v/v). Solvents are known to accumulate in and disrupt the bacterial cell membrane thus affecting the structural and functional integrity of the cell. Although there are some micro-organisms which can assimilate these toxic organic solvents, they do so only when the solvent concentration is very low. Any medium containing large volumes of organic solvents seems an extreme environment for micro-organisms and hence for many years it was believed that no micro-organism could withstand such a harsh environment [187, 365]. The first report of an organic solvent tolerant bacterium was by Inoue and Horikoshi in 1989. They discovered a strain of *Pseudomonas putida* (IH-2000) which could actively grow and multiply in the presence of 50% (v/v) toluene. This surprising observation was confirmed by others and the search to uncover the mechanisms behind this extraordinary characteristic began [187].

A large number of the reported organic solvent tolerant bacteria are *Pseudomonas* strains [9,14,187] especially *P. putida*. Organic solvent tolerant mutants, tolerant to p-xylene, have been constructed from *E.coli* K-12 [11,12]. Since gram- negative bacteria have an additional outer membrane made of phospholipids and lipopolysaccharides compared to the single cytoplasmic membrane of gram-positive bacteria, it was assumed that gram –negative bacteria are better equipped to cope with solvent induced shock [365]. But recently, strains of gram–positive

bacteria like *Bacillus, Rhodococcus* and *Arthrobacter* tolerant to benzene have been reported [1,91,201,269, 308].

1.3B.2 Isolation of organic solvent tolerant bacteria :

Organic solvent tolerant *Pseudomonas* strains have been isolated from mud samples, garden, forest and humus soils [9,187]. Kato et al report that organic solvent tolerant bacteria are over 100 times more abundant in deep-sea mud samples than in terrestrial soils. Most of the gram-positive organic solvent tolerant microbial strains such as *Bacillus* DS-1906 and DS-994 and *Arthrobacter* ST-1 have been isolated from deep sea and marine mud samples. In addition other organic solvent tolerant species like *Flavobacterium* DS-711 and a yeast (*Candida* Y-40) have also been obtained from marine mud, indicating a greater biodiversity of such bacteria in the marine environment [201]. Several laboratory strains have been found to adapt to high solvent concentrations [87,421] e.g. *Pseudomonas putida* Idaho to p-xylene and *Pseuomonas putida* S12 to styrene. It has been found that *Pseudomonas* strains growing in the presence of short chain fatty acids like acetate have a lower membrane fluidity which prepares them for growth in presence of supersaturating amounts of toxic non-metabolisable solvents like toluene. *Pseudomonas putida* S12 could adapt to grow on styrene in a 2-phase styrene-water system. Acetate was toxic for *Pseudomonas* S12 but cells were able to adapt to higher concentrations of it [421,192]. *Escherichia coli* mutants tolerant to cyclohexane and p-xylene have been constructed from parent strains having a solvent sensitive phenotype. The parent strain *E.coli*

JA300 tolerated only n-hexane and diethyl ether. Spontaneous cyclohexane tolerant mutants were obtained at a frequency of one in a million after growth in an equivalent mixture of cyclohexane and p-xylene. A p-xylene tolerant mutant OST 3121 was isolated from the mixture after 1-methyl, 3-nitroso -guanidine treatment [11,12].

Kato et al have described the following method for isolation of organic solvent tolerant bacteria that degrade crude oil, polyaromatic hydrocarbons or cholesterol or utilise sulphur compounds from deep sea sediment. Benzene was added to artificial sea-water containing deep-sea sediment to a concentration of 50 % (v/v) and the cultures were incubated at room temperature on a rotary shaker for one week. After incubation, the benzene layers were carefully separated from the sea-water layers, and a portion of each benzene layer was spread on a suitable nutrient medium. Colonies that grow on the medium after incubation for 2 days at 25 or 30 degrees C were isolated and purified. Growth in presence of organic solvents in most organisms is known to lead to a decrease in growth rate and yield. The reduction in yield is due to energy consuming adaptations as well as the uncoupling effects of solvents [201]. Some reported strains of OSTB and their characteristics have been summarised in **table 1.5**.

1.3B.3 Physiological basis of solvent toxicity and the concept of organic solvent tolerance :

The primary site of action of organic solvents is the cell membrane. The cytoplasmic membrane of bacterial cells, a phospholipid bilayer, is a

matrix in which various enzymes and transport proteins are embedded. It plays a vital role in solute transport, maintaining the energy status of the cell, regulation of the intracellular environment, turgor pressure, signal transduction and energy transducing processes [365]. Solvents partition into and disrupt the lipid bilayer thus compromising cell viability. It has been proved that it is not the chemical structure of the solvent, but the concentration to which it accumulates in the cell membrane that plays a crucial role in determining toxicity.

Physiological investigation of microbes has revealed a co-relation between solvent toxicity and its log P value. The parameter log P is defined as the partition coefficient of the given solvent in an equimolar mixture of octanol and water. The greater the polarity, the lower the log P value and the greater the toxicity of the solvent [**table 1.6**]. Generally, solvents with log P values below 4 are considered extremely toxic as their degree of partitioning into the aqueous layer (which contains cells) and from there into the lipid membrane bilayer is high. The greater the degree of accumulation of the solvent in the membrane, the higher its toxicity [91,187].

Table 1.5 : Organic solvents and their log P values

Solvent	Log P value
n-decane	5.6
Decalin	4.8
Diphenyl ether	4.3
Cyclo-octane	4.2
Propyl benzene	3.8
Tetralin	3.8
Methyl cyclohexane	3.7
Hexane	3.5
Cyclo-hexane	3.2
Ethyl benzene	3.1
p-xylene	3.0
Styrene	3.0
Octanol	2.9
Carbon tetrachloride	2.7
Toluene	2.5
Heptanol	2.4
Dimethyl phthlate	2.3
Fluorobenzene	2.2
Benzene	2.0
Chloroform	2.0
Cyclohexanol	1.5
n- butanol	0.8

Each organism has its own intrinsic tolerance level for organic solvents, which is determined genetically and is also influenced by environmental factors [219]. Organic solvent tolerance is believed to be a strain specific property [11]. The tolerance level of each micro-organism is represented by two terms, the index solvent and the index value. The index value is the log P value of the most toxic organic solvent among those that can be tolerated by the organism [187,219]. Each bacterium can grow on agar media overlaid with any one of the organic solvents having a log P value greater than the index value. However, under such conditions, the growth of bacteria is suppressed by organic solvents having log P value near the index value.

1.3B.4 Tolerance mechanisms in gram-negative bacteria :

Since the destructive effect of solvents is primarily on the cytoplasmic membrane, it is expected that most adaptations should occur at the membrane level. Elucidation of tolerance mechanisms has been done mainly by comparison of the solvent tolerant mutants with the solvent sensitive parent strains and also the solvent adapted cells with non-adapted cells. The two main methods of enhancing tolerance in gram-negative bacteria are: a) creation of a low permeability barrier and b) solvent efflux. To decrease cell permeability to the solvent, cells modify their cell membrane in various ways. One of the first changes observed is the conversion of the cis- unsaturated fatty acids into their more rigid trans isomer by the enzyme cis-trans isomerase (Cti).

Table 1.6 : Some reported strains of organic solvent tolerant bacteria and their characteristics

Strain	Organic solvent tolerance	Index value	Additional information
P.putida IH-2000	Cyclohexane, xylene, styrene, heptanol	2.5	First organic solvent tolerant bacterium reported, does not metabolise toluene
P. putida DOT-T1E	90% toluene	2.5	Degrades toluene
P.putida S12	Cyclohexane, ethyl benzene, p-xylene, styrene, octanol, toluene, heptanol, dimethyl phthalate	2.3	Degrades styrene, octanol and heptanol
P.putida Idaho	p- xylene, m – xylene, toluene	2.5	Degrades p-xylene, m-xylene, toluene
E.coli JA300 Mutants of JA 300			Solvent tolerant mutants produced from a sensitive parent
OST3408	Cyclohexane	3.2	
OST3301	n-pentane		
OST3121	p-xylene	3.0	
P. putida ST-200	p-xylene	3	cholesterol degrader
Bacillus OS-1906	Hexane, cyclohexane, benzene	2	Marine polyaromatic hydrocarbon degrader
Bacillus DS-994	p-xylene, toluene, benzene	2	Utilises sulphur compounds like thiophene in water petroleum 2-phase systems
Flavobacterium DS-711	p-xylene, toluene, benzene	2	Degrades crude oil
Arthrobacter ST-1	p-xylene, toluene, benzene	2	Marine origin, cholesterol degrader, accumulates 1,4 andro-stadiene 3,17 dione

In addition to this, increased chain length of fatty acids, increase in the proportion of saturated fatty acids, changes in head group composition of fatty acids, increase in the phospholipid content as well as in the basal rate of phospholipid synthesis, modifications in the lipopolysaccharides and lack of certain porins is also observed. Organic solvent tolerance is enhanced in presence of magnesium ions. This is because Mg is an important cation in L.P.S. stabilisation. Organic solvent tolerant bacteria are known to have a faster rate of membrane repair. A quantitative increase in L.P.S. is observed with no significant chemical alterations. It is observed that *E.coli* mutants which are organic solvent tolerant have a lower cell surface hydrophobicity which in turn adversely affects binding of the solvent molecules to the cells and protects cells from damage. In case of solvents like tetralin, specific enzymes play a role in enhancing solvent tolerance.

However, all these changes are static changes and will not protect the membrane for a prolonged duration of solvent exposure. Physicochemical changes may be important in coping with exposure to the solvent, but the organism will be under great stress if it is forced to function with high levels of solvent in its membrane, which is unavoidable considering the lipophilic nature of the solvents and their tendency to accumulate in the lipid bilayer. Toluene and xylenes can reach half – equilibration across the outer membrane of these cells in a few seconds, a very short time considering the doubling time of the organisms. Hence, it was felt that a more dynamic mechanism would be involved in allowing the cells to grow.

The amazing levels of solvent tolerance in most *E.coli* and *Pseudomonas* cultures are now believed to be mainly owing to the presence of active efflux pumps which play a role in extrusion of the solvent. This is an energy-dependent mechanism and hence the cell yield in presence of solvents is lower than under normal conditions. Isken and de Bont used ^{14}C labelled toluene to obtain evidence of an energy dependent export system functioning in the toluene resistance of *P. putida* S12. This has now been confirmed with other solvent tolerant strains of *Pseudomonas* and *E.coli*. Efflux systems vary in their degree of specificity. Multi-drug efflux pumps with a broad substrate specificity make a major contribution to intrinsic and acquired multiple antibiotic resistance, e.g. Mex-AB-Opr M in *P.aeruginosa* and Acr AB in *E.coli*. The integrity of cell surface structures is found to be essential for the organic solvent exclusion system to functions. Recently, a novel toluene elimination mechanism has been reported in *P.putida* IH-2000. The culture was found to release membrane vesicles composed of phospholipids, lipopolysaccharides, and very low amounts of outer membrane proteins. The toluene molecules adhering to the outer membrane are eliminated by shedding of these vesicles, and this system appears to play an important role in the toluene tolerance mechanism. These vesicles contain a much higher concentration of toluene than the membrane and such shedding of vesicles is not observed in the toluene-sensitive mutants. The tolerance mechanisms of gram-negative bacteria have been extensively studied. [8,10, 12,13,15,16, 19,21,72, 98,158, 159,160,161,173,177,190,191,193,

194,208,209,210,219,221,241,247,270,280,281,300,310,325,326, 327,358,364,365, 366,367, 391, 419,420,421]

1.3B.5 Probable tolerance mechanisms of gram- positive bacteria :

While the gram –negative bacteria show tolerance to high concentrations of toluene (log P =2.5), the reported gram-positive organic solvent tolerant isolates like *Rhodococcus* sp., *Arthrobacter* ST-1, *Bacillus* DS-944 and DS-1906 show excellent tolerance to benzene (log P=2.0) which is much more toxic than toluene. [201] However, very little information is available on what makes these cultures tolerant to benzene. There is a difference between the cell membranes of gram-positive and gram-negative bacteria, and the additional outer membrane is missing in gram-positive bacteria, which however possess a more extensively linked peptidoglycan [365]. Studies need to be undertaken to determine whether the molecular mechanisms of solvent tolerance elucidated in gram-negative bacteria are also conserved in gram-positive bacteria. It has been proposed that the mechanisms of solvent tolerance of the benzene tolerant *Bacillus* DS-1906 and the toluene tolerant *P.putida* IH-2000 are different, due to differences in cell surface components. Many of these benzene tolerant bacteria also have the potential to degrade this solvent. It is believed that organic solvent emulsifying/ deactivating/ solubilising enzymes/ substances could play a very important role in diminishing solvent toxicity in gram-positive bacteria [1].

Table 1.7: Organic solvent tolerance mechanisms of bacteria

I.Gram-negative bacteria: E.g. some strains of *Pseudomonas* and certain *E. coli* mutants	Known mechanisms: 1) modifications in cell envelope to increase cell membrane rigidity and decrease permeability: a) cis-trans isomerisation of membrane fatty acids by cis-trans isomerase b) decreased cell surface hydrophobicity c) changes in chemical composition/ proportions of membrane lipids and proteins 2) increased rate of membrane repair enzymes 3) special solvent inactivating enzymes 4) active efflux of solvents by means of solvent efflux pumps (*tol C / mar/ rob /sox S / acr AB* genes) 5) release of membrane vesicles with solvent molecules adhering to them 6) production of phage shock protein (stress protein in *E.coli*)
II.Gram- positive bacteria E.g. some strains of *Bacillus*, *Rhodococcus*, *Arthrobacter*	Proposed mechanisms: 1) protection rendered by endospores? 2) induction of general stress regulon, leading to the production of general stress proteins ? 3) solvent deactivating or emulsifying enzymes?

With reference to *Bacillus* species, it would be interesting to compare the effect of organic solvents on spores and vegetative cells. Spores have been recognised as the hardiest life-forms on earth and display an almost unbelievable resistance to adverse conditions. *Bacillus* spores are known to withstand heat, uv and oxidative damage, dessication and chemical agents like acids, bases, phenols, alcohols, alkylating agents etc. Factors important in spore resistance to chemicals are: impermeability of the spore coat to hydrophilic chemicals, low spore core water content which keeps the enzymes in an inactive state and protection of spore DNA by alpha-beta SASP proteins. *Bacillus* endospores have survived in 95%(v/v) ethanol for prolonged periods [288,353]. However, the growth rate of *B. subtilis* is found to be lowered but the final yield remains unchanged when ethanol is present in the growth medium. At concentrations allowing growth at half-maximal rate, practically no spores are formed. Post exponential events such as excretion of certain enzymes and modification of RNA polymerase are altered or suppressed in presence of ethanol. Sensitivity to ethanol is much greater for the sporulation process than growth, since a concentration of 0.7 M may reduce the yield of spores to the extremely low value of 10^{-5}, although it reduces growth rate only by half. It is possible that similar effects on sporulation may be induced by organic solvents.

Another angle which needs to be investigated, is the involvement of the general stress regulon, in solvent tolerance of gram- positive bacteria. It has been established that cells are under great stress in presence of

organic solvents and various genes undergo activation when subjected to solvent shock or other stress stimuli which threaten the cell membrane. This can be demonstrated by various instances.

In *E.coli*, organic solvent tolerance levels of strains are improved by over-expression of the stress response genes marA, robA and soxS. The AcrAB efflux system which is triggered during solvent shock is also stimulated by other stress conditions like salt and ethanol exposure. *E.coli* K12 strains express the phage shock protein A (pspA) under various stress conditions like heat shock, hyperosmotic shock, salt or ethanol treatment, prolonged stationery phase incubation, during inhibition of protein, fatty acid and ATP synthesis. Psp A is believed to play a role in maintaining proton-motive force under stress conditions. It was found that introduction of a multi-copy plasmid vector carrying psp operon into *E.coli* improves the survival frequency of cells on sudden exposure to organic solvents like n-hexane, but not the growth rate [219].

Hence, the possibility that general stress proteins could be induced on solvent shock in gram – positive bacteria also can not be neglected. Like the sigma S of *E.coli, Bacillus subtilis* also controls a large stationery phase regulon called the sigma B regulon . Sigma B is a secondary sigma factor of *Bacillus subtilis*. The RNA polymerase containing sigma B transcribed a sub-set of genes expressed after heat shock or on the onset of stationery phase Initially it was believed that the sigma B regulon of *Bacillus subtilis* is induced only in the stationery phase. However, it is now known that sigma B activity is induced both upon entry into the stationery phase and by environmental stresses such as salt and heat

stress and ethanol shock ,during logarithmic growth. Data suggest that the sigma B regulon of *Bacillus subtilis* together with the associated regulatory network provides a less extreme alternative to sporulation under growth limiting conditions [33,34,35,36,37,54,55, 154,155,123, 260,263,404,405,406,407]. This may become critically important under environmental conditions that do not support sporulation. Since ethanol represses sporulation in *B.subtilis*, [45] it is possible that organic solvents could exert a similar effect.

Extensive studies have been done on the general stress regulon of *Bacillus subtilis*. It is known that sigma B is required for the induction of approximately 100 genes after imposition of a whole range of stresses and energy limitations (exposure to heat, acid, ethanol, salt stress, deficiency of either glucose, phosphate or oxygen). Sigma B null mutants unable to induce the regulon following shock displayed at least a 50 to 100 fold reduction in survival after exposure to heat (54 degrees C), ethanol (9%), salt (10%), acid (pH 4.3) as well as freezing and desiccation compared to the wild type. Preloading cells with sigma B dependent general stress proteins prior to subjecting to the stress response confers considerable protection. Loss of sigma B reduces the viability of stationery phase cells grown in alkaline or acidic media and also decreases the cell yield in Luria broth containing high concentration of ethanol. It is interesting to note that sigma B is very strongly induced by ethanol shock, and ethanol is known to have multiple effects on the cell membrane, hence the site is damage is the same as that of organic solvent. A large

number of csb products appear to be associated with the cell envelope and it has been hypothesised that loss of sigma B function would become evident under environmental conditions that challenge envelope functions]. It is tempting to speculate that certain sigma B proteins induced strongly by ethanol shock, such as Gsp 9,33,67, 70,71,80 and GtaB which confer a survival advantage on the cell when the membrane is affected could play a vital role in protecting from organic solvent shock, which inflicts similar damage. Another interesting feature is that the extent of the sigma B dependent stress response varies from strain to strain, among specific strains used in analysis. For instance, *B. subtilis* 168 displayed a less pronounced induction of sigma B dependent stress genes than *B. subtilis* IS58. It is possible the degree of solvent tolerance increases with the strength of the stress response. The crucial role of multi-drug efflux transporters, whose expression is induced by structurally divergent compounds such as antibiotics, inhibitors and other toxic compounds in organic solvent tolerance of *E.coli* and *Pseudomonas* strains has been extensively researched. In *B. subtilis*, the bmrUR operon, which encodes proteins that may contribute to resistance to multidrug compounds is regulated by sigma B. It is highly probable that multi-drug efflux proteins of *Bacillus* could also be involved in solvent tolerance.

1.3B.5 Conclusions and future trends:

It is evident that organic solvent tolerant bacteria are an interesting group of extremophiles with unique adaptations, cell components and enzymes

capable of protecting the cells and allowing them to function in solvent saturated environments [115]. This property can be exploited in several industrial processes. Studies done on the solvent tolerance mechanisms of bacteria like *E.coli* and *Pseudomonas* have enriched our understanding on what makes cells withstand such severe stress. However, there is a large void in available data on such mechanisms in gram – positive bacteria. More studies need to be undertaken in this direction. Also, fresh habitats need to be explored to isolate other species displaying such tolerance.

C) Industrial applications of organic solvent tolerant bacteria

The toxic effects of organic solvents on bacteria are well-known. Organic solvent tolerant bacteria are a relatively novel group of micro-organisms which combat these destructive effects and thrive in presence of high concentrations of organic solvents due to various adaptations. Their potential in industrial and environmental biotechnology is tremendous, since their enzymes have the potential to function in the media saturated with organic solvents. This property could be exploited to carry out bio-remediation and biocatalysis in presence of organic solvents. Since a large number of substrates used in industrial chemistry, such as steroids are water –insoluble, their bioconversion rates are affected by poor dissolution in water. This problem could be overcome by carrying out the process in a biphasic organic-aqueous fermentation system, wherein the substrate is dissolved in the organic phase and provided to cells present in the aqueous phase. In bio-processing of fine chemicals like cis-diols and epoxides using such cultures, organic solvents can be used to extract a toxic product from the aqueous phase, improving the efficiency of the process. Bacterial strains reported which grow on and utilise supersaturating concentrations of organic solvents like toluene could revolutionize the removal of such pollutants. The role of solvent stable enzymes in non-aqueous biocatalysis needs to be explored and could result in novel applications.

1.3C.1 Biphasic / non-aqueous systems in bio-catalysis:

Advantages of carrying out biotechnological processes in presence of organic solvents:

1. Water is not the ideal medium for the majority of organic reactions. Many reactants such as molecular oxygen, steroids and lipids are more soluble in organic solvents than in water and some products may be quite labile in an aqueous environment .

2. Accomplishing reactions with such substrates (e.g aerobic oxidation of oestrogens catalysed by fungal laccase, cholesterol transformation) in non-aqueous media allow a much increased volumetric activity to be achieved.

3. Microbial contamination is much less of a problem in such solvents, and the consequent absence of microbial proteases may lead to an apparent stabilisation in the biocatalyst.

4. Some polymerising reactions such as the polymerisation of phenols catalysed by peroxidase, will produce a higher molecular weight product when carried out in a solution more able to dissolve the product (ie. oligomers) initially formed.

5. Under normal physiological conditions, hydrolytic enzymes catalyse the degradation of polymers ie. hydrolases are transferases normally transferring a moeity to the acceptor, water. Water is normally present in a vast molar excess over other potential acceptor molecules so no reaction occurs other than hydrolysis. Also the normal concentration of water (about 55.5 M) is much greater than its typical Km (about 50 mM) and the rate of hydrolysis will not be affected as the hydrolysis proceeds. By

greatly reducing the water activity in these systems, they can be used to transfer to other acceptors. An example of this can be found in the transesterifiction reactions of esterases and lipases. Lipolytic enzymes are often used in non-aqueous media because these media can increase substrate solubility and facilitate product recovery and are favorable for reactions such as ester synthesis and transesterification, both of which are thermodynamically unfavourable in water. Enzymatic reactions using protease in the presence of organic solvents have been extensively studied for the synthesis of peptides and esters. If organic solvents can be used as media for enzymatic reactions, the reaction equilibria of hydrolytic enzymes can be shifted towards completion of the reverse reaction of hydrolysis, that is the synthetic reaction.

6. For bioconversion of organic compounds with low solubilities in water, large volumes of appropriate medium are required for solubilisation of the compounds. This consumption of media and water and the inevitable treatment of waste-water constitute one of the major cost factors in bioconversion fermentation. If the water insoluble compounds were suspended in a small volume of the medium, it would take a very long time to complete the bioconversion. The solubility of most steroid compounds as well as the products expected from the bioconversion in water is extremely low (10^{-2} to 10^{-3} grams/ litre of water). Cholesterol is usually suspended in bioconversion systems containing surfactants to affect the bioconversion rate, but this does not prevent the formation of solid particles. Some organic solvent tolerant bacteria capable of transforming cholesterol in a biphasic system containing cholesterol

dissolved in the organic phase and cells suspended in aqueous phase have been discovered. *Pseudomonas* sp. strain ST-200 effectively oxidised the C3 and C6 positions of cholesterol by introduction of a hydroxyl or ketone group in presence of a mixed organic solvent (p-xylene and p-diphenyl methane 3:7 v/v). The ST-200 enzyme is constitutive and extracellular in nature. Its cholesterol consumption rate in presence of benzene, toluene, p-xylene, propyl benzene or diphenylmethane was 3 to 3.5 fold higher than in absence of organic solvent. *Arthrobacter* ST-1 shows a very high percentage of cholesterol degradation when n-decane and n-dodecane are employed as organic phase. Androsta-1,4-diene-3,17 –dione was the product obtained.

7. Another important advantage of having an organic phase in the system lies in elimination of toxic products from the fermenter. Product toxicity of fine chemicals is a problem in several biotechnological processes. In many instances, a second phase of the organic solvent can extract the toxic product from the aqueous phase during the fermentation. *Pseudomonas oleovorans* can convert 1,7-octadiene into both 7,8 –epoxy-1-octene and 1,2 –7,8 –diepoxy-octane when grown on octane. Epoxides are very toxic to cells and hence high concentrations of the products could not be reached in the aqueous phase. By using cyclohexane as the second phase, the production of epoxides was enhanced because the monoepoxide preferentially partitioned into the cyclohexane phase. The use of organisms in production of lipophilic compounds in 2-phase systems is limited by the range of solvents that the organism can withstand as a second phase. It is here that solvent tolerant

bacteria play a vital role by allowing a new degree of freedom in coping with toxic products. They can tolerate solvents that are much less lipophilic and these can be used to partition out toxic products from the aqueous phase. Many important fine chemicals including catechols, phenols, aldehydes and ketones, low molecular weight epoxides and diepoxides, medium chain alcohols and terpenoids are in low lipophilicity range.

There is ample proof to show that enzymes of organic solvent tolerant bacteria can perform stably in presence of an organic solvent [103, 104,296,297,298,416]

Extensive research has been done in the field of non-aqueous bio-catalysis and enzymes in biphasic systems [7,27,31,71,72,66, 67,90,91, 100,101,102,113,149,152,153,148,146,203,207,216,217,218,259,262, 304, 341,342, 381,383,390,428, 440,441,442].

1.3C.3 Role of organic solvent tolerant bacteria in bio-remediation and waste-water treatment

Many aromatic hydrocarbon degrading strains have been isolated, but they are usually solvent sensitive and degradation of aromatic hydrocarbons occurs only when these compounds are supplied at low concentrations. Organic solvent tolerant bacteria with the requisite catabolic potential can be of vital importance in clean-up operations. For instance, A *Rhodococcus* sp. strain 33 isolated from a contaminated site in Sydney can degrade benzene at concentrations of 200 ppm and tolerate high concentrations of benzene. This culture also grows in

presence of 6% NaCl and at temperatures from 0-37 degrees C, which are necessary characteristics for a culture if it has to be used in cleaning up marine oil spills [308]. Abe et al (1995) isolated an organic solvent tolerant bacterium from deep-sea sediment samples after treatment with 50% v/v benzene. This strain, *Bacillus* DS-1906 showed polyaromatic hydrocarbon degrading ability in presence of organic solvent. It degraded 48% of naphthalene solubilised in n-hexane and the amount degraded was more in the presence of solvent [1]. Huertas et al (1998) assayed the tolerance of three different toluene degraders to organic solvents in soil. The toluene tolerant *Pseudomonas putida* DOT-T1E recovered from the shock faster than *P.putida* F1 and hence became established at higher densities in the polluted sites. Their studies show that *Pseudomonas* strains are more resistant to solvents in soils than in liquid culture medium, which may explain why these microbes deal with these pollutants in soil and in biofilms. However, the level of tolerance in soil is related to the level of tolerance in liquid medium. The higher the tolerance in the liquid medium, the faster the recovery of the strain in soil after solvent shock. Therefore, in sites heavily polluted by aromatic hydrocarbons, solvent tolerant strains would be expected to become established first, to colonise the site and become predominant in the removal of such compounds [166]. The catabolic potential of *P. putida* was expanded to include m- and p-xylene and related hydrocarbons by transfer of the TOL plasmid pWW0-Km [327].

Chapter 2

Screening of L- asparaginase producing and cholesterol transforming bacteria from coastal and estuarine habitats

Section A : Screening of L-asparaginase producing bacteria from coastal and estuarine samples

L-asparaginase is an amino-hydrolase enzyme which cleaves asparagine to release aspartic acid and ammonia. This enzyme is a crucial component of the synergistic therapy used in remission induction protocols of certain cancers especially acute lymphoblastic leukemia (ALL). Several clinical studies have been performed on this enzyme which confirm its powerful anti-leukemic activity. Leukemic cells have a low asparagine synthetase activity and very high requirement for asparagine, as compared to normal cells. Asparaginase destroys exogenous asparagine, an essential growth factor and halts mRNA translation, which eventually leads to apoptosis or cell suicide, selectively, in cancer cells [189, 283]. Currently, the sources for industrial manufacture of this enzyme are certain strains of *E.coli* and *Erwinia.* However, due to hypersensitivity reactions induced by their enzymes, low yields and extensive purification procedures, the search for new strains of L-asparaginase producers continues. Presently, the industrial manufacture of this enzyme is being done through certain strains of *E.coli* and *Erwinia.* [320,352]

The search for new asparaginase producers continues due to several reasons. The industrially used strains being gram-negative bacteria, extreme care is required in purification and complete removal of endotoxin components, hence an extra-cellular enzyme from natural sources would be ideal. Besides, hypersensitivity to L-asparaginase on prolonged exposure is developed, due to the

production of anti-enzyme antibodies which necessitates the administration of a different serological entity [295,211]. There is always scope to improve upon the yields / activity presently given by the strains in use [86].

Recent reports on L-asparaginase producing bacteria isolated along the Indian coastline are promising [323,324,352, 361]. Hence, this work was undertaken to isolate L-asparaginase producing bacteria from marine and estuarine sediment. The amino-hydrolase enzyme glutaminase is also known for its anti-leukemic activity [338], hence presence of glutaminase activity among the L-asparaginase producing bacteria was also determined.

Materials and Methods:

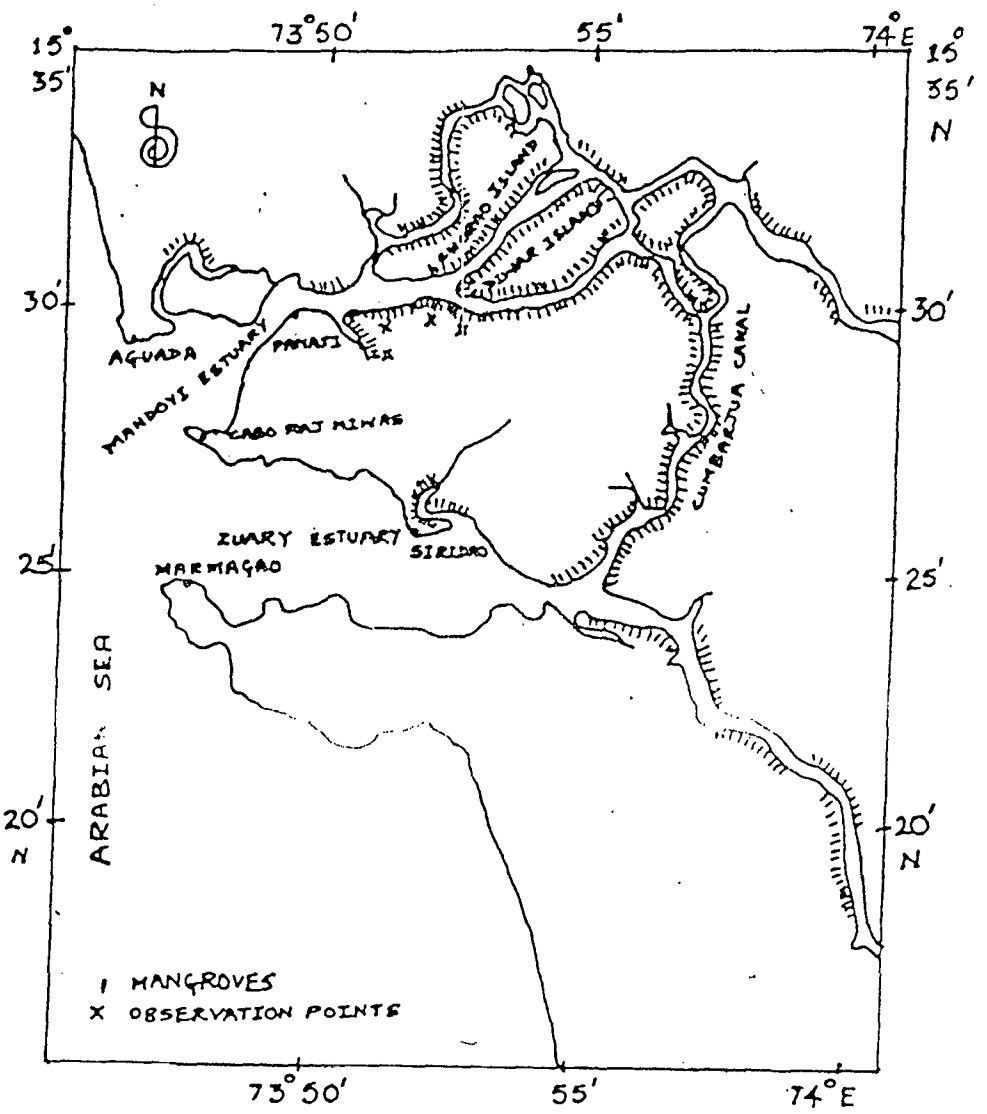
2A.1 Sample collection: The state of Goa extends over 3611 km and lies on the West Coast of India surrounded by the Arabian sea on the west, touching Maharashtra on the north and Karnataka in the east and south. It lies between the north latitudes 14 degrees 53`5``–15 degrees 57` 5``and east latitudes 73 degrees 40` 5``-73 degrees 20`11``. The mean maximum and minimum temperature ranges from 32 degrees C and 21.55 degrees C with humid tropical climate. Goa has a very rich mangrove forest association, the total extent of mangroves being about 2000 ha; 900 ha along Zuari estuary, 700 ha in Mandovi estuary, 200 ha along Cumbharjua canal and remaining in Chapora, Talpona, Galgibaga and Tiracol river estuary **(fig 2.1)**. To exploit the rich biodiversity from these habitats, estuarine samples were collected from Mandovi estuary, along the Panjim - Ribander highway **(fig 2.2)** and along the coast-line.

2A.2 Screening for L-asparaginase producing bacteria from coastal and estuarine samples:

A total of 8 coastal and estuarine sediment and water samples were screened for L-asparaginase producing bacteria by Gulati et al`s novel semi-quantitative rapid plate assay method [144].

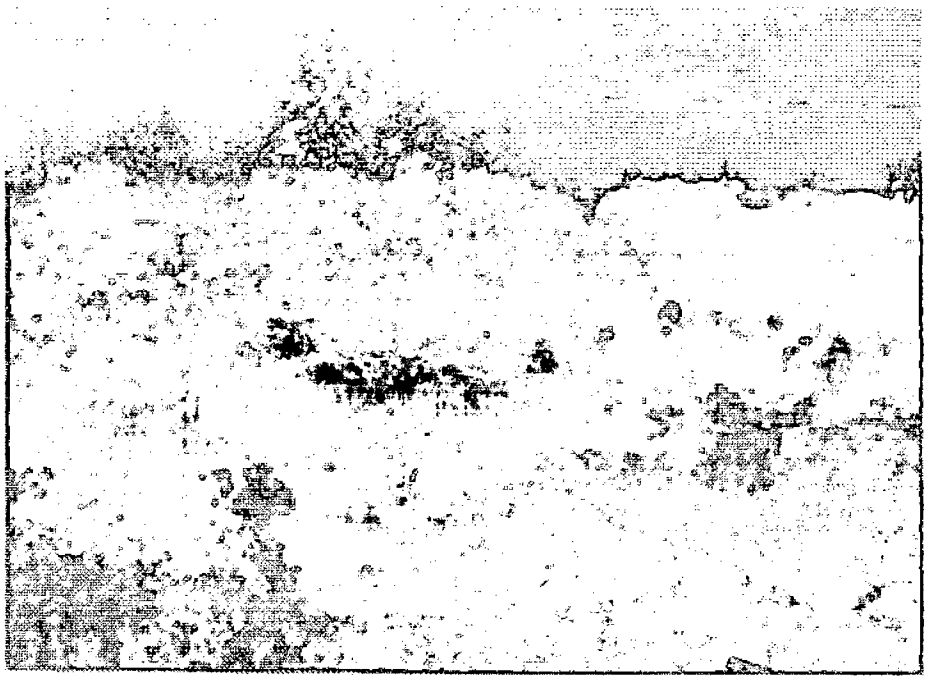
This involved inoculating the samples on M9-asparagine medium containing phenol red as indicator. Colonies producing a pink zone around them were isolated and purified.

Fig 2.1: Map of Goa showing estuaries along the coast-line



MAP OF GOA SHOWING MANGROVES IN MANDOVI AND ZUARY ESTUARIES AND INTERCONNECTING CUMBARJUA CANAL

Fig 2.2: A Sampling Site in the Mandovi Estuary, Goa.



Plates were incubated at 37 degrees for 24-48h. Control plates were maintained replacing the amino acid with sodium nitrate as the nitrogen source instead. Cultures obtained were purified by repeated streaking on the same medium and their colony characters recorded. Pure cultures were spot-inoculated on the centre of the M9-asparagine–phenol red agar and zone sizes recorded. Gram staining of the isolates was done.

2A.3 Effect of concentration of asparagine on zone size:

To detect the bacteria producing L-asparaginase with the lowest Km value (enzyme able to act at very low asparagine concentrations), plates of M9-sea water-asparagine-phenol red agar were prepared with varying asparagine concentrations of 0.05, 0.10,0.25 and 0.50 %. Each culture was spot-inoculated in the centre of the plates having the different asparagine concentrations. The plates (pH 7.4) were incubated at 37 degrees C for 24 hours and zone sizes were recorded. The selected cultures were routinely grown on M9-sea water-asparagine agar.

2A.4 Detection of glutaminase producing bacteria:

To detect glutaminase producers, the L-asparaginase producing bacteria were spot-inoculated on M9 – sea water- phenol red medium prepared in which asparagine is substituted with L-glutamine (0.5%).The plates were incubated at 37 degrees C for 24 hours. Glutaminase activity was indicated by the appearance of a pink zone.

2A.5 Determination of sea-water requirement: All the isolates were inoculated on M9-phenol red –asparagine agar prepared in distilled water instead of sea water and incubated at 37 degrees C for 5 days. Growth of the cultures was noted as an indication that the cultures do not have an obligate requirement for sea water.

2A.4 Identification of the L-asparaginase producing bacteria: The identification of the selected L-asparaginase producing bacteria A1, A2 and F2 was done on the basis of biochemical characteristics, as per Bergey`s Manual of Systematic Bacteriology [372].

Results and Discussion:

Estuarine and coastal samples were collected from eight different sites in Goa and screened for Lasparaginase production. **[table 2A.1].**

The screening procedures used led to the detection and isolation of 11 bacterial cultures producing L-asparaginase, with 7 being gram – positive and 4 gram – negative. Morphological and cultural characteristics were noted and are recorded in **table 2A.2**. All the samples showed a larger proportion of gram- positive rods as compared to other morphological types.

Traditionally, screening for L-asparaginase producing bacteria has been a labour-intensive and time-consuming process, as it has involved detection of ammonia in culture supernatants of random individual isolates by Nessler`s reaction [178,411,412]. However, in the present work, Gulati`s novel semi-quantitative rapid plate assay method was

adapted [144]. This method is a pH and dye based semi-quantitative plate assay procedure for detection and isolation of bacteria capable of producing an enzyme of potential clinical utility. This was accomplished by directly plating diluted samples on modified M9-asparagine-sea water – phenol red agar having asparagine as the sole nitrogen source. Growth of the culture as well as appearance of a pink zone around the L-asparaginase producing colony was taken as a clear indication of enzyme activity [**fig. 2A.1, A-D**]. Gulati et al have shown that the zone size obtained on these plates can be directly co-related with the enzyme activity in the culture supernatants. The criteria used in the selection of potentially useful cultures were;

a) good growth on M9 medium and nutrient agar
b) ability to produce zones on plates having low asparagine concentrations
c) L-asparaginase activity at pH 7.4 and 37 degrees C [144] ,since, L-asparaginase for clinical utility needs to be active under these physiological conditions

Colonies producing pink zones were isolated, purified by streaking and the enzyme activity was confirmed by the spot-inoculation of the pure culture on the same medium and checking for pink zones [**table 2A.3**].

Fig 2A.1: Zone obtained on M9-Asparagine-phenol red Agar due to the Asparaginase Activity of the Bacterial isolate A1

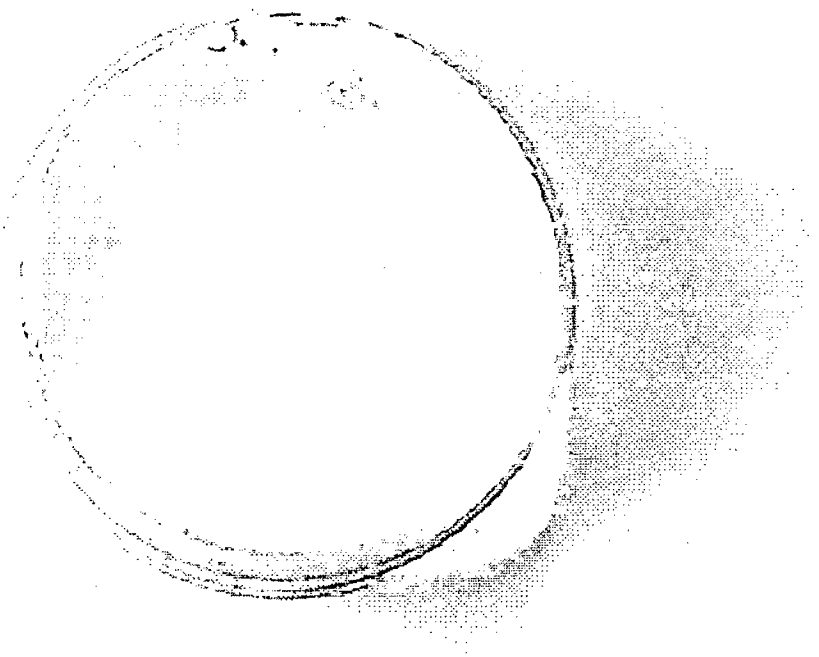


Fig 2A.1(B): Growth of *Bacillus F2* on M9-Asparagine-phenol-red agar

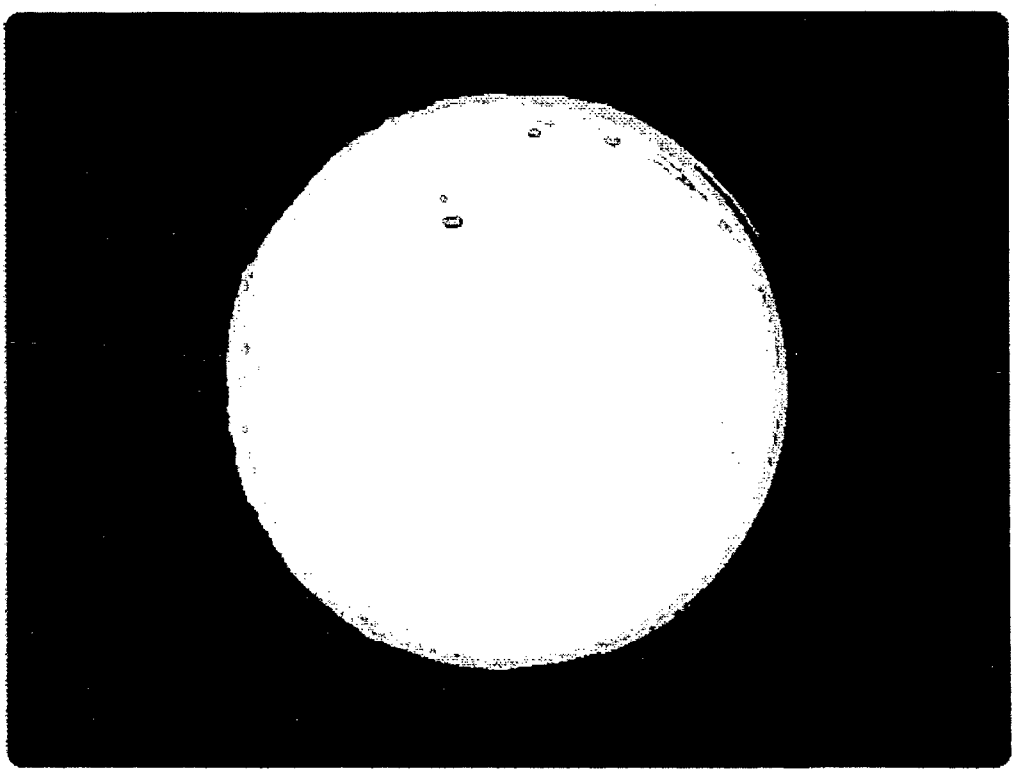


Fig 2A.1(C): Growth of an L-Asparaginase Producing Bacterium On Control plate containing Sodium Nitrate instead of L-Asparaginase

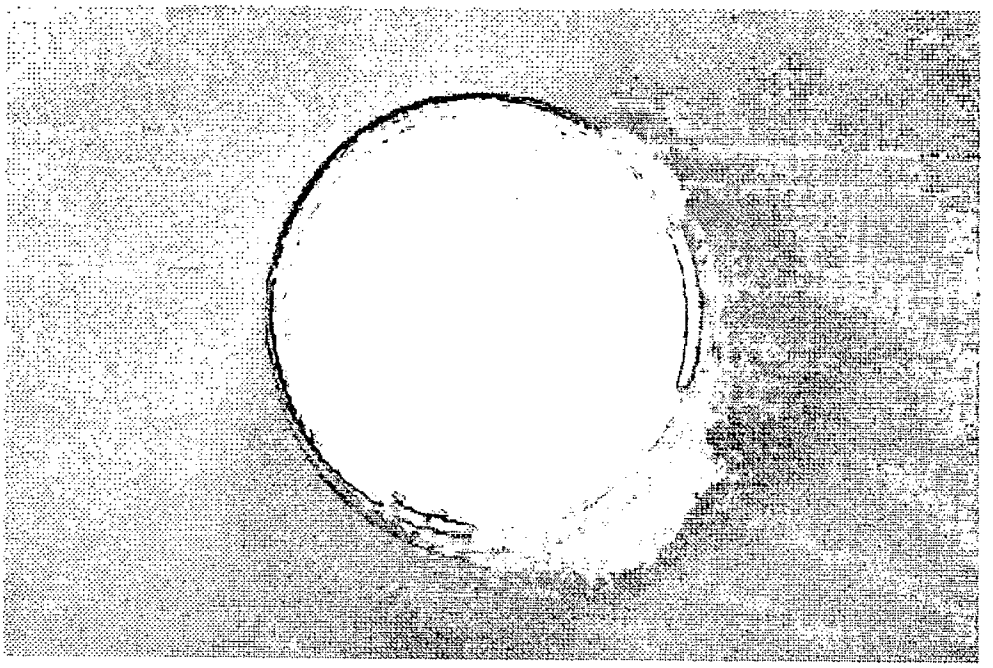


Fig 2A.1(D): Plate Inoculated with *Bacillus A1* turns pink after 48 hours.

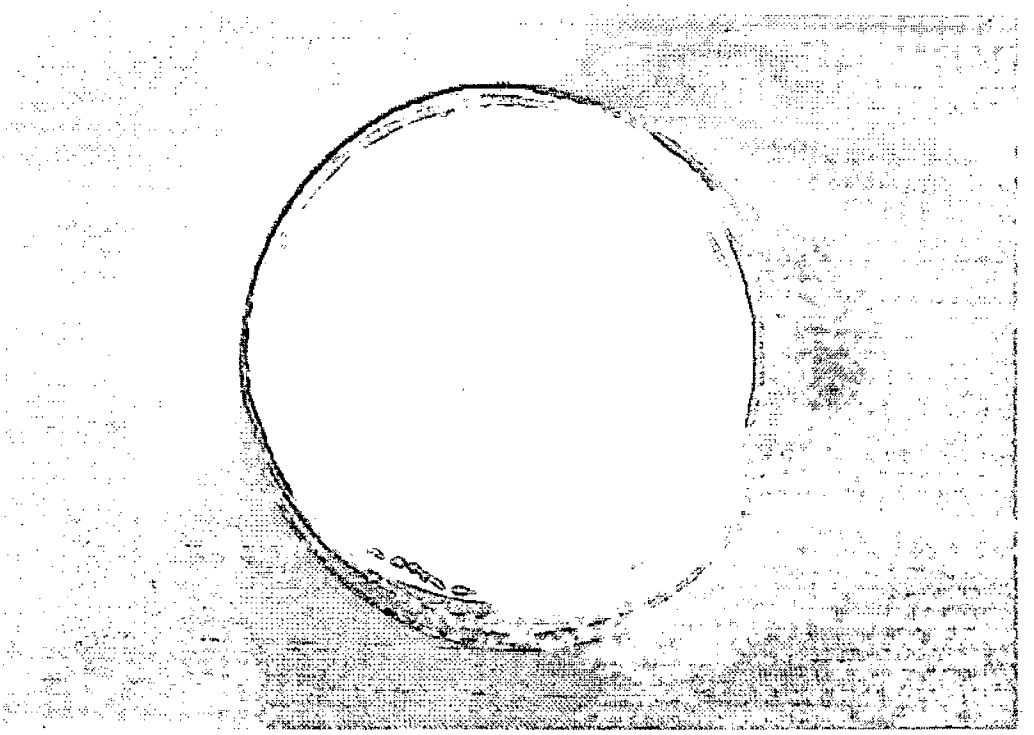


Table 2A.1: Screening and isolation of asparaginase producing bacteria

Sample name	Nature	Cultures isolated
A	sediment	A1, A2, A3
B	sediment	B1
C	sediment	C1
D	water	-
E	Sediment	E1, E2, E3
F	Sediment	F1, F2, F3
G	Water	-
H	Water	-

A total of 11 bacterial cultures were obtained by screening of 8 samples. Samples A-D are of coastal origin, whereas E-H were taken from Mandovi estuary.

Table 2.2:
A) Morphological and Cultural characters of the bacterial isolates producing L-asparaginase on M9-asparagine –sea water agar:

Cultures of coastal origin

Culture name	A1	A2	A3	B1	C1
Gram character and morphology	Gram positive rods	Gram negative short rods	Gram positive long rods in chains	Gram positive rods	Gram positive rods
Colony characters :					
Size	Large	Medium	Small	Medium	Large
Shape	Irregular	Circular	Circular	Circular	Circularspreading
Colour	White	White	Cream	White	White
Consistency	Slimy turning dry	Butyrous	Butyrous	Butyrous	Slimy
Opacity	Opaque	Opaque	Transparent	Opaque	Opaque
Elevation	Flat	Convex	Raised	Raised	Raised
Margin	Rough	Entire	Entire	Entire	Entire

Cultures of estuarine origin

Culture name	E1	E2	E3	F1	F2	F3
Gram character and morphology	Gram positive rods	Gram positive rods	Gram negative short rods	Gram negative fine rods	Gram positive long rods	Gram negative cocco-bacilli
Colony characters :						
Size	Small	Small	Pinpoint	Medium	Small	Pinpoint
Shape	Circular	Circular	Circular	Circular	Circular	Circular
Colour	White	Yellow	White	White	Yellow	White
Consistency	Dry	Butyrous	Butyrous	Butyrous	Dry	Dry
Opacity	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque
Elevation	Flat	Raised	Flat	Raised	Raised	Raised
Margin	Entire	Entire	Entire	Entire	Entire	Entire

2A.2 B) Colony characteristics of bacteria producing L-asparaginase on nutrient agar:

Colony characters	A1	A2	A3	B1	C1
Size	Medium	Small	Small	Small	Spreading
Shape	Wrinkled	Circular	Circular	Circular	Irregular
Colour	Irregular	Cream	Cream	Cream	Cream
Consistency	Slimy turning dry	Butyrous	Butyrous	Dry	Butyrous
Opacity	Opaque	Opaque	Transparent	Transparent	Transparent
Margin	Rough	Entire	Entire	Entire	Entire
Elevation	Flat	convex	Convex	Convex	Raised

Colony characters	E1	E2	E3	F1	F2	F3
Size	Small	Medium	Small	Small	Medium	Small
Shape	Circular	Circular	Circular	Circular	Circular, wrinkled	Circular
Colour	Cream	Creamish yellow	Cream	Creamish yellow	Yellow	Cream
Consistency	Butyrous	Butyrous	Butyrous	Butyrous	Butyrous	Butyrous
Opacity	Transparent	Opaque	Transparent	Transparent	Opaque	Transparent
Margin	Entire	Entire	Entire	Entire	Entire	Entire
Elevation	Raised	Flat	Raised	Raised	Convex	Raised

To select the cultures having L-asparaginase with the highest affinity for asparagine , pure cultures were spot-inoculated on plates with varying asparagine concentrations , 0.05, 0.1, 0.25 and 0.5% and the comparative zone sizes on the modified M9 medium noted. All the cultures produced pink zones on plates having 0.5 and 0.25 % asparagine, whereas no zones were seen on plates with 0.05% asparagine [**table 2A.4].** The isolates A1, C1, E1, E2, F2 produce zones with 0.1 % asparagine indicating that their L-asparaginases have a potentially higher affinity (lower Km) for the substrate, asparagine. Among all the L-asparaginase producers, C1 produced the largest zone on M9-asn agar, due to the spreading nature of the colonies. The other two cultures, A1 and F2 showing good zones i.e. 12 and 20 mm respectively were selected for further studies. A2 was selected because it was one of the few gram-negative bacterial isolates obtained from these samples. In addition to L-asparaginase activity it also exhibited very high glutaminase activity and showed very good salt tolerance (excellent growth in presence of 10% NaCl**).[table 2A.5]** Like the aminohydrolase enzyme asparaginase, L-glutaminase which degrades glutamine is also known to exhibit anti-leukemic activity [338]. Hence, screening for glutaminase activity was done by spot-inoculation of the 11 isolates on M9 medium with glutamine as the carbon source.

Table 2A.3: Detection of L-asparaginase and L-glutaminase activities of the isolated cultures by Gulati`s semi-quantitative plate assay

Culture	Zone size on asparagine agar	Zone size on glutamine agar
A1	65mm	-
A2	30mm	+(full plate pink)
A3	20	-
B1	30mm	-
C1	65mm #	-
E1	20mm	-
E2	25mm	+(10mm)
E3	15mm	-
F1	20mm	-
F2	25mm	+(20mm)
F3	20mm	-

The large zone size of C1 is on account of the tendency of the colonies to spread throughout the plate.

The zone sizes were recorded after incubating the plates, pH 7.4 for 48 hours at 37 degrees C.
None of the cultures show an obligate requirement for sea water, although all are halotolerant.

Medium used was M9-phenol red agar with either asn or gln as the sole N source.

Table 2A.4: Relation between asparaginase concentration and zone size formed by the cultures

Culture name	0.05% asn	0.10% asn	0.25% asn	0.50% asn
A1	+	12	20	65
A2	+	+	12	30
A3	+	+	10	20
B1	+	+	15	30
C1#	+	40	60	65
E1	+	6	20	20
E2	+	10	15	25
E3	+	+	12	15
F1	+	+	15	20
F2	+	20	20	35
F3	+	+	10	20

+ refers to growth but no zone formation.# large zone sizes are on account of spreading colonies

Among the 11 bacteria isolated, only four cultures exhibited L-glutaminase activity namely A1, F2 and E2 which were gram-positive rods and A2, a gram-negative rod. None of the cultures had an obligate requirement for sea water, although all the cultures showed good growth on sea water agar, indicating their halotolerance.

Identification of the selected isolates A1, A2 and F2 based on biochemical tests as per the Bergey`s Manual of Systematic Bacteriology [372] revealed the cultures A1 and F2, which are aerobic, endospore forming, catalase positive , gram positive rods to be species of *Bacillus*, whereas A2 , a gram negative non-saccharolytic rod was identified as *Alcaligenes* species **[table 2A.6].**

It has been reported in prior studies that L-asparaginase producing bacteria were found in concentrations of upto 10^5 per ml or gram from marine environments on the west coast of India of which species of *Aeromonas, Bacillus, Vibrio, Pseudomonas* and *Acinetobacter* were the main L-asparaginase producing bacteria.

Table 2A.5: Characteristics of potential selected isolates

Culture	Selection criteria
A1	Gram +ve rod of marine origin showing good asparaginase activity at 37 degrees and pH 7.4, exceptionally good activity at low asparagine concentration compared to other cultures, rapid and good growth on M9 and nutrient agar
A2	Gram –ve rod of marine origin, showing asparaginase and glutaminase activities at 37 degrees and pH 7.4 with tolerance to 10% NaCl, rapid and good growth on M9 and nutrient agar
F2	Gram +ve rod of estuarine origin, showing asparaginase and glutaminase activities at 37 degrees and pH 7.4, good activity at low asparaginase concentrations, rapid and good growth on M9 and nutrient agar

Table 2A.6) : Biochemical and physiological characteristics of the selected asparaginase producing bacteria :

Characteristic	A1	A2	F2
Gram character and morphology	Gram +ve small rods	Gram –ve rods	Gram +ve long rods
Spores	+	-	+
Swelling of cells	-	-	-
Oxidase	+	+	+
Catalase	+	+	+
Glucose fermentation	+(acid produced, no gas)	- (alkali produced)	+(acid produced, no gas)
Nitrate reduction	+	+	-
Aerobic growth	+	+	+
Anaerobic growth	+	+	+
Methyl Red	-	-	-
Voges Proskauer	+	-	-
Indole	-	-	-
Starch hydrolysis	++	-	+
Casein	+	+	+
Gelatin liquefaction	-	-	+
Growth at 50 degrees C	+	-	-
Growth at 40 degrees C	+	+	+
Growth initiation at pH 5.6	+	+	+
Growth in presence of NaCl			
0.5%	+	+	+
2%	+	+	+
5%	+	+	+
7%	+	+	_
10%	_	+	_
Obligate requirement for NaCl or KCl	-	-	-
Citrate	+	+	-
Growth factor requirement	-	-	-
Arginine dihydrolase			
Motility	+	+	+
Pigments	-	Very light pink	Yellow , cell-bound
Triple sugar iron agar: H2S production	Yellow butt and slant	Growth but no fermentation	Yellow butt and slant
Fermentation of	Acid from	Acid from	Acid from

other sugars:	arabinose, lactose, fructose and sucrose	arabinose, sucrose and fructose but not lactose	arabinose, lactose, fructose and sucrose
Special characteristics	Slime produced , colonies attached firmly to the agar	Extremely good salt tolerance , but no obligate requirement, does not ferment glucose	Nutrient agar becomes brownish on incubation
Tentative Identification	Bacillus licheniformis	Alcaligenes denitrificans	Bacillus megaterium

In this study, two of the potent strains fulfilling all the preliminary criteria for L-asparaginase having clinical potential, A1 and F2 were found to be *Bacillus* species. Various studies performed in Indian marine habitats corroborate the importance of this environment as a rich source of L-asparaginase producing bacteria.

Benny and Kurup (1991) reported varying amounts of L-asparaginase in many genera of marine bacteria. Shome and Shome (2001) have assayed L-asparaginase of bacterial strains from mangroves of Andamans. They found that only 54% of the isolates could show L-asparaginase activity at pH 7.4 and 37 degrees C, of which only two strains were potent for large-scale production.

Ramaiah and Chandramohan (1992) examined luminous bacteria i.e. *Vibrio* and *Photobacterium* species from various sources in the Arabian sea for L-asparaginase activity and found that the amounts of this enzyme secreted by luminous procaryotes is generally higher than that reported for other bacterial species. Selvakumar (1991) screened 740 bacterial isolates from coastal environments of Porto Novo for L-asparaginase activity and selected a potent strain of *Vibrio* isolated from the gut of *Telescopium* sp, which was found to be effective in suppressing Yoshida ascites sarcoma in Wistar rats.

Streptomyces plicatus isolated from the gut of *Gerres filamentosus* was found to produce a potent L-asparaginase. The open sea is believed to be an oligotrophic (nutrient poor) environment and hence a good source for oligotrophic bacteria. The salient feature of these bacteria is that their

enzymes generally have high affinity for their substrates (low Km values) so as to enable their survival under nutrient poor conditions. For a clinically effective L-asparaginase, high affinity for the substrate L-asparagine is an essential requisite.

There are conflicting reports as to whether selection of effective L-asparaginase producers depends on the habitat. However, it has been established that strains isolated from sea water particularly off shore regions appear to be potential sources of asparaginase. Not all strains of a particular species produce L-asparaginase and differences occur in the quantities of asparaginase produced by different strains of the same species. Also, it is essential that the enzyme produced should have a sufficiently high activity and fulfill criteria required for clinical utility. It has been seen that the presence of this enzyme in natural habitats is random regardless of the source of origin. The ocean has various ecological niches and all of these such as estuarine, coastal and oceanic sediment and water, algae, microflora of marine plants and animals, gut content of fishes etc needs to be explored to yield new strains. More studies directed to understanding eco-physiological aspects as well as those involving strain improvements are needed to evaluate the potential of marine bacteria in the production of L-asparaginase. [224,323,352,361] However, this study shows that the coastal and estuarine habitats of Goa are a rich source of L-asparaginase producing bacteria.

Section B: Screening for cholesterol transforming bacteria from coastal and estuarine sample

Steroids are important therapeutic agents being used to treat rheumatic arthritis, allergies, inflammatory diseases, for contraception and hormonal insufficiencies. To meet such a large demand for a diverse range of products, microbiological transformations play a vital role. The discovery of the ability of microbes to bring about site-specific changes in virtually any unactivated centre in the carbon skeleton of a steroid molecule to generate intermediates which can serve as precursors for other steroids, served as a turning point in the microbiological transformation of steroids. The major rate-limiting factor in the biotransformation process is the extremely poor dissolution of steroids in water (10^{-2} to 10^{-3} g/ml), which increases costs. Microbial cholesterol conversion occurs poorly in aqueous media because the reactants are in a solid state and therefore the reaction rate is hampered by limited availability of the substrate. In industrial microbial cholesterol degradation processes, some detergents such as Tween and Span, have been used to form stable suspensions of the water insoluble substrate during fermentation and to prevent simultaneous formation of solid particles. Although detergent addition helps to increase the degradation rate, it does not prevent the formation of solid particles. For bioconversion of organic compounds with low solubilities in water, large volumes of appropriate medium are used for solubilisation of these compounds. This consumption of medium or water and the inevitable treatment of waste- water constitutes one of the major cost factors in bioconversion fermentation. Accordingly, as a reactant reservoir to keep the product as well as the substrate in a soluble form till the degradation is complete, the use of organic solvents is thought to be a

logical way to prevent simultaneous formation of solid particles. Steroids like cholesterol are completely soluble in some organic solvents such as chloroform and n-butanol. Hence, a biphasic system wherein the cells are present in the aqueous phase and steroids dissolved in the organic phase is an ideal set-up .The major problem here is the fact that most bacteria and their enzymes are inactivated or destroyed in presence of these toxic organic solvents [14,269,252,370].

It has been established that cholesterol dissolved in some organic solvents at a high concentration is converted when immobilized enzymes or resting cells are suspended in the water phase of water-organic solvent two-phase system.

Solvent tolerant bacteria are reported to be present in much higher numbers in the marine ecosystem as compared to soil [201]. Mangrove sediment being a rich source of cholesterol can be expected harbour a large number of cholesterol degraders. Hence, the present work was undertaken to isolate and identify cholesterol transforming bacteria tolerant to organic solvents from the marine ecosystem.

Materials and Methods:

2B.1 Isolation of cholesterol transforming bacteria in presence of chloroform: Coastal sediment samples A, B, C and estuarine sediment sample F (from Mandovi estuary) were screened for the presence of bacteria utilising cholesterol dissolved in organic solvent. 1 gram each of the samples was suspended in 8 ml mineral salts medium which was then supplemented with chloroform (2 ml) containing dissolved cholesterol as

a carbon source. The cholesterol concentration was 1 mg/ml of the medium and the organic solvent concentration was 20 % v/v. The tubes were incubated on a rotary shaker (180 r.p.m) at 28 degrees C for 2 days. Aliquots of 0.2 ml from each sample tube were plated out on mineral medium agar containing cholesterol as the sole carbon source. The plates were incubated at 28 degrees C for a week and the colonies obtained were purified by streaking on mineral medium with cholesterol and on Luria agar. Cultural and morphological characteristics of pure colonies obtained were recorded and the cultures maintained on mineral medium agar with cholesterol as carbon source and on Luria agar.

2B.2 Isolation of n butanol tolerant cholesterol degrading bacterium by enrichment of butanol tolerant micro-organisms from mangrove sediment:

The culture was enriched from a mangrove sediment sample collected from Mandovi estuary in Goa by soaking 10 grams of sediment in 10 ml of n butanol for a period of one month at 30 degrees C. Aliquots of this sample (one gram) were transferred to flasks containing artificial sea water supplemented with 20% (v/v) of benzene or butanol. The flasks were incubated at 30 degrees on a 120 r.p.m. rotary shaker. After one week, the organic layers from these flasks were transferred to a nutrient rich medium containing 50% (v/v) of the organic solvent or incubated on a shaker for 2 d. 0.1 ml aliquots of the organic layers were plated on L.B.M.G. agar and the plates were incubated at 30 degrees C for 2 days. The isolates obtained were purified and maintained on Luria agar.

2B.3 Identification of selected bacterial isolates: The selected cholesterol transforming solvent tolerant bacteria SB1 and BC1 were identified based on their morphological, physiological and biochemical characteristics, as per Bergey`s Manual of Systematic Bacteriology.

Results and Discussion:

For a successful bio-transformation, it is absolutely necessary that the substrate molecules come into contact with the enzyme [252,370]. Cholesterol utilising bacteria capable of tolerating solvents were isolated by enrichment in presence of chloroform and n-butanol , using two different procedures.

Various coastal and marine sediment samples were enriched for **chloroform tolerant, cholesterol utilising bacteria** by inoculation of samples into mineral medium containing cholesterol as carbon source in presence of 20 % (v/v) chloroform (**table 2B.1**). 11 bacterial cultures were obtained, most of which were aerobic, gram-positive rods (**table 2B.2**). The marine sediment samples A, B, C and estuarine sample F all showed the presence of cholesterol degrading bacteria. Cholesterol degradation was confirmed by production of pure colonies on mineral salts agar with cholesterol as carbon source. Of these cultures, BC1, an aerobic gram-positive endospore forming rod tentatively identified as *Bacillus megaterium* (**table 2B.3**), was found to show the fastest growth on cholesterol agar.

(within 48 h) and produce relatively larger colonies. The other cholesterol degrading bacteria grew slowly producing colonies after 4-5 days of incubation and their colony size was smaller than BC1.

Fig 2B.1: Growth of the cholesterol utilising bacterium SB1 on mineral medium with cholesterol as the sole carbon source.

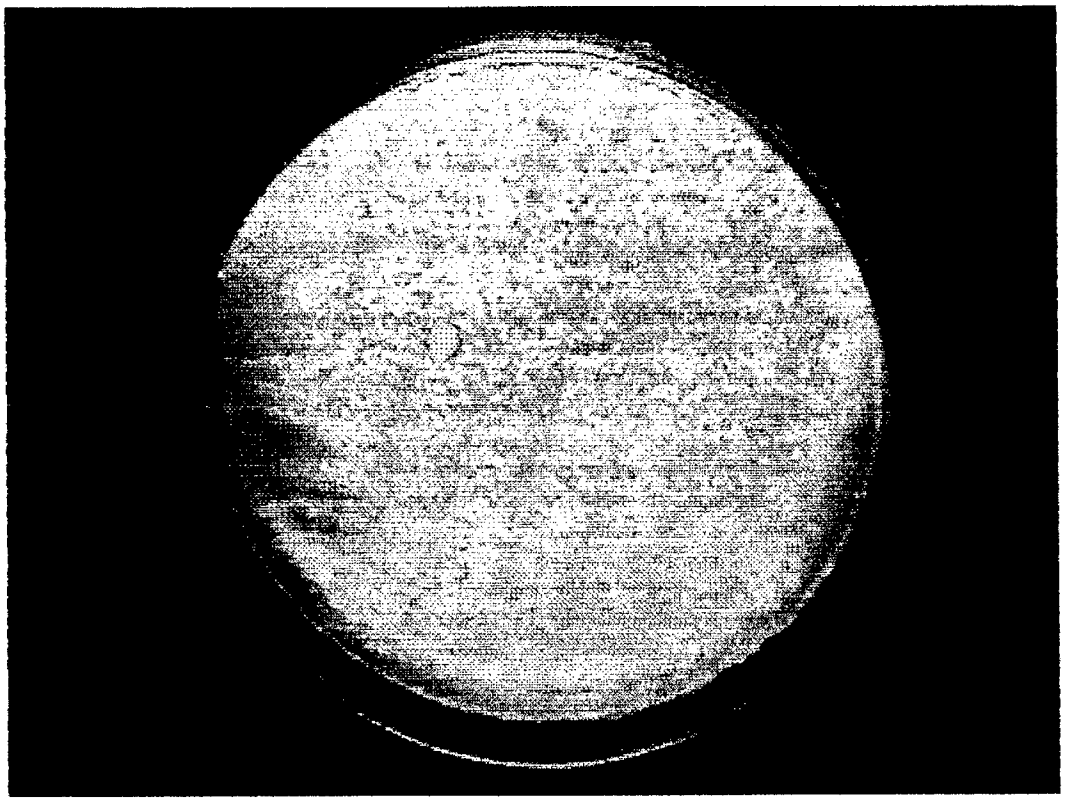


Table 2B.1: Isolation of cholesterol degrading bacteria

Sample name	Nature of the sample	Names assigned to cultures isolated
A	Marine sediment	AC1,AC2, AC3
B	Marine sediment	BC1, BC2
C	Marine sediment	CC1, CC2
F	Estuarine sediment	FC1, FC2, FC3, FC4

Table 2B.2: Characteristics of some cholesterol utilising bacteria

Culture	AC1	BC2	BC1	CC1	CC2
Colony characters on Luria agar					
Size	Large	Pinpoint	Small	Medium	Small
Shape	Irregular	Circular	Circular	Circular	Circular
Colour	White	Yellow	White	Cream	Cream
Consistency	Butyrous	Butyrous	Butyrous	Slimy	Butyrous
Opacity	Opaque	Opaque	Opaque	Opaque	Opaque
Elevation	Flat	Convex	Flat	Raised	Raised
Margin	Rough	Smooth	Smooth	Smooth	Smooth
Gram character and morphology	Gram positive branching rods , fine long and short	Gram negative rods	Gram positive rods	Gram positive rods	Gram positive rods
Colony description on cholesterol mineral medium agar	Small, white and circular	Medium , circular, white, showing rapid growth on cholesterol medium and causing depressions on the plate	Circular, pink colonies	Small,white and circular	Small, white, circular

Culture	FC1	FC2	FC3	FC4
Colony characters on Lurla agar				
Size	Large	Medium	Large	Large
Shape	Wrinkled, irregular	Circular	Circular	Irregular
Colour	Creamish white	Orange	Cream	Cream
Consistency	Dry	Butyrous	Slimy	Butyrous
Elevation	Flat	Raised	Flat	Flat
Opacity	Opaque	Opaque	Transparent	Opaque
Margin	Entire	Entire	Entire	Entire
Gram character and morphology	Gram positive rods	Gram positive rods	Gram positive rods	Gram positive rods
Colony description on cholesterol mineral medium agar	Small, white, circular	Small, white	Small, white	Small, white

*Characteristics of AC2 and AC3 were the same as AC1 and hence are not recorded. Colony appearance of all 3 cultures changes with age.

Table 2A.3 : Biochemical and physiological characteristics of the selected cholesterol degrading bacteria

Characteristics	BC1	SB1
Gram character and morphology	Gram positive rods	Gram positive rods
Spores	+	+(central, no swelling)
Oxidase	+	+
Catalase	+	+
Glucose fermentation	Acid , but no gas	Acid , but no gas
Fermentation of other sugars	Acid from fructose, but not arabinose, lactose or sucrose	Acid from fructose, sucrose, arabinose and lactose
Anaerobic growth	+	-
Growth at 50 degrees C	-	+
Growth at 43 degrees C	+	+
Growth at 40 degrees C	+	+
Initiation of growth at pH 5.6	+	+
Obligate requirement for NaCl / KCl	-	-
Growth in presence of NaCl		
2%	+	+
5%	+	+
7%	+	+
10%	-	+
Methyl red	-	-
Voges Proskauer	-	+
pH in V.P. broth	6	6
Indole	-	-
Degradation of starch	-	++
Gelatin liquefaction	-	+
Degadation of casein	+	+
Citrate utilisation	+	+
Nitrate reduction	-	+
Growth on TSI slant	Growth but no fermentation , no H2S	Acidic butt and slant, no H2S
Arginine dihydrolase		
Colony characters on Luria agar	large, white, butyrous colonies	Large, irregular, wrinkled colonies ,slimy turning dry
Production of flourescent pigments	-	-
Motility	+	+
Tentatively Identified As	Bacillus megaterium	Bacillus subtilis

n-Butanol tolerant bacteria were enriched from mangrove sediment by prolonged incubation in presence of butanol, followed by growth \ cultivation on media supplemented with butanol. This procedure led to the isolation of a single bacterial culture called SB1, producing large wrinkled creamish colonies white colonies. The colony appearance changes with age. Mucoid white colonies on prolonged incubation on prolonged incubation acquire a dry wrinkled creamish appearance on L.B.M.G. agar. The culture forms a wrinkled pellicle in liquid media like nutrient broth. This culture tolerated direct exposure to undiluted butanol in the plate assay and 50% (v/v) butanol in liquid media and showed growth in mineral medium containing cholesterol as the sole carbon and energy source [**fig 2B.1**].

Identification studies revealed that SB1 is a gram-positive, aerobic, catalase positive, endospore producing rod belonging to Genus *Bacillus.* [372] (**table 2B.3)** It produces small rods which are seldom in chains , colonies which are irregular and become dull and wrinkled with age. Colonies spread on moist agar. The cell material grown on agar does not disperse in saline. Besides, in presence of 1 % glucose, the growth becomes thick and viscous, probably due to polysaccharide production. No growth factors are required and the culture grows well in simple minimal medium with glucose. Growth is also seen at 50 degrees and with 7 % NaCl. It produces acid from glucose and other sugars but no gas further, it degrades starch, gelatin, casein, citrate. These characteristics attributed to *Bacillus subtilis* are exhibited by SB1.

Cholesterol is an oxygenated tetracyclic isoprenoid derivative and a complex molecule to degrade. It is the precursor for all the steroid hormones of the body, except retinoic acid, and hence an ideal substrate to obtain useful intermediates. Bio-transformations catalysed by microbial enzymes have been used for a considerable period of time in the synthesis of pharmaceutically important steroids. The advantages of microbial conversion as opposed to comparable chemical processes lies in the regio and stereo- selectivity of the enzymes and their mild reaction conditions. Cholesterol and phytosterols are known to be degraded by fungi and bacterial genera represented by *Arthrobacter, Pseudomonas, Brevibacterium, Mycobacterium, Corynebacterium, Nocardia, Rhodococcus, Streptomyces* and *Bacillus* [3, 250,251,252].
Both the cholesterol degrading bacteria selected in this study, SB1 and BC1 are *Bacillus* sp [372].

Bio-transformations are employed when a given reaction step is not easily accomplished by chemical means. The ideal substrate should be soluble in the fermentation medium and pass the cell membrane without being toxic to the organism. Cholesterol is not a water soluble substrate, hence dissolution in an organic solvent can be done to speed up the rate of conversion. However, organic solvents on account of their toxicity are lethal to most bacteria even in low concentrations. This problem can be overcome by utilising organic solvent tolerant cholesterol degrading bacteria, which can carry out the bio-transformation of cholesterol while

dissolved in the organic phase of an organic –aqueous biphasic system [14].

SB1 and BC1 have the traits of cholesterol degradation and organic solvent tolerance, hence are ideal candidates for use in such a biphasic system.

Solvent toxicity is graded by log P values, where P is partition coefficient of a given solvent in an equimolar mixture of octanol and water. The lower the log P value, the greater its polarity and hence its toxicity [187].

SB1 tolerates chloroform (log P=2) and butanol (logP=0.8) , two of the most toxic organic solvents, whereas BC1 tolerates chloroform but not butanol. Chloroform has earlier been used as a solvent of choice in cholesterol transformation. Since both the cholesterol degrading bacteria selected in this study are chloroform tolerant, it can be assumed that their enzymes are capable of functioning in the presence of this solvent. The development of a biphasic [organic-aqueous] bio-conversion system using SB1 and BC1 has been discussed Chapter 4.

Chapter 3: Study of the L-asparaginase producing bacterium *Bacillus A1*

Microbial L-asparaginase is an aminohydrolase enzyme which halts protein synthesis in cancer cells. Being an important component of the synergistic therapy in acute lymphoblastic leukemia, industrial production of the enzyme through microbial means has gained importance [320,352]. In Chapter 2, the isolation of L-asparaginase producing bacteria and the identification of the isolates A1 and F2 as *Bacillus* sp. and isolate A2 as *Alcaligenes* sp. has been described.

Initial isolation and studies resulted in the selection of *Bacillus* A1 for further studies, since this culture exhibits good asparaginase activity under physiological conditions (pH 7 and 37 degrees C) and at low concentrations of asparagine. Also, it does not exhibit glutaminase co-activity as A2 and F2. Studies on the optimisation of enzyme production as well as enzyme activity are discussed here.

Materials and methods:

3.1 Determination of growth profiles of *Bacillus* sp. A1 and F2:

The cultures A1 were grown in M9-asparagine medium with 0.5 % asparagine in side-arm flasks incubated on a rotary shaker (150 rpm) at 30 degrees C. Aliquots from the flasks were diluted and plated on Luria agar at periodic intervals and viable counts determined. Optical density (600 nm) was determined periodically with Elico-CL157 colorimeter.

3.2 Morphological study of A1 by electron microscopy:

The morphology of *Bacillus* sp. A1 was studied by electron microscopy [374].

Procedure for Scanning Electron Microscopy: The cultures were taken after 24 h growth. The cells were washed twice in 0.05 M phosphate buffer (pH7) and centrifuged at 5000 rpm for 5 minutes to separate the cells. The cells were fixed by suspending the pellet in 2 ml of 2.5% glutaraldehyde fixative (pH 7.2-7.4) overnight at room temperature. The cells were centrifuged at 5000 rpm for 5 minutes and the supernatant was removed. The cells were washed twice with 0.05 M phosphate buffer (pH 7) and separated by centrifugation at 5000 rpm for 5 minutes. The cell pellet was vortexed and to the cells , 1 ml of 30% acetone was added. The eppendorf was vortexed again and allowed to stand for 10 minutes. The cells were separated by centrifugation and the dehydration procedure repeated with 50, 70 and 90 % acetone in succession for 10 minutes each. This is followed by incubating the cells with 100% acetone for 30 minutes. The cells which are in 100% acetone are placed onto the stub with double sided adhesive tape and immediately put in a critical point drying device for drying. During critical point drying the acetone is replaced by liquid carbon dioxide at high pressure and then is evaporated by raising the temperature to 45 degrees C wherein the liquid carbon dioxide is converted into gaseous carbon dioxide. The procedure takes about 1 hour. After drying, the stub with the specimen was

placed on the sputter coater (spl module) specimen holder which is mounted on to the stage by a screw. The position of the stage Is set in such a way that the specimen is approximately 50 mm from the bottom of the sputter head. The glass work chamber was replaced and the sputter head was placed on top of the chamber. The timer is set to around 120 seconds. The leak value is fully closed. The argon pressure is set to psi. Both the power switches are put on, the rotary pump starts Immediately and the vacuum Is indicated on the meter. When the pressure falls to approxlmately 600 –400 millitorr the ready light goes on. The gas leak valve is partially opened and the work chamber is flushed with argon gas for about 10-15 seconds. The leak valve is closed and the pressure is brought down to 80 millitor. The gas leak valve Is opened till the pressure just begins to rise by intermittently depressing the test buttons and adjusting the leak valve, the plasma current is set to required 18 ma. A visible discharge is observed in the chamber. The stat button is depressed and gold is sputtered on the specimen for the set time. The plasma switches are put off and air is admitted to the chamber using the vent valve on top of the sputter head. The sputtering Is done to procedure a thin film (10-15 nm) of gold. The stub is then placed into the electron microscope sample chamber and the specimen observed with JEOL – 5800 LV scanning electron microscope.

3.3 Optimisation of growth and enzyme production of *Bacillus* A1:

To determine the optimum conditions for growth and enzyme production, the cultures were grown in M9-asparagine medium prepared in half-strength sea-water and distilled water with different asparagine concentrations ranging from 0.1 to 2%. Increase in growth was determined by turbidity and enzyme production was monitored by measuring protein concentration of the cell-free supernatant using Folin-Lowry`s method. Since asparagine production is accompanied by release of ammonia, concentration of ammonia in the supernatent and its pH were also determined. Nessler`s reagent was used to determine the concentration of ammonia based on the standard curve prepared using ammonium sulphate.

3.4 : Determination of L-asparaginase activity of *Bacillus* A1 by Wriston`s Assay:

The reaction mixture taken is as follows;

0.5 ml of enzyme solution (cell-free crude supernatant) , 1ml of buffer and 0.5ml of 0.04M asparagine solution prepared in buffer are taken in a test-tube and incubated at 37 degrees C in an incubator for 30 minutes. The buffer used is phosphate buffer (pH 7). At the end of the incubation period, 0.5 ml of 15% TCA (tri-chloroacetic acid) solution is added to the tube to stop the reaction. The contents are centrifuged. One ml of the reaction mixture is taken in a test-tube to

which 4 ml of distilled water and 0.1 ml of Nessler`s reagent are added and mixed. The tube is kept at room temperature for 20 minutes and the optical density is determined by an Elico-CL157 colorimeter at 470 nm.

Enzyme activity: One international unit (i.u) is the amount of enzyme that releases one micromole of ammonia per minute under standard assay conditions. L-Asparaginase specific activity is generally expressed as i.u / mg protein. [433]

3.5 Optimisation of factors affecting L-asparaginase activity of *Bacillus* A1:

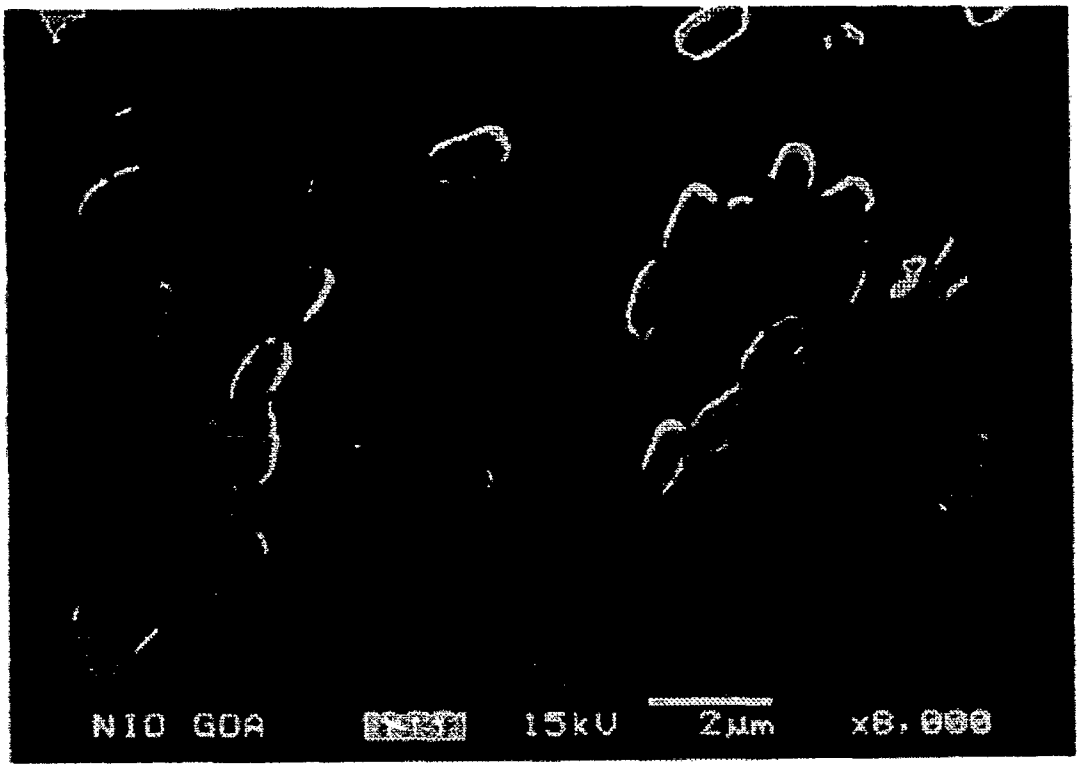
Optimisation of factors affecting the L-asparaginase activity of *Bacillus* A1 was done by carrying out the enzyme assay using Wriston`s method . The enzyme activity was determined under varying conditions of pH (5.6-10), temperature (30-50 degrees C), asparagine concentration (0.03125-0.5 M) and reaction time (0-60 min).

Results and Discussion :

Bacillus A1, a culture isolated from coastal sediment samples as described in chapter 2, was found to produce an extracellular L-asparaginase enzyme (detectable in the culture supernatant). This asparaginase was found to be active at 37 degrees, pH 7.4 and low asparagine concentrations. Also, this enzyme exhibit no glutaminase activity, lack of which is desirable for in medical application. Hence, it was an ideal candidate for optimisation studies. The selection of a stable, high affinity enzyme that operates efficiently under physiological conditions ensures effective depletion of the circulating amino acid [410].

Electron micrographs reveal the culture to be a rod of 1.5 x 0.66 micron size [**fig. 3.1**]. The culture was found to have a generation time of 57 minutes in M9-medium with asparagine (0.5%)[**fig 3.2**] . Optimisation studies revealed that maximum enzyme production occurs during the stationery phase of growth and in M9 medium containing 1% asparagine when grown on a shaker at 30 degrees C. 1% asparagine gives a higher growth rate as well as enzyme production [**fig.3.3**]. There are contradictory reports on the ideal medium for L-asparaginase production. It has been reported that synthetic media with asparagine as a nitrogen source stimulates more enzyme production by some cultures than natural media. Also, presence of glucose in the medium is sometimes known to repress enzyme synthesis. In some cultures nitrogen catabolite repression of enzyme synthesis is also seen [106, 271,295], however, in case of

Fig 3.1: Electron Micrograph of the L-Asparaginase producing bacterium *Bacillus A1*



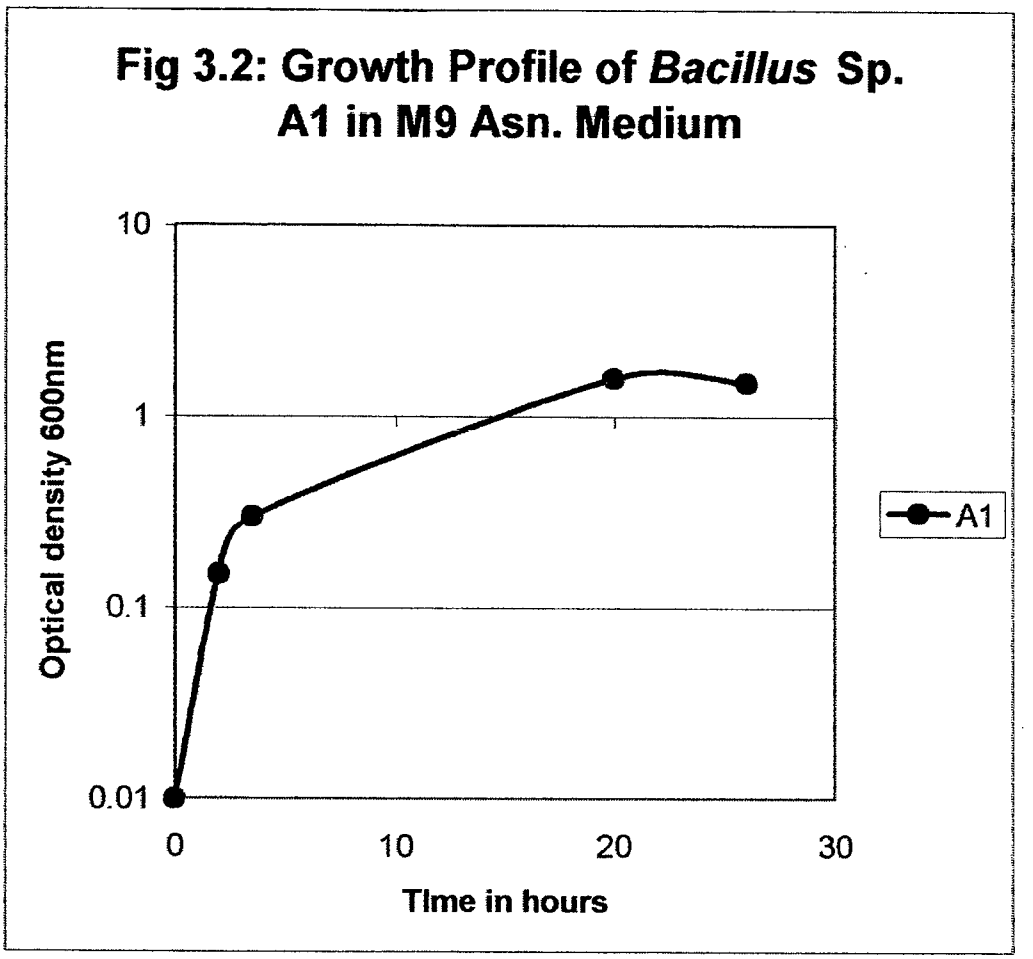


Fig 3.2: Growth Profile of *Bacillus* Sp. A1 in M9 Asn. Medium
10
1
0.1
0.01
Optical density 600nm
0
10
20
30
TIme in hours
A1

Fig 3.3 A : Effect of Asparagine Concentration on Growth of *Bacillus* A1

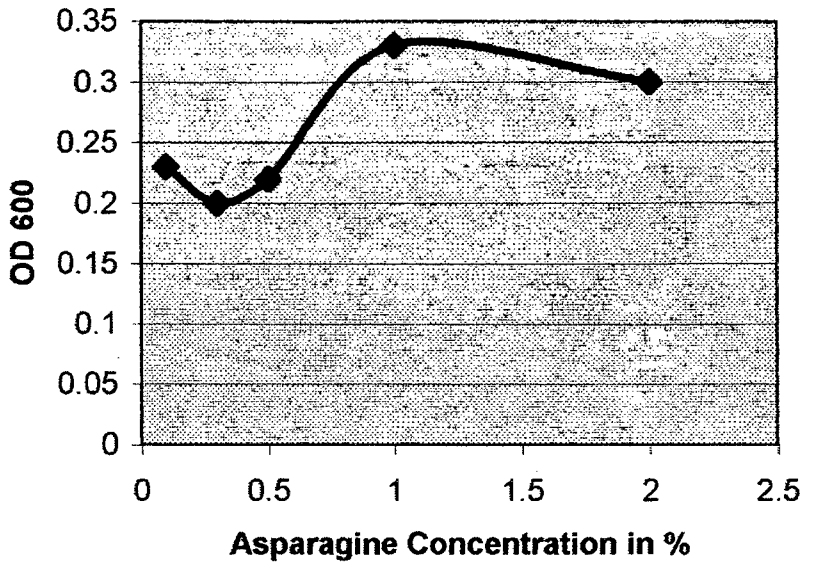
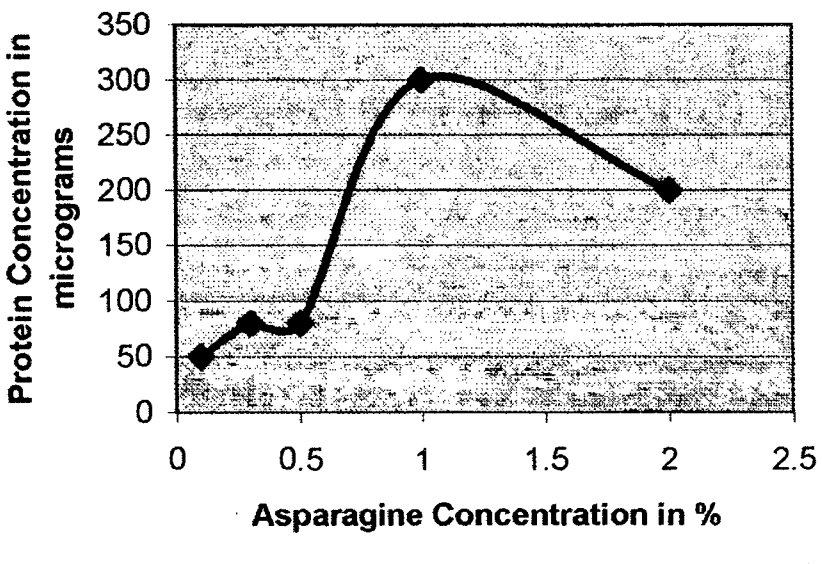


Fig 3.3 B : Effect of Asparagine Concentration on Enzyme production by *Bacillus* A1



E.coli B cells, 1-3% peptone is added as a primary nitrogen source [147], the preferred peptone being a pancreatic digest of casein. Use of fermentation media containing peptone as the primary N source is believed to be important in providing a dependable process for production of L-asparaginase with anti-tumor activity by the growth of *E.coli*. Since A1 is capable of growing on M9 medium with L-asparagine as the sole source of carbon and nitrogen, it was grown under these conditions [246]. It is interesting to note that growth takes place even when there is no measurable activity of L-asparaginase. Since this culture is of marine origin, the studies were carried out using media prepared in sea - water as well as distilled water, however, no difference was seen in growth or enzyme production indicating that the culture does not exhibit any dependence on sea-water. It has been reported that even with coastal and estuarine strains, L-asparaginase is found to be synthesised at low concentrations of sodium chloride. Reduced activity at high concentration id due to the formation of certain inhibitors in the growth medium or due to the inhibitory nature of the increased sodium chloride level [224,323].

Enzyme activity was monitored using the Wriston`s assay [433], which measures released ammonia, a product of the reaction catalysed by asparagine. The activity of L-asparaginase is expressed in terms of the micromoles of ammonia released per minute per mg of protein.

The assay was carried out with the crude cell-free supernatant as enzyme source. The enzyme activity was found to be 0.055 iu / mg protein at pH 7.4 and 37 degrees C with 0.04 M asparagine as substrate (standard assay conditions). The specific activity of the industrially used *E. coli* B crude extract asparaginase is reported to be 0.2 units /mg protein [147]. After a 5-step purification process involving column chromatography and electrophoresis, the specific activity increased from 0.2 to 300-400. With *Serratia marcescens* the activity increases from 0.4 iu/mg to 300. L-asparaginase from a marine vibrio was purified 19 fold to give enzyme activity of 14 iu/mg [433,434]

The optimum temperature for theA1 asparaginase to function was found to be 37 degrees C (0.055 iu /mg). The enzyme is also active at 30 degrees C (0.035 iu / mg), but not at 15 and 50 degrees C .

The activity of the enzyme was found to be stable between pH 7 and 9 respectively where the activity remains the same (0.055 iu / mg). However, the enzyme also shows significant activity at pH 5.6 (0.4 iu / mg) and 10 (0.038 iu / mg) . Most of the reported bacterial asparaginases have pH optima in the alkaline range usually 8.5, but some exhibit appreciable activity at pH 7 e.g. *E. coli* , *Vibrio* strains [411, 412, 358, 352] The optimum reaction time was found to be 30 minutes [**table 3.1 ,A-D and fig.3.4 A-C**]. To determine the ideal substrate concentration, the enzyme activity was determined over a wide range of substrate concentrations ranging from 0.5 to 0.015 M.

Fig 3.: Optimisation of factors affecting the L-asparaginase activity of *Bacillus* A1

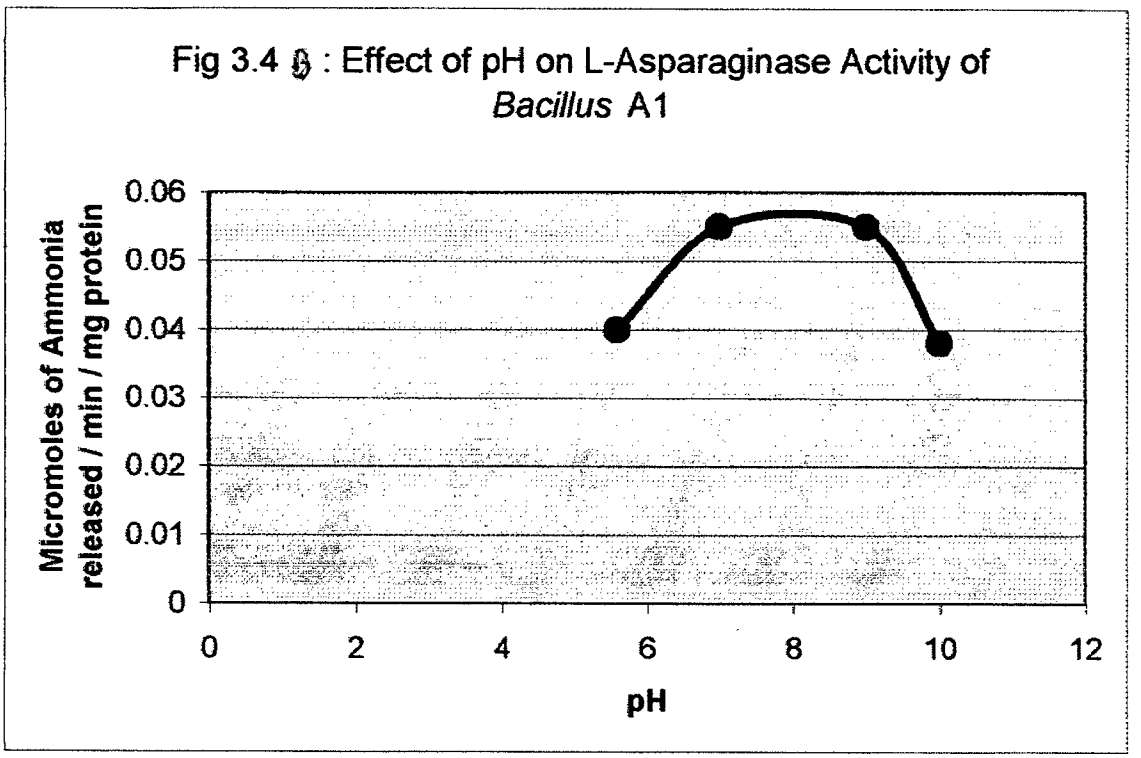


Fig 3.4 A : Effect on temperature on L-Asparaginase activity of *Bacillus* A1

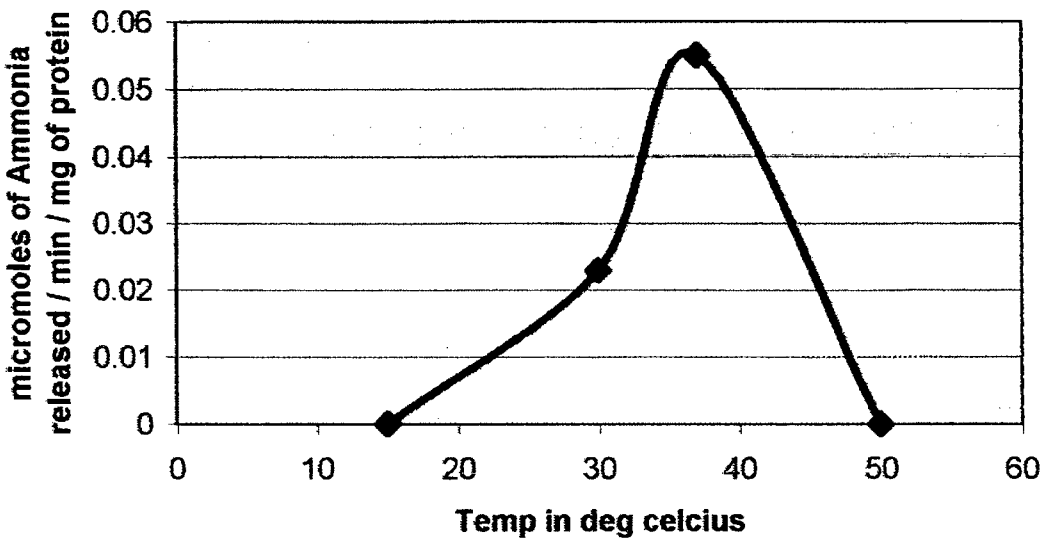


Fig 3.4C : Effect of reaction time on L-Asparaginase Activity of *Bacillus* A1

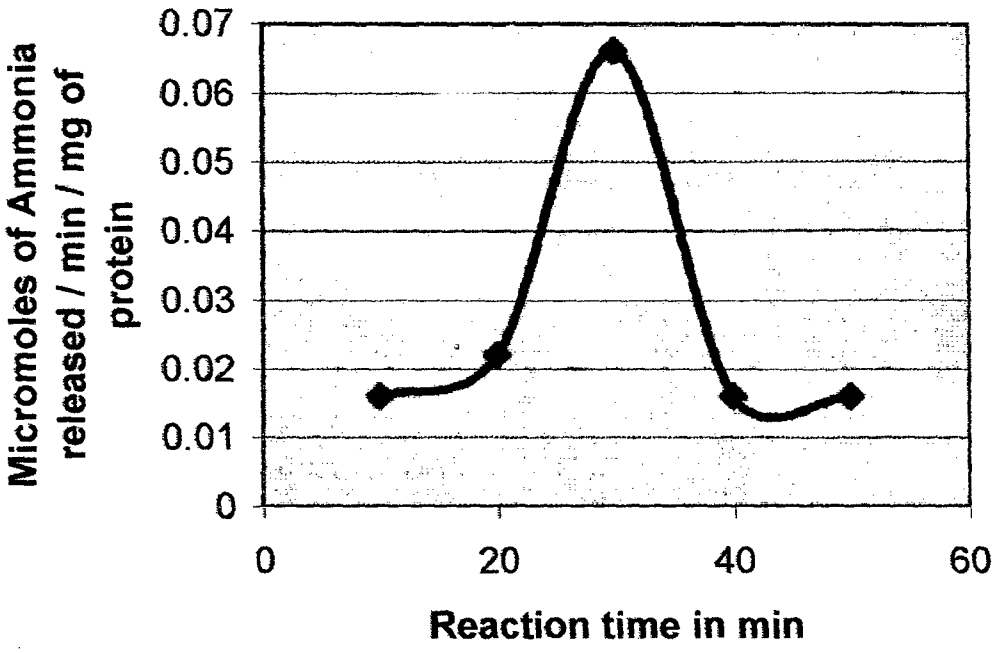
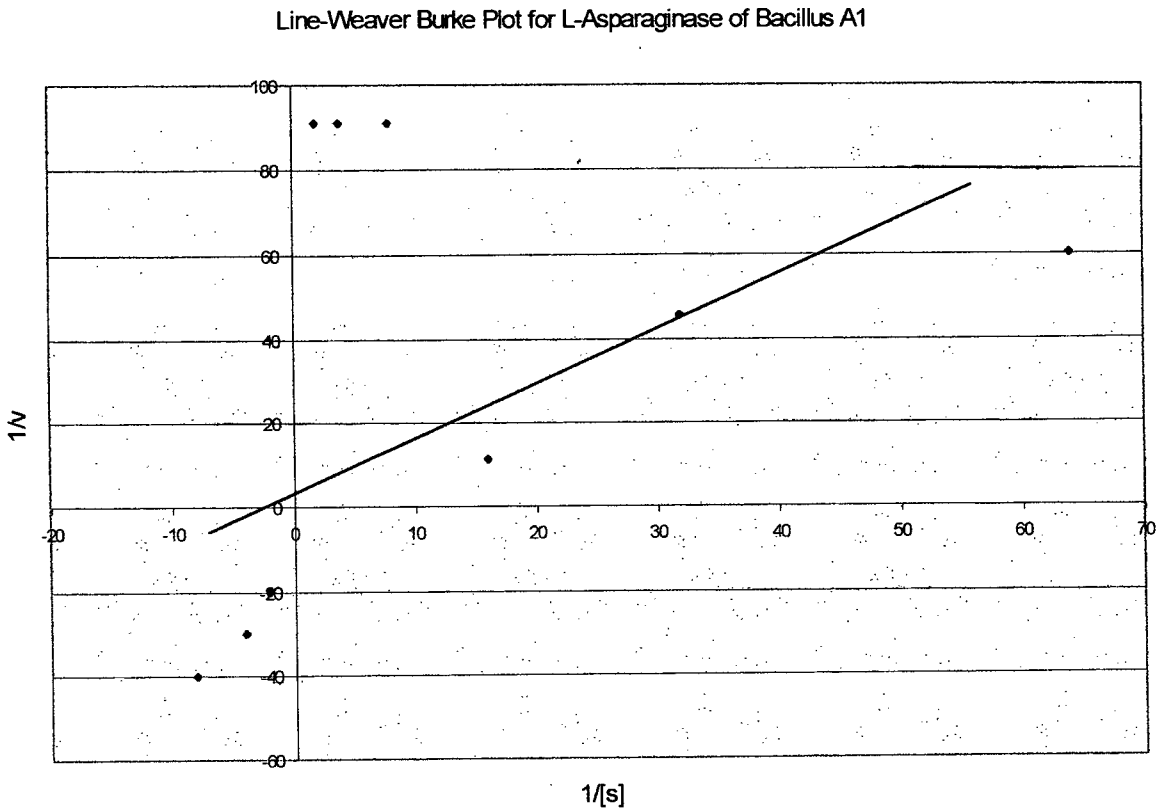


Fig 3.5


Line-Weaver Burke Plot for L-Asparaginase of Bacillus A1
1/V
1/[s]
100
80
60
40
20
0
-20
-40
-60
-20
-10
0
10
20
30
40
50
60
70

The highest enzyme activity (0.088 iu / mg) was seen when the substrate concentration is 0.0625 M. However, the enzyme can function at substrate concentrations as low as 0.01563 M showing activity (0.0166 iu / mg) protein(tables 3.2 D). The Km value (substrate concentration at half maximum velocity) as per the Line-Weaver Burke plot is 0.08M [**fig.3.5**]. Since, the crude extract shows promising activity, it is conceivable that after multi-step purification procedures, the activity should increase manifold as seen in case of the *E. coli* B enzyme [433]. Purified enzymes are known to show high affinity for L-asparaginase with Km below 10^{-5} M.

Table 3.1: Optimisation of enzyme activity of *Bacillus* A1

3.1 A)Effect of temperature on L-asparaginase activity of *Bacillus* A1

Temperature (degrees C)	Total activity (micromoles of ammonia released per min)	Total protein (micrograms)	Specific activity (iu/mg)
15	-	-	-
30	2.5×10^{3}	70	0.035
37	0.0166	300	0.055
50	-	-	-

3.1 B)Effect of pH on L-asparaginase activity of *Bacillus* A1

pH	Total activity (micromoles of ammonia released per min)	Total protein (micrograms)	Specific activity (iu/mg)
5	0.0133	300	0.044
7	0.0166	300	0.055
9	0.0166	300	0.055
10	0.0175	300	0.038

3.4 C) Effect of reaction time on L-asparaginase activity of *Bacillus* A1

Reaction time in minutes	Total activity (micromoles of ammonia released per min)	Total protein (micrograms)	Specific activity (iu/mg)
10	2.5×10^{-3}	150	0.0166
20	3.3×10^{-3}	150	0.022
30	0.01	150	0.066
45	2.5×10^{-3}	150	0.0166
60	2.5×10^{-3}	150	0.0166

3.5 D) Effect of substrate concentration on L-asparaginase activity of *Bacillus* A1

Asparagine concentration (M)	Total activity (micromoles of ammonia released per min)	Total protein (micrograms)	Specific activity (iu/mg)
0.5	1.66×10^{-3}	150	0.011
0.25	1.66×10^{-3}	150	0.011
0.125	1.66×10^{-3}	150	0.011
0.0625	0.0133	150	0.088
0.03125	3.3×10^{-3}	150	0.022
0.01563	2.5×10^{-3}	150	0.0166

The manufacture or processing of enzymes for use as drugs is a minor but important facet of today`s pharmaceutical industry. The development of microbial enzymes for cancer therapy presents difficulties not commonly experienced with biological drugs. The development of the enzyme asparaginase from *E.coli* in the USA and the plant pathogen *Erwinia* has not only added to the choice of anti-leukemic drugs but also provided a valuable guide to the selection and development of new therapeutic enzymes [410]. Recently initiated clinical trials have confirmed the eminent value of asparaginase in combination therapy of A.L.L. and in some sub-types of non-Hodgkin`s lymphoma, and its important role as an essential component of multi-modal treatment protocols [6,387,392]. Enzyme induced asparagine depletion of the serum constitutes the decisive cytotoxic mechanism. However, treatment response may only be expected if malignant cells are unable to increase their asparagine synthetase activity to an extent providing enough asparagine to the cells [105]. The limiting factors in asparaginase use are the severe immunological reactions and the short serum half-life associated with this enzyme. Immobilisation in a bio-compatible matrix has been found o greatly decrease the immunogenicity, increase its half-life in vivo and its therapeutic value [373,380]. It has been established that different L-asparaginase preparations are not readily interchangeable and changes in dosage, preparation, schedule require careful monitoring [49] .

The L-asparaginase produced by *Bacillus* A1, fulfills all required preliminary criteria for medical use and is a good candidate for further tests. The studies have also projected the wide dimensions and implications of bacteria for the marine ecosystem for producing metabolites which can be potential bio-active substances of desired specification. Purification, determination of likely persistance, toxicity, efficacy of the enzyme in clinical use etc are required so as to arrive at meaningful biological tests for quality control of the finished product.

The following chapter deals with another facet of marine bacteria in evolving bio-active compounds by transformation of other organic compounds.

Chapter 4

Studies on the organic solvent tolerant cholesterol transforming bacteria – *Bacillus* strains SB1 and BC1

Organic solvent tolerant bacteria (OSTB) are a new group of extremophilic bacteria which thrive in presence of organic solvents, which are ordinarily very toxic to bacterial cells.

OSTB are of great academic as well as industrial interest. Being a novel extremophilic group, they are bound to have new cell components and adaptive mechanisms. The acute industrial interest in OSTB is on account of the stability of their enzymes, particularly the externally secreted enzymes, to organic solvents. This makes the cultures extremely viable in areas like non-aqueous bio-catalysis and biphasic organic- aqueous bio-transformation systems [9,14,269]. Hence, OSTB could prove to be significant in the synthesis of steroidal compounds, fine chemicals like cis-diols etc. Since the discovery of the first such culture by Inoue and Horikoshi in 1989 [187], several other strains of *Pseudomonas* tolerant to toluene have been reported. *E.coli* mutants tolerant to p-xylene have been constructed from solvent sensitive parents [11]. Some gram-positive bacterial strains of *Bacillus, Arthrobacter, Rhodococcus* of marine origin have been reported to tolerate benzene [9,201]. However, unlike the gram-negative bacteria, their tolerance mechanisms have not been elucidated.

There are several advantages in using organic solvent tolerant bacteria to catalyse steroid transformations in bi-phasic organic-aqueous bio-conversion systems, since the problems arising from poor water solubility of steroids can be solved by using an organic phase (chapters 1,2). Initial isolation techniques resulted in the

isolation of cultures capable of transforming cholesterol. This is directed to study the organic solvent tolerance of the cultures *Bacillus* sp. SB1 and BC1 and develop a proper bi-phasic organic-aqueous bio-conversion system for cholesterol transformation. Since n-butanol tolerance is a novel trait displayed by SB1, it has been studied in detail.

This chapter has been divided into 2 sections :

Section A describes the studies done on the organic solvent tolerance of the cultures, particularly the n-butanol tolerance of *Bacillus* SB1.

Section B covers the transformation of cholesterol by resting cells in a biphasic system with 50% (v/v) chloroform.

Section A: Studies done on organic solvent tolerance of the Bacillus sp., particularly SB1

Materials and Methods:

4A.1 Effects of n- butanol on the growth of SB1:

(i)Effect of various butanol concentrations on the growth rate of SB1 and

***(ii)the minimum inhibitory concentration of butanol (M.I.C)* for its growth were determined.**

18 hour old culture grown in L.B.M.G. broth was used as inoculum. The cells were inoculated in different concentrations of n butanol (0.1 to 10% v/v) in L.B.M.G. broth and growth was monitored by measuring the absorbance at 600 n.m. using an Elico CL-157 colorimeter and by determining the viable counts on L.B.M.G. agar at periodic intervals.

The growth rate and generation times were determined as per the formula;

g= t/ 3.3(log 10 Nt- log 10 No), where g is generation time , t is time in hours, No is the initial bacterial population and Nt is the viable count obtained after time interval t. Growth rate (r) is the reciprocal of generation time (r=1/g).

(iii) Determination of inhibition of growth rate of the culture by solvents:

Effects of solvents are expressed as the ratio of maximal growth rate obtained from the growth curves (Gs and Go) , where Gs is the growth rate with solvent and Go is the growth rate without solvent.

(iv)Effect on butanol concentration on growth yield of SB1 :

The growth yield of SB1 in presence of various butanol concentrations and in its absence absence was determined by comparing the optical density values (600 nm) at the end of the stationery phase with the initial optical density. Growth yield is expressed as :

O.D. 600 at the end of the stationery phase - initial optical density.

The following controls were maintained: 1)Throughout the study, distilled n butanol was used. The sterility of the butanol used was determined from time to time by inoculating small quantities (0.2 ml) in sterile Luria medium and checking for appearance of growth. 2) To determine the comparitive tolerance level of other cultures to toxic organic solvents like chloroform and n butanol, standard laboratory strains of *E.coli, Staphylococcus aureus, Pseudomonas aeruginosa* and *Bacillus subtilis* were tested for growth in presence of Luria broth

4A.2 Organic solvent tolerance assays with BC1 and SB1 :

A) Plate method

To determine the range of organic solvents to which the cultures are tolerant, the plate assay was used, wherein 18 hour old cultures grown in L.B.M.G. broth were spread plated on an L.B.M.G. agar plate to get approximately 10^5 colonies per plate. The plates were then overlaid with various organic solvents (3 ml),

sealed with parafilm, wrapped in paper and incubated at 30 degrees in an incubator [11,219,296]. This procedure slows down the rate of evaporation of the solvents and all solvents used in this study were found to persist on the plate for at least 24-48 hours. Solvents like n -butanol and xylene remain for 5 days and more. Survival on direct exposure to solvents and appearance of colonies on a plate overlaid with undiluted (100%) organic solvent is considered as an indication of tolerance. To determine the colony forming frequency of the culture in presence of the various organic solvents, viable count of the cultures on L.B.M.G. plates with and without organic solvent were determined.

B) Liquid medium Organic Solvent Tolerance Assays with SB1:

18 hour old cells of SB1 grown in L.B.M.G. broth at 30 degrees C on a rotary shaker were used as inoculum in 250 ml flasks containing :

(i) L.B.M.G. broth with 10, 50 and 90% (v/v) of toluene, benzene, chloroform and n butanol.

(ii) Mineral salts medium flasks with 10, 50 and 90 % (v/v) of toluene, benzene, chloroform and n butanol.

The flasks were incubated on a rotary shaker (120 rpm) at 30 degrees C and viability was tested by spread plating 0.1 ml aliquots from each flask on L.B.M.G. agar plates immediately after inoculation and after 1h, 3h, 6h, 24h.

(iii) Recovery of viable cells from 95 and 99 % v/v of butanol: SB1 was inoculated into 250 ml flasks containing 95 and 99 %(v/v) of distilled butanol respectively. The flasks were monitored for viability by plating on L.B.M.G. agar as described above and this was done for 30 days.

4A.3 Role of spores in butanol tolerance:

Effect of n-butanol on the survival of spore-bearing cells and vegetative cells of *Bacillus* sp. SB1:

i) Determination of effect of n butanol concentrations on the cell viability of vegetative cells of SB1:

Vegetative cells of SB1 in the logarithmic phase of growth were inoculated in a 100 ml flask containing L.B.M.G. broth to give a final cell count of 5×10^5 cfu/ml. The flasks were overlaid with n butanol in concentrations ranging from 1 to 10 % (v/v). The flasks were incubated on a rotary shaker (120 rpm) at 30 degrees C for 24 h. Viable counts were determined by plating on L.B.M.G. agar after 24 h incubation in presence of n-butanol. The plates were incubated at 30 degrees for 48 h. One control flask was maintained which contained culture inoculated in broth in the absence of butanol.

ii) Determination of number of spore-bearers in the inoculum :

To determine whether spore-bearers exist in the inoculum and their numbers if present, 0.1 ml of the culture suspension in saline was

transferred to a sterile test tube and heated at 80 degrees C for 10 minutes, after which the tube is cooled. 9.9 ml of sterile saline was added to it and mixed (hundred-fold dilution).0·1 ml of this heated sample was plated on L.B.M.G agar and count taken after 24 h of incubation. This treatment destroys vegetative cells and allows only spore bearers to survive. This was done to determine the exact number of spores present in the inoculum. For the experiment described above, the inoculum was chosen such that the number of spore-bearers is zero. The absence of spores was confirmed by endospore staining as described by Schaeffer and Fulton (Sneath, P., 1986).

iii) Effect of 99% n-butanol on spores and vegetative cells of SB1:

A 24 h old culture of SB1 grown in L.B.M.G. broth was diluted hundred-fold and inoculated into a flask containing 99 % (v/v) of distilled n butanol (99 ml butanol and 1 ml of L.B.M.G.broth).The flask was incubated on a rotary shaker and viable counts were taken on L.B.M.G. agar immediately after inoculation and mixing, and after 24h and 5 days. To determine the fate of spore bearing cells in the population, an aliquot of the sample was subjected to heat treatment (80 degrees C for 10 minutes). The difference between the total cell count and spore count gives the total number of vegetative cells.

iv) Effect of n-butanol on spore germination: A five day old culture of SB1 on LBMG agar slant was used to prepare a

suspension in saline. 1 ml of the suspension was transferred to a sterile test tube which was then heated in an water bath set at 80 degrees for a period of 10 minutes. 0.2 ml allquots of the heated samples were transferred to L.B.M.G. broth tubes which were overlaid with 1.5 %(v/v) and 3% (v/v) n butanol. 0.1 ml aliquots were plated on L.B.M.G. agar. The plate was overlaid with 3 ml of n-butanol and incubated. The tubes and plates were incubated at 30 degrees C for 5 days and checked for appearance of growth.

v) Effect of n-butanol on sporulation: 18 h old inoculum of SB1 was added to flasks with L.B.M.G. broth to get a uniform initial count per flask. The flasks were overlaid with 1.5 and 3% (v/v) n-butanol and one flask was maintained as a control (without addition of n-butanol). The flasks were incubated on a rotary shaker (120 rpm) at 30 degrees C. The viable count of spores and vegetative cells was determined after periodic time intervals.

4A.4 Detection of metabolic removal / degradation of organic solvents by utilisation of the solvents as a sole source of carbon and energy:

SB1 was inoculated in sterile liquld mineral medium supplemented with 0.5 % glucose in a 250 ml flask. The flask was incubated on a rotary shaker (120 rpm) at 30 degrees C. The cells were harvested by centrifugation (8000 rpm for 20 minutes) while in the logarithmic phase of growth and inoculated in flasks containing mineral medium supplemented with 1% (v/v) of benzene, toluene and n

butanol respectively. The flasks were incubated on a rotary shaker (120 rpm) at 30 degrees C and absorbance at 600 nm was monitored at periodic intervals. A 0.1 ml aliquot from each flask was plated out on mineral medium agar plates which were then overlaid with benzene, toluene and butanol (1 ml), as the sole source of carbon and energy. The plates were sealed with parafilm, wrapped in paper and incubated at 30 degrees for 48 hours. Increase in absorbance in the liquid medium and appearance of colonies on plates would indicate utilisation of the organic solvent as a sole source of carbon and energy .

4A.5 Study of factors affecting organic solvent tolerance and n butanol tolerance of SB1:

(i) ***Composition of the growth medium*** : To determine whether the composition of the growth medium can affect the butanol tolerance of SB1, the culture was grown in modified Luria broth in presence of 1% (v/v) n butanol and corresponding control flasks without butanol were also maintained for comparison. The Luria broth used was with the following modifications:

a) L.B : i.e. regular Luria broth containing tryptone , NaCl and yeast extract,

b)L.B.M. : Luria broth supplemented with 10 mM $MgSO_4$

c)L.B.G. : Luria broth supplemented with 0.1% glucose and

d)L.B.M.G ı Luria broth with glucose and $MgSO_4$ supplements.

The flasks were incubated on a rotary shaker (120 rpm) at 30 degrees C. Growth was monitored by checking the absorbance at 600 nm using an Elico CL-157 colorimeter at periodic intervals. Growth profiles were plotted based on this data. Similarly, viable counts were taken on L.B.M.G. agar of SB1 grown in these media in presence of 1.5 % v/v butanol.

To determine whether use of n butanol as a carbon source affects the maximum concentration of butanol in which growth of SB1 occurs : Logarithmic phase cells of SB1 grown in mineral medium with n butanol (0.2%) as a carbon source were used as inoculum in flasks containing mineral medium. The flasks were supplemented with 0.1, 0.5, 1 and 1.5% (v/v) butanol as a sole source of carbon and energy. The flasks were incubated on a rotary shaker (120 rpm) at 30 degrees C and absorbance at 600nm was determined at periodic intervals . This data was used to plot the growth profiles of SB1 in presence of the butanol concentrations stated.

(il) ***Temperature of incubation:*** Logarithmic phase cells of SB1 were inoculated in 20 ml L.B.M.G. broth in 100 ml side arm flasks. The culture was grown in presence of 1 % (v/v) n butanol at temperatures of 30, 37 and 48 degrees C (under stationery conditions) Control flasks were maintained for each temperature without butanol addition. The absorbance at 600 nm was monitored at periodic intervals using an Elico CL-157 colorimeter. The effect of

temperature of incubation on the growth of the culture in presence of butanol was determined by comparing the growth rate and yield in presence and absence of n butanol at various time intervals.

(iii) ***pH of growth medium :*** Logarithmic stage cells of SB1 were inoculated in buffered and unbuffered L.B.M.G. broth medium having pH values of 7 (neutral) , pH 5 (acidic) and pH 9 (alkaline). Growth was monitored by comparing growth rates of SB1 at these pH values in presence (1% v/v) and absence of butanol. Also the pH of unbufferred and buffered media was determined at periodic time intervals using pH paper.

(iv) ***Inoculum density***: To determine whether amount of inoculum added affects growth in presence of 1% and 1.5% v/v n butanol, logarithmic phase cultures of SB1 were inoculated in 20 ml L.B.M.G. broth present in sterile 100 ml side-arm flasks . The inoculum level in each flask was varied (10^4, 10^5, 10^6 cfu/ml). The exact number of cells added per flask was monitored by determining the viable count on L.B. agar and by taking the O.D.600. The lag phase taken before the culture enters logarithmic phase of growth was recorded. The same was also done with butanol free control flasks. The flasks were incubated at 30 degrees C on a rotary shaker (120 rpm) and O.D. 600 determined at periodic intervals.

(v)***Adaptation of cells to butanol:*** To study the role of adaptation to butanol on n butanol supplemented growth and butanol tolerance of SB1 the following studies were undertaken; a) SB1 was repeatedly sub-cultured over 12 times on L.B. agar slants in complete absence of butanol or any other organic solvents. The culture obtained from the 12th such transfer was inoculated on a L.B.M.G agar plate which was subsequently overlaid with butanol and incubated.

4A.6 Effect of butanol on cell capsulation, morphology and ultrastucture:

(i) To determine whether growth in presence of butanol affects normal processes like capsulation and whether morphological changes are induced; capsules were demonstrated by negative staining with nigrosine and cellular morphology was studied by gram staining. This was done with cells grown in presence of 1, 2 and 3 % (v/v)n butanol and cells grown in absence of butanol in L.B.M.G. broth. Cells in various stages of growth were observed.

(ii) To determine whether growth in presence of organic solvent induces any visible changes in cellular ultrastructure: Cells of SB1 and BC1 were grown on L.B.M.G. agar plates overlaid with n butanol and chloroform respectively. Control plates with cultures grown in absence of organic solvents

were also prepared. Electron micrographs were prepared with cells grown in presence and absence of n- butanol (procedure described in chapter 3, 3.2).

4A.7 Bacterial Adherence to Hydrocarbon (B.A.T.H.) Assay:

Cells of SB1 were grown in L.B.M.G. broth at 30 degrees C on a rotary shaker (120 rpm) and harvested in the mid-logarithmic and stationery phases of growth by centrifugation at 10,000 rpm for 20 minutes. This procedure was carried out with cells grown in presence of 1 and 2 % v/v butanol and in absence of butanol. To determine whether bacterial cells adhere to hydrocarbons (possess a hydrophobic cell surface), the cells were washed with phosphate buffered saline (P.B.S. , pH 7) and resuspended in it to get an O.D. 600 in the range of 0.25 –0.35. 3 ml of cell supension was then transferred to 3 glass test tubes. The O.D.600 of each tube was determined and recorded as A1. To the 1st tube 0.5ml of hexadecane was added , to the 2nd tube 0.5 ml of n -butanol was added and no addition was made to the 3rd tube. The tubes were cyclomixed for 2 minutes and allowed to stand for 15 minutes. Eventually, the organic and aqueous layers separate. The O.D. 600 values immediately on agitation (A2) and after 20 minutes (A3) are recorded. The B.A.T.H. value is the % decrease in the turbidity of the saline phase after agitation in presence of solvent. Hexadecane or n-octane are the organic solvents generally used to perform the B.A.T.H. assay. However, in this study, an additional

tube was maintained containing n butanol. This was done to determine whether the cells of SB1 adhere to n butanol.

BATH value or % adherance= A3-A2 /A1 x 100.

Results and Disussion: Section A

The cultures *Bacillus* SB1 and *Bacillus* BC1 isolated from estuarine and coastal sediment samples respectively were studied for their solvent tolerance and cholesterol degradation traits.

In order to devise a proper biphasic organic-aqueous fermentation system for cholesterol transformation, it is first essential to understand the level of solvent tolerance of the isolates. Hence, organic solvent tolerance was determined by the plate assay. Here, cultures are streaked on L.B.M.G. agar and submerged with the respective solvent and incubated. Appearance of colonies signifies resistance to the solvent [219].

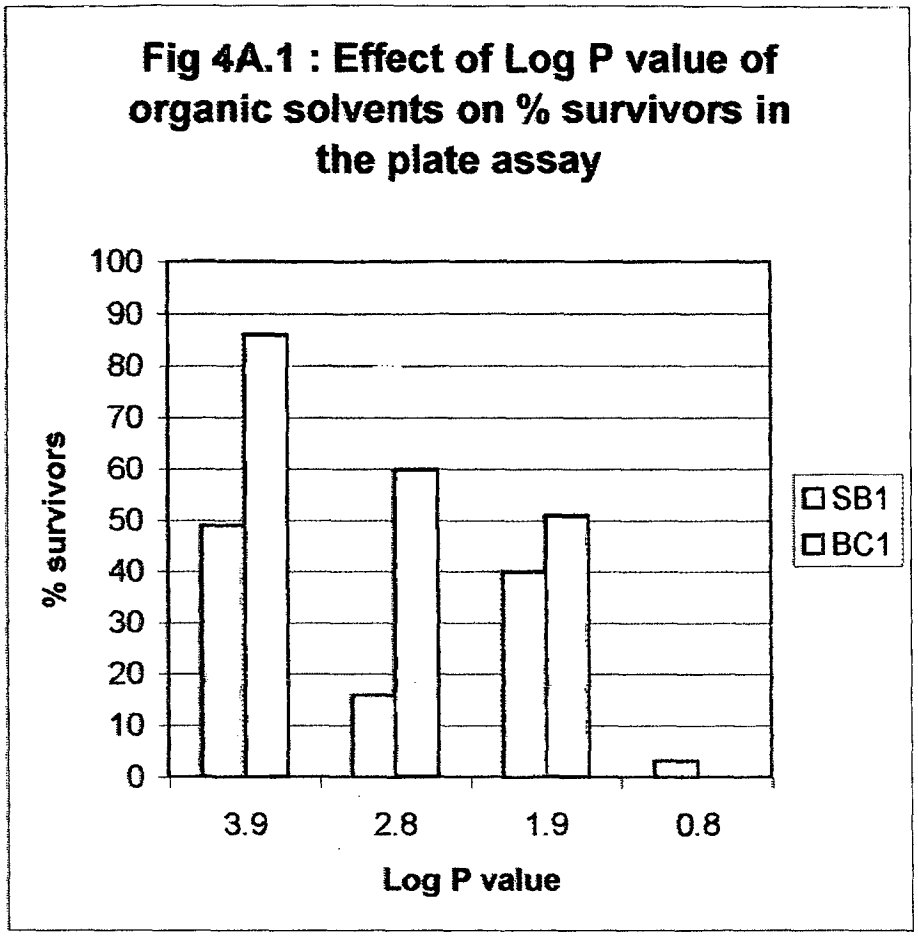
Solvent toxicity is graded based on log P values, where P is the partition coefficient of an equimolar mixture of octanol and water. The greater the polarity of the solvent, the lower the log P value, the greater the toxicity. This is because the more polar solvents partition into the aqueous phase to a greater extent and from there penetrate into and accumulate in the lipophilic cell membranes of bacteria causing lethal damage [91]. **The assays reveal that both SB1 and BC1 exhibit an exceptional level of organic solvent tolerance which has not been reported in any other bacterial strains**. The assay was carried out with a wide range of organic solvents with log P values ranging from 6 to 0.8. It has been established that solvents with log P values below 4 are highly

toxic. The most toxic organic solvent which is tolerated by a culture is called the index solvent and its log P value is called the index value. The organic solvent tolerant strains reported in literature have index values of 3 (*E.coli* mutants tolerant to xylene) [11], 2.5 (*Pseudomonas* strains tolerant to toluene) and 2 (some gram-positive strains tolerant to benzene) [19, 201]. Our strains *Bacillus* sp. SB1 and BC1 tolerate n-butanol and chloroform respectively, conferring on them index values of 0.8 and 2 **[tables 4A.1, 4A.2]**. Both chloroform tolerance and n- butanol tolerance are highly unusual and novel traits, which have not been reported in any other cultures. Although the log P value of chloroform (2) is similar to that of benzene, its toxicity is much greater than that of benzene, on account of the chlorine atom. Chloroform is tolerated by both SB1 and BC1. On the other hand, n-butanol which is an amphiphilic alcohol (8% solubility in water) [290] is one of the most toxic organic solvents. It is known to damage the cell membrane, cause leakage of potassium ions, protons and cell components and affect inter-dependent activities like pH homeostasis and respiration, resulting in cell death [182]. The only other culture reported to exhibit some degree of tolerance to this solvent is *Clostridium acetobutylicum*, the producer organism in acetone-butanol fermentation. But even this culture shows 99% reduction in growth yield in presence of 1.23 % (v/v) butanol and butanol toxicity is known to be the limiting factor in acetone-butanol fermentation [53,164, 198].

Table 4A.1: Organic solvent tolerance assay (Plate Method)

Organic solvent	Log P value	Colonies of SB1	Colonies of BC1
Control plate without organic solvent	-	312	200
n-decane	6	++	++
Iso-octane	4.8	++	++
n-hexane	3.9	154	172
Cyclohexane	3.4	++	++
Xylene	3.0	44	++
Toluene	2.8	52	120
Benzene	2.1	35	++
Chloroform	1.9	124	102
n-butanol	0.8	10	No growth

++ refers to a spreading film of growth over the entire plate (uncountable).



- The log P values 3.9, 2.8, 1.9 and 0.8 correspond to n-hexane, toluene, chloroform and butanol respectively.
- BC1 does not grow at log P =0.8.

Table 4A.2: % Survivors of SB1 and BC1 by Plate Assay of Organic Solvent Tolerance (colony forming frequency of the cultures when overlaid with 100% organic solvent):

Organic solvent	Log P value	% survivors of SB1	% survivors of BC1
Hexane	3.9	49	86
Toluene	2.8	16	60
Chloroform	1.9	40	51
n- butanol	0.8	3.2	0

Bacillus BC1 does not tolerate n-butanol but SB1 is found to resist high concentrations of n-butanol which confers on it the lowest ever reported index value. It is interesting to note that both BC1 and SB1 can tolerate a wide range of organic solvents such as decane, iso-octane, hexane, toluene, xylene in addition to chloroform [**table 4A.3**]. There appears to be a co-relation between % survivors and log P value of the solvent. The lower the log P value, the lower the % of survivors. The only exception to this is SB1 and chloroform. SB1 appears to tolerate chloroform (log P =2) better than toluene (logP =2.5) and xylene (log P=3) [**fig.4A.1**].

Generally, solvents are known to exert toxicity by their action on the bacterial cell membrane. They accumulate in the membrane disrupting its structural and functional integrity [365].
Solvent tolerant bacteria have mechanisms to counter this damage and the adaptive mechanisms of *Pseudomonas* strains have been extensively studied, the principal ones being a) the existence of an active efflux pump which pumps out the solvent from the membrane, b) rapid membrane repair mechanisms, c) changes in the cell envelope composition to decrease solvent adherance and cellular permeability to the solvent, d) shedding of membrane vesicles along with the adhered solvent molecules [160,161,192,308, 325,221]. However, there is no data on the possible solvent tolerance mechanisms of gram +ve bacteria like the *Bacillus* sp.

Table 4A.3: Fate of SB1 in liquid medium organic solvent tolerance assays:

Organic solvent	Growth of SB1	Viable cells recovered
Toluene		
10	-	++
50	-	++
90	-	++
Benzene		
10	-	++
50	-	++
90	-	++
n butanol		
10	-	++
50	-	++
90	-	++

Since n-butanol tolerance is a novel unreported trait, it was studied in detail in case of SB1.

In order to determine whether the cultures can utilise the solvents as a sole carbon and energy source, SB1 and BC1 were inoculated in mineral salts medium with the solvents as the sole carbon source. SB1 was found to degrade its index solvent n-butanol, in addition to aromatic solvents like benzene and toluene at 1% v/v concentration. Similarly, BC1 was seen to be capable of degrading its index solvent chloroform. This suggests that the cultures have the required enzymes for metabolic removal/ degradation of their index solvents. However, this can not be the primary means of solvent tolerance, although it may contribute to improving the overall solvent tolerance. A distinction has been made between solvent tolerant bacteria and solvent assimilating bacteria. Most solvent assimilating bacteria are destroyed at concentrations over 0.3 % (v/v) of organic solvents [166,307,327]. Solvent degradation does not help to overcome the initial stress induced by solvent accumulation in the membrane, as this is a quick destructive process which occurs in a few seconds [325]. Abe et al (1995) have suggested that the mechanisms of solvent tolerance in gram-positive and gram-negative bacteria seem to differ. It has been stated that solvent inactivating or emulsifying enzymes could play a larger role in solvent tolerance of gram-negative bacteria. In fact, most of the reported gram positive solvent tolerant strains like *Rhodococcus, Bacillus* and

Arthrobacter tolerant to benzene are also benzene degraders [1,201].

Studies on effect of butanol on SB1:

The effect of the butanol concentration on growth rate and yield was analysed. SB1 has a generation time of 24 minutes in LBMG broth which increases to 84.5, 295 and 862 minutes respectively in presence of 1,2 and 3% (v/v) butanol . The minimum inhibitory concentration of butanol for SB1 appears to be between 3 and 4 % (v/v) butanol, as there is 97.2% reduction in growth at 3% butanol and no growth is seen at 4% (v/v) **[table 4.4 A-D]**. The decrease in yield which is calculated from decrease in optical density is 6.2%, 18.6%, 33.5% at 1,2, and 3% (v/v) butanol respectively. Hence it can be concluded that the concentration of butanol significantly affects both the growth rate and yield of the culture **[fig 4A.2, 4A.3]**. This could be attributed to some damage induced by butanol as also the involvement of certain energy-consuming adaptations to counter solvent stress [182,199]. It has been proved that it is not the chemical structure of the solvent, but the dose to which it accumulates in the cell membrane which is the deciding factor governing lethality of the solvent [192]. It is clear from the studies done here that above a concentration of 3% (v/v), the dose of butanol accumulated in the membrane of SB1 is capable of inducing significant damage, thereby causing retardation of growth.

f ig 4A.2:Growth profiles of *Bacillus* SB1 in Luria broth with various butanol concentrations

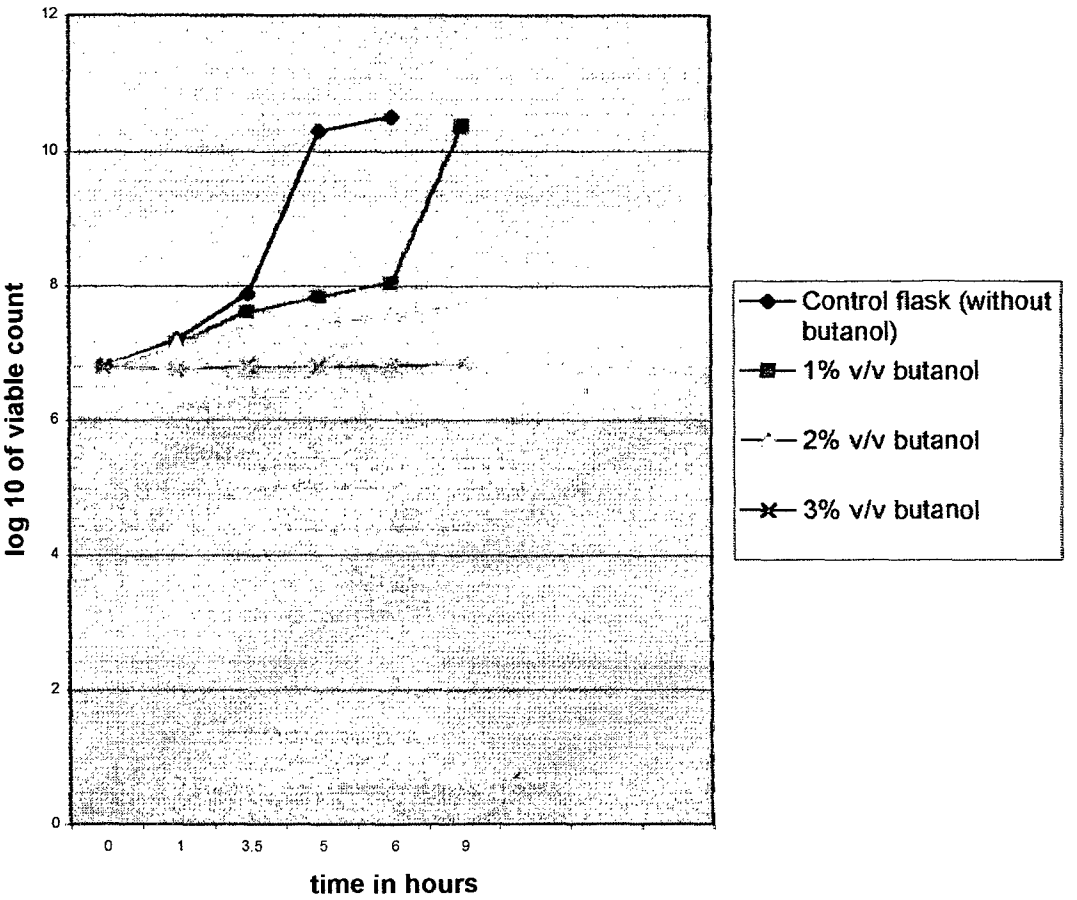
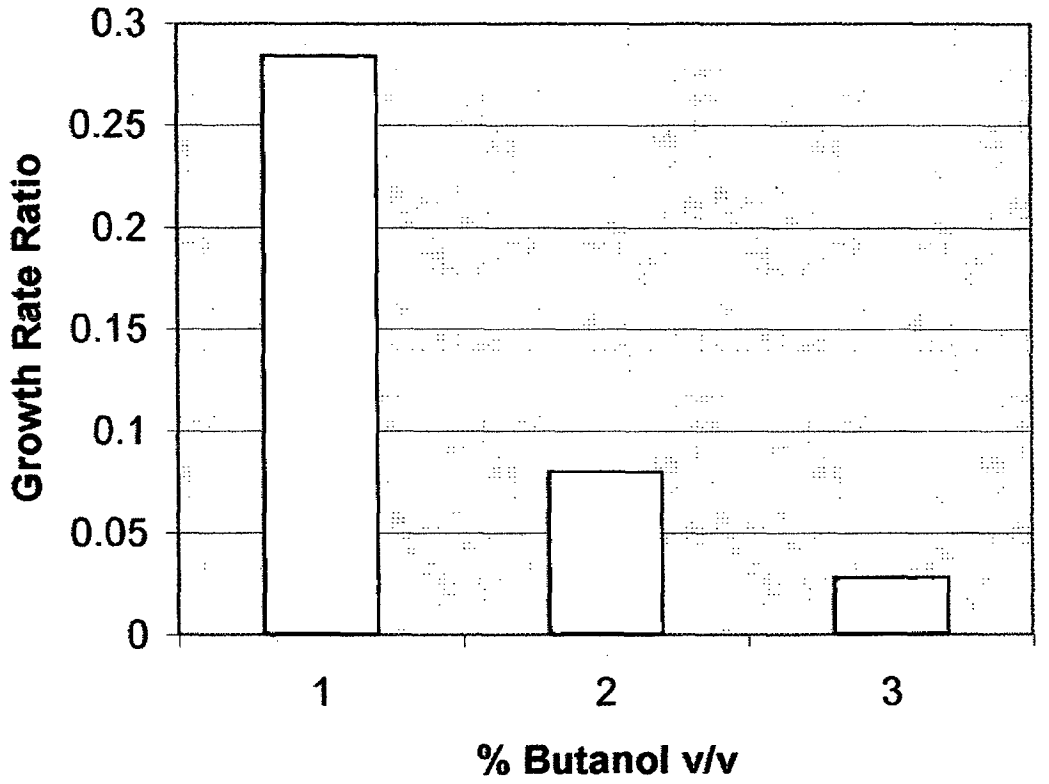
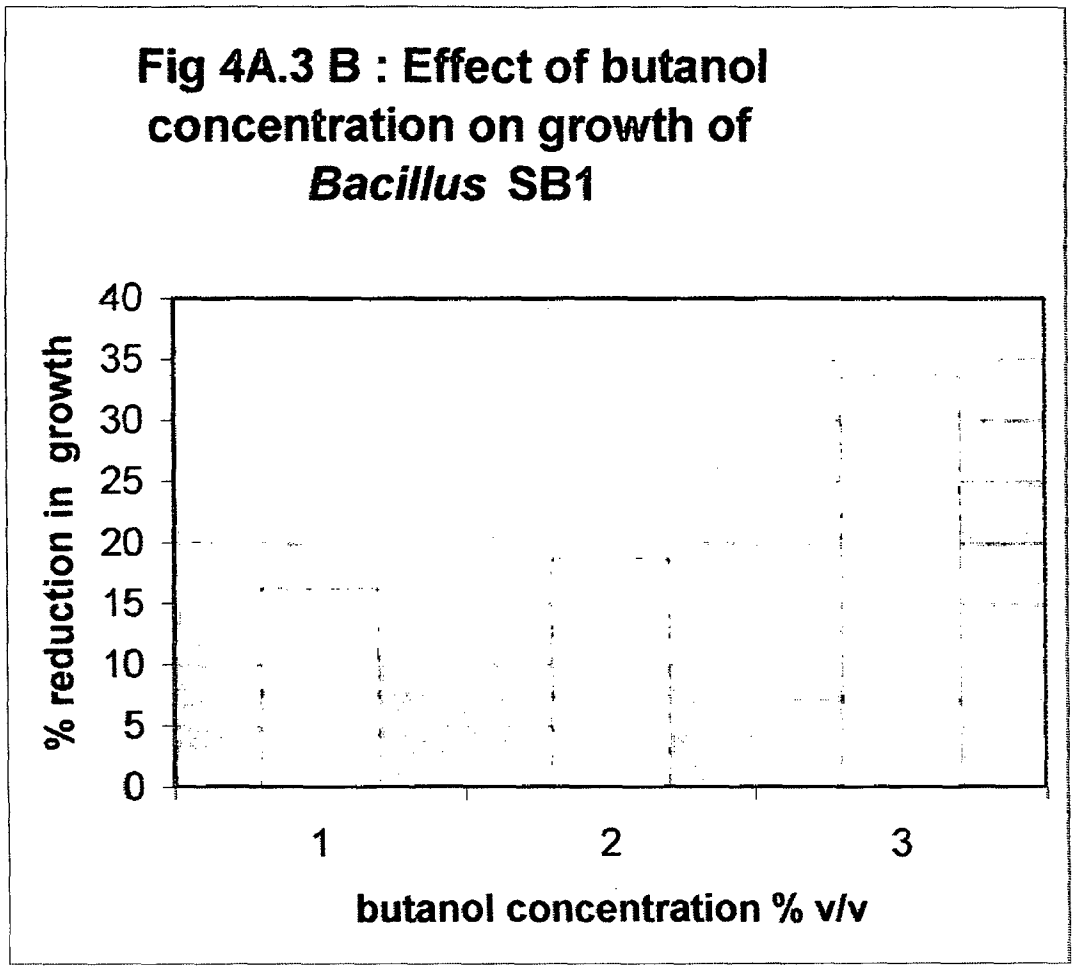


Fig 4A.3 (A) : Effect of Butanol Concentration on Growth Rate ratios on *Bacillus* SB1





Growth was measured by determining the optical density at 600 nm.

Table 4A.4 A): Effect of butanol concentration on growth rate of SB-1 in L.B.M.G. broth

Butanol concentration in %(v/v)	*Growth rate (r) h^{-1}*	*% reduction in growth rate*
Control flask without butanol	2.5	-
1	0.710	71.6
2	0.20	92
3	0.07	97.2
4	No growth	100

Table 4A.4 B): Effect of n butanol concentration on growth rate ratios of SB1:

Butanol concentration	Growth rate ratio of SB1
1%v/v (0.1093M)	0.284
2%v/v (0.218 M)	0.08
3%v/v (0.327M)	0.028

* The maximal growth rate of SB1 in absence of solvent (Go) is 2.5 h^{-1}.

Growth rate ratio is calculated by the formula Gs/Go where Gs is the growth rate in presence of organic solvent.

Table 4A.4 C):Concentration of n butanol (Relation between molarity & % v/v):

% (v/v) of n butanol	Concentration in M	Concentration present in aqueous phase *
1	0.1093	8.74×10^{-3}
2	0.218	0.01744
3	0.327	0.026

- **n butanol is an amphiphilic alcohol, which has a solubility of 8 % (v/v) in water.**

Table 4A.4 D)Effect of n- butanol concentration on the yield of SB1

Butanol concentration in % (v/v)	Initial O.D.600	Final O.D. 600	Difference between initial and final O.D. values	% reduction in growth yield compared to butanol free control
0	0.09	1.70	1.61	-
1	0.09	1.60	1.51	6.2
2	0.09	1.40	1.31	18.6
3	0.09	1.16	1.07	33.54

Sample calculation : % reduction in growth yield (for 1% v/v butanol)=1.61-1.51/1.61 x 100= 6.2%

The effect, however, appears to be static and not cidal, as viable cells persist in butanol saturated media containing 95 and 99 % (v/v) butanol even after 30 d of incubation although they can not multiply. Viable cells of SB1 have also been recovered from liquid media containing 10 , 50 and 90% of toluene, benzene and n-butanol [**table 4A.5**].

To obtain a comparitive estimate, the effect of the organic solvents chloroform and butanol on selected laboratory strains such as *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa* and *Salmonella* was determined. After 24 h exposure to 10% (v/v) butanol and 50% (v/v), no viable cells could be detected of any culture indicating that the test cultures are killed. The only exception to this was *Pseudomonas aeruginosa*, where a few viable cells were found after chloroform exposure [**table 4A.6**]. These results indicate that BC1 and SB1 which tolerate high levels of chloroform and butanol respectively are exceptional in that respect, as all ordinary bacteria are destroyed by these solvents.

Table 4A.5: Recovery of viable cells of SB1 from Butanol saturated medium after prolonged incubation:

Recovery of viable cells after incubation in butanol saturated media:	After 24h exposure	After 5 days	After15 days	After 30 d
95%(v/v)	+++	+++	++	+
99%(v/v)	+++	+++	++	+

Table 4A.6: Effect of the organic solvents n -butanol and chloroform on some selected laboratory strains:

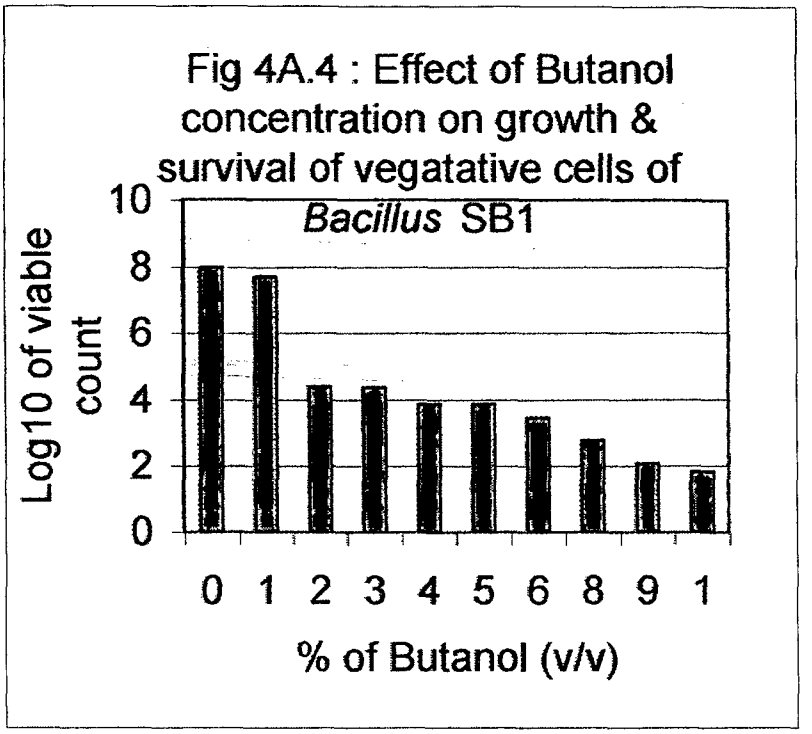
Culture	Viable cells after incubation with 50 % v/v chloroform	Viable cells after incubation with 10% v/v butanol
E.coli	-	-
Staphylococcus aureus	-	-
Pseudomonas aeruginosa	Few cells	-
Salmonella	-	-
SB1	++	++
BC1	++	

Since *Bacillus* SB1 is an endospore producing culture, the role of spores in butanol tolerance and the effect of n-butanol on sporulation, germination and survival of spores and vegetative cells was investigated. Spores are known to be the hardiest life-forms on earth which show resistance to many adverse conditions such as heat, uv light, radiations, oxidative damage, dessication and chemical agents. Factors important in spore resistance to chemicals are impermeability of the spore coat to hydrophilic chemicals, low spore core water content which keeps the enzymes in an inactive state and protection of spore DNA by alpha-beta SASP proteins. *Bacillus* endospores have been shown to survive in presence of 95% (v/v) ethanol for prolonged periods of time [288,353].

Earlier studies done on other strains of *Bacillus* have shown that the growth rate is lowered but the final yield remains the same when ethanol is present in the growth medium. At concentrations allowing growth at half the maximal rate, practically no spores are formed. Post exponential events such as excretion of certain enzymes and modification of RNA polymerase are altered or suppressed in presence of ethanol. Thus, the sensitivity to ethanol is found to be much greater for the sporulation process rather than growth [45]. Our experiments on *Bacillus* sp. SB1 suggest a similar sensitivity of the sporulation process to n-butanol. Presence of butanol appears to inhibit sporulation in SB1. However, spores are found to germinate in media with concentrations of butanol which enable growth and multiplication. It is interesting to note that while

the % of surviving vegetative cells decreases with increase in butanol concentration, this pattern does not appear when a mixed inoculum is used containing both vegetative cells and spores. This is probably due to the fact that spores have a much slower rate of death as compared to vegetative cells when exposed to butanol **[table 4A.7 A-D and 4A.8].**

Butanol being toxic exerts stress on cells for their survival. Sporulation is one of the chief means by which *Bacillus* spores survive stressful conditions. However, studies show that exposure to alcohols inhibits the sporulation process. Hence, it is possible that cells revert to some other means to face this challenge. One possibility is the involvement of the general stress regulon, producing specific stress proteins. *Bacillus* is known to contain a large sigma B regulon which now known to be induced upon entry into stationery phase and by environmental stresses such as salt, heat stress and ethanol shock in the logarithmic phase of growth. The general stress regulon is a less extreme alternative to sporulation under growth limiting conditions and may become critical under conditions that do not support sporulation. A large number of sigma B products appear to be associated with the cell envelope and it has been hypothesised that the loss of sigma B would become evident under conditions that challenge envelope function [33,34, 35,36,37, 55,123,154, 155,260,263, 404,405, 406,407]. Like ethanol, butanol also appears to inhibit sporulation. However, cells still appear to withstand the solvent. Stress proteins



The initial number of cells in all the flasks was $5x10^5$ cfu/ml (log10 = 5.7). Growth was seen only in 0 & 1% flasks.

like the phage shock protein A have been found to appear in *E.coli* mutants on exposure to hexane, although their exact function is not clear [56,219]. It would be interesting to determine whether the general stress regulon of *Bacillus* is involved in tolerance to organic solvents.

While butanol inhibits sporulation, germination of spores however is not affected at concentrations below the minimum inhibitory concentration for growth. Vegetative cells of SB1 are adversely affected by butanol concentration and show a decline in number which is proportional to the concentration of butanol **[fig. 4A.4]**. There appears to be difference in rate of death of vegetative cells and spores of SB1 on exposure to n-butanol. After exposing a mixed inoculum to 99% n-butanol for 5 d, it was found that there is 95% reduction in the number of vegetative cells. However, % reduction of spores was only 47%, indicating that spores are destroyed at a slower rate **[table 4A.8].**

The effect of various factors such as temperature of incubation, pH, growth media, inoculum density and adaptation to butanol , on growth of SB1 in presence of butanol was determined.

Table 4A.7 A) Effect of n-butanol on sporulation and germination by *Bacillus* SB1

n-Butanol concentration (% v/v)	Sporulation	Germination
0 (control)	+	+
1.5	–	+
3	–	–
99	–	–

Table 4A.7B) Effect of n -butanol concentration on vegetative cells of SB1:

Concentration of butanol in %(v/v)	Concentration of butanol in M	Viable counts of SB1 in cfu/ml after 24 h exposure to butanol	Log 10 of viable count
0.00(control flask)	0.00	1×10^{8}	8.00
1	0.1093	5×10^{7}	7.7
2	0.218	2.6×10^{4}	4.4
3	0.327	2.4×10^{4}	4.38
4	0.437	7.2×10^{3}	3.86
5	0.546	4.8×10^{3}	3.68
6	0.656	3×10^{3}	3.47
8	0.874	6×10^{2}	2.78
9	0.984	1.2×10^{2}	2.08
10	1.093	70	1.84

- Each flask was inoculated with 5×10^{5} cfu/ml of vegetative cells of SB1 in the logarithmic phase of growth.

Table 4A.7 C) Relation between % survivors of vegetative cells of SB1 and n- butanol concentration:

Concentration of n butanol in % (v/v)	% reduction in the number of vegetative cells after 24 h exposure to butanol	% survivors
2	95.2	4.8
3	95.2	4.8
4	98.56	1.44
5	99.04	0.96
6	99.40	0.60
8	99.88	0.12
9	99.976	0.024
10	99.986	0.014

Table 4A.7D) Effect of butanol concentration on spore count and total cell count of SB1:

Butanol conc (%v/v)	Total count Cfu/ml	Spore count Cfu/ml	Effect on spore count
Control (no butanol)	1.2×10^{9}	3×10^{7}	10^{2} fold increase
1.5%	3×10^{9}	6.6×10^{4}	10^{1} fold decrease
3%	6×10^{6}	2×10^{3}	10^{2} fold decrease

Table 4A.8 Effect of incubation in 99 % (v/v) n -butanol on the spore count and total cell count of SB1:

Time for which incubated in presence of 99% n butanol	Total cell count cfu/ml	Spore count cfu/ml	Number of surviving vegetative cells
0min.	72	17	55
24 h	12	9	3
5d	12	9	3

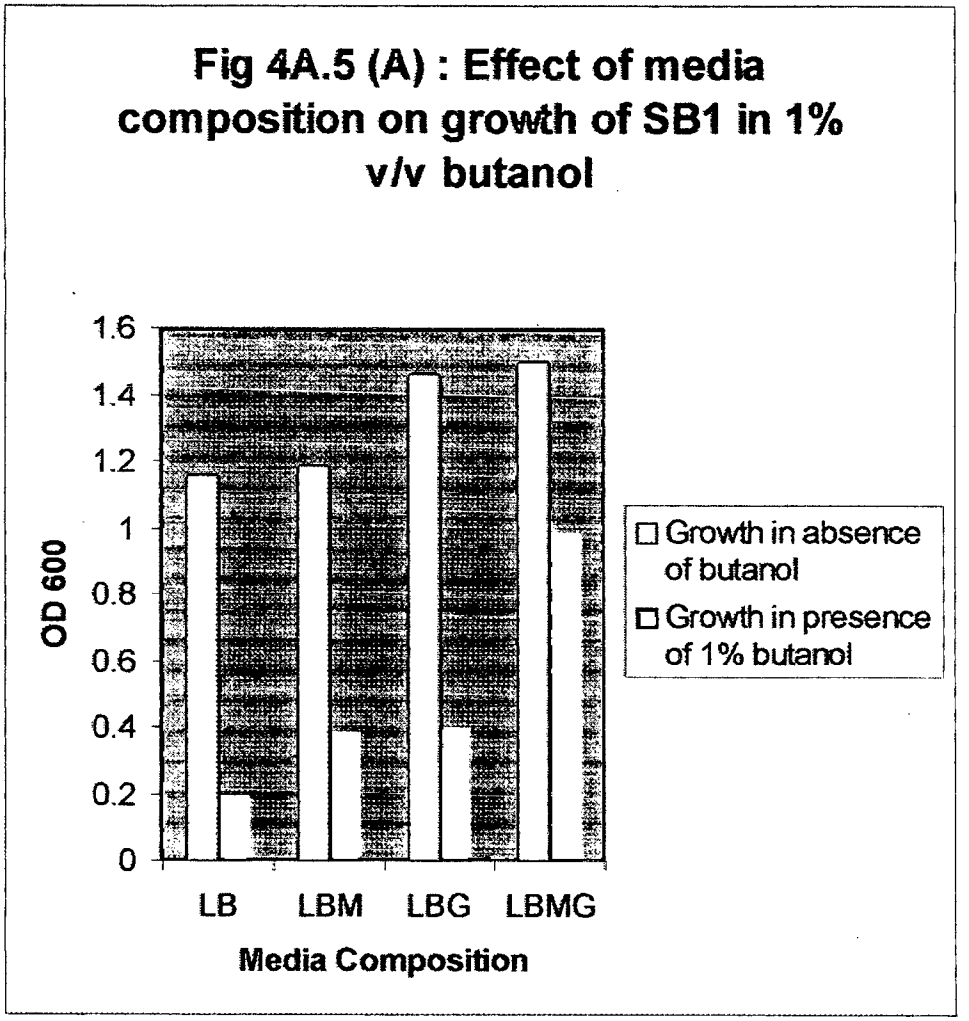
% reduction in number of vegetative cells in 5 days = (55-3) / 72 x 100= 95%

% reduction in the number of spore bearers in 5 days = (17-9) / 17 x 100 = 47%

% reduction in overall population in 5 days = (72-12) / 72 x 100 = 83.33%

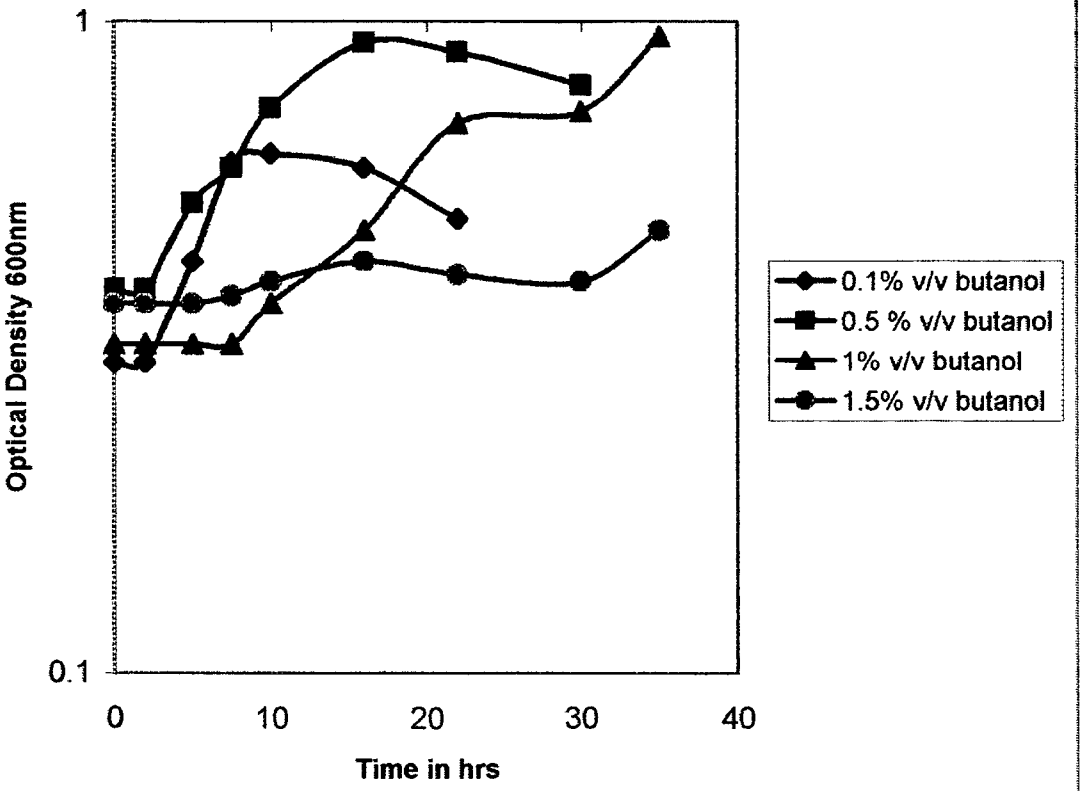
Firstly, the growth medium appears to play a role in tolerance to butanol. The culture was grown in Luria broth (LB) and in Luria broth with supplements; LBM (having 10mM MgSO4), LBG (having 0.1% glucose) and LBMG (having both supplements) [185,186] and also in mineral medium with butanol as the sole carbon source. The best growth (in terms of high growth rate) was seen in LBMG broth, which is incidentally the most nutritive medium [**fig.4A.5 A**]. The role of Mg ions in solvent tolerance is believed to be significant. Mg is the co-factor for several enzymes required in membrane repair, and this activity has to be enhanced to promote growth in solvents. In case of gram-negative bacteria which possess lipopolysaccharides (LPS) in their envelope, Mg is believed to be an important cation in L.P.S stabilisation [186]. The effect of butanol concentration (0.1, 0.5, 1 and 1.5%) on the growth of SB1 in presence of mineral medium using butanol as the carbon source was determined. Growth was found to be severely retarded at a concentration of 1.5 % (v/v). Good growth was seen at 0.5 and 1% (v/v). In case of 0.1% butanol, the cells enter decline phase sooner probably due to exhaustion of the carbon source butanol [**fig.4A.5 B**].

Temperature of incubation is one vital factor which plays an important role in influencing growth in presence of butanol. Ordinarily, for growth in absence of butanol, 48 degrees C is the optimum temperature of incubation for SB1 where it exhibits the



LB refers to Luria Broth , LBM is LB with 10 mM $MgSO_4$, LBG is LB with 0.1% glucose, LBMG is LB with both $MgSO_4$ and glucose.

Fig 4A.5(B) : Growth of *Bacillus* Sp. SB1 in mineral medium with butanol as carbon source



highest growth rate of 1.6 h^{-1}. The growth rate at 30 and 37 degrees C is the same ie. 1.2 h^{-1}.

However, when the culture is grown in presence of 1% (v/v) butanol, it is seen that no growth occurs in the flask incubated at 48 degrees C, even after 24 h incubation. This could be on account of the fact that the changes in membrane fluidity (fatty acid composition) dictated by growth at high temperature are not compatible with the membrane changes required to grow in presence of solvents like butanol [180,181,182]. There is no difference in the growth of butanol at 30 and 37 degrees C whether in the presence or absence of butanol [**fig. 4A.5C and table 4A.9A**].

When grown in unbuffered LBMG broth (pH 7), it was found that SB1 rapidly makes the medium alkaline. Hence, the effect of pH of incubation on growth in butanol was studied by growing SB1 in buffered LBMG medium. It was found that SB1 was incapable of growth at pH 5 irrespective of presence and absence of butanol. There is no difference in growth rate of SB1 when grown at pH 7 and 9 in presence or absence of butanol [**table 4A.9 B**]

The initial inoculum density added to the flask affects the duration of the lag phase in presence of 1.5 (v/v)% butanol. Low inoculum size (10^3) prolongs the lag phase. Recent studies have shown that low density and high density cultures respond differently to environmental stimuli. They are now believed to be as different as lag phase and stationery phase cultures [244,245]. The minimum

Fig 4.7: Factors affecting growth of Bacillus SB1 in presence of 1 % v/v butanol.

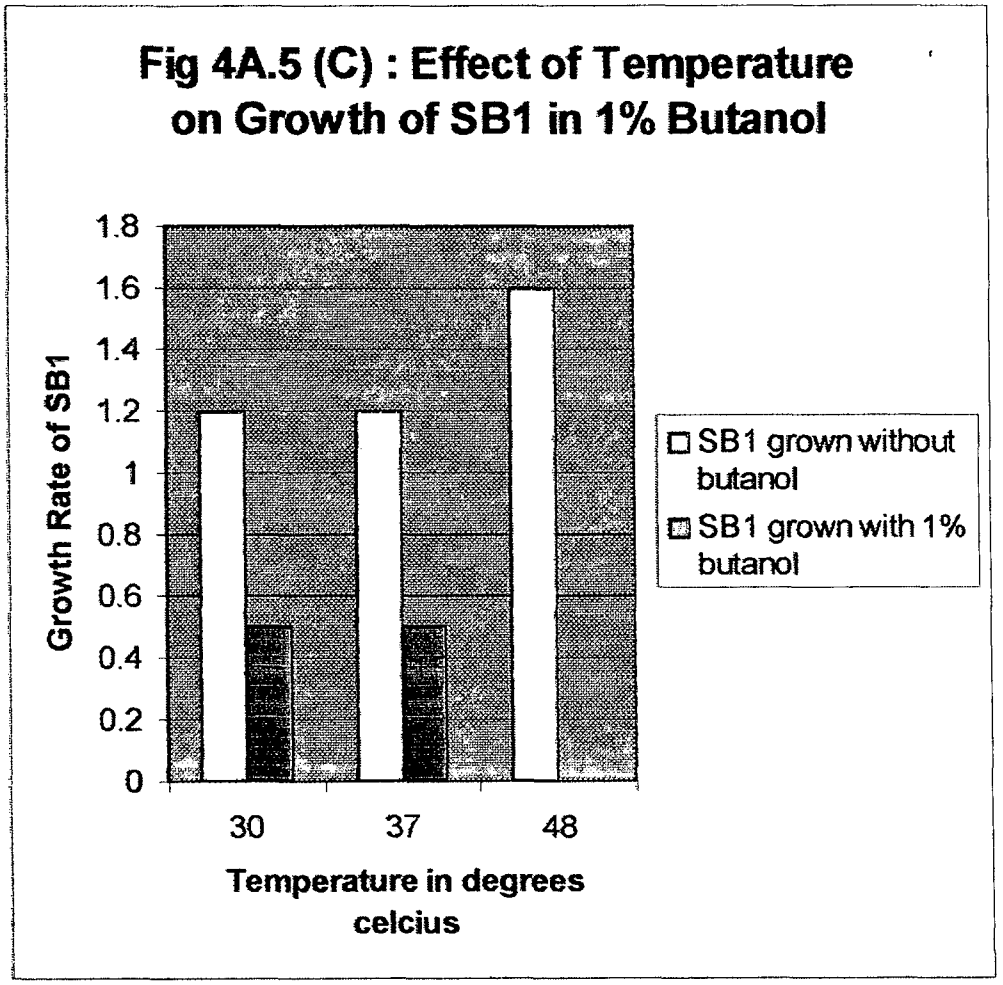


Table 4A.9A): Effect of temperature of incubation on growth rate and yield of SB1 in presence and absence of butanol:

Butanol concentration (%v/v)	Temperature(in degrees C)	Growth rate (h^{-1})	Yield (dry weight in mg/ml)
0	30	1.2	2.8
1	30	0.503	1
0	37	1.2	2.8
1	37	0.503	1
0	48	1.6	2.9
1	48	No growth till 24 h incubation*	-

*Growth was observed after 48 h incubation.

Table 4A.9B): Effect of pH on growth of SB1 in presence of butanol:

pH of buffered L.B.M.G medium	Growth rate of SB1 in control flasks without butanol	Growth rate of SB1 in flasks with 1%v/v butanol
5	No growth *	No growth*
7	2.5	0.710
9	2.5	0.710

*It was found that SB1 is not capable of growth at pH 5 when grown in buffered medium. However , growth does occur in presence and absence of n butanol in unbuffered L.B.M.G. broth, where it Is found that culture rapidly changes the pH of the medium making it alkaline.

Table 4 A.9 C) Effect of inoculum size on growth of SB1 in presence of butanol

Inoculum size (initial cfu/ml per flask)	Duration of lag phase in control flask (without butanol)	Duration of lag phase in presence of 1 % v/v butanol
7×10^{4}	3h	>14 h
5×10^{5}	1h	2h
5×10^{6}	1.5h	1.5h

Table 4A.9 D)Effect of inoculum size on duration of lag phase in presence of 1.5% v/v butanol:

Inoculum size (cfu/ml)	Duration of lag phase in h
1×10^{3}	15
4×10^{5}	14
4×10^{6}	10

inhibitory concentration is known to be affected by inoculum size dependency [229]. It is also possible that initial addition of butanol may destroy or inactivate a certain fraction of the population. Hence, starting with a low initial density may result in fewer surviving cells in the flask, which may take a longer time to produce a detectable optical density which leads to a longer lag phase [**table 4A.9 C,D].**

It has been stated that organic solvent tolerance is a strain - specific property which is determined by genetic and environmental factors [11]. It appears that growth media composition and conditions of incubation play a vital role in growth in presence of butanol.

Unlike certain strains of *Pseudomonas* sp. where prior cultivation in presence of small concentrations of organic solvent is a pre-requisite for solvent tolerance [191,192], *Bacillus* SB1 does not appear to depend upon prior adaptation to butanol to withstand butanol exposure. Even after 12 repeated sub-cultures in absence of any organic solvent, it was found that SB1 could grow well on LBMG medium supplemented with butanol. This indicates that butanol tolerance is a stable phenotypic property of the culture.

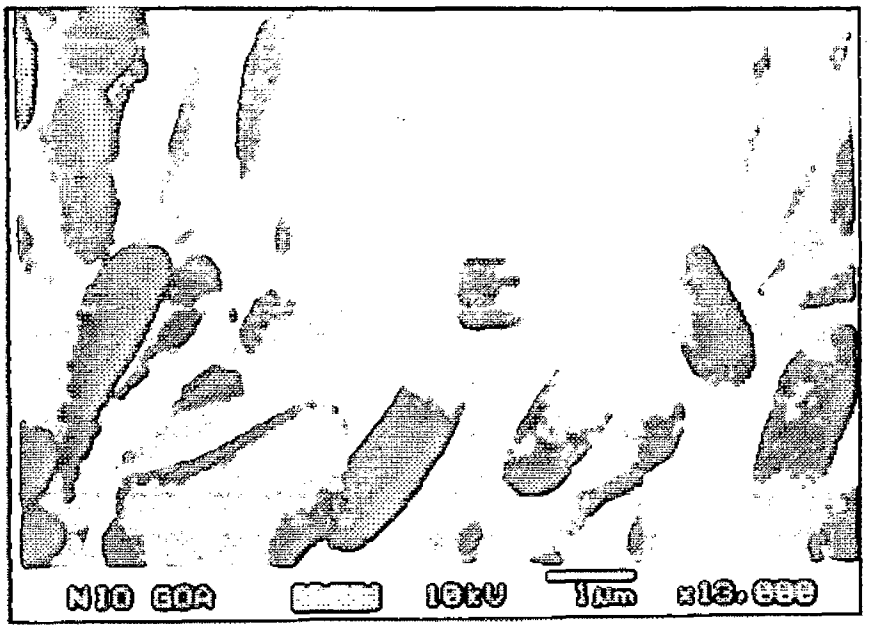
In order to determine whether growth in the presence of butanol affects normal processes like capsulation and induces morphological changes, the cells were stained and studied microscopically. Capsule production does not seem to be affected

by presence of butanol and the size of the capsules appears to be the same when cells are grown in the presence or absence of butanol. Electron micrographs of SB1 and BC1 when grown in presence of their index solvents, butanol and chloroform respectively were prepared and compared with those of cells grown on LBMG agar in the absence of any organic solvents. It was interesting to note that *Bacillus* SB1 shows a decrease in cell size when grown in presence of n-butanol, from 3 to 1.8 microns in length. It has earlier been stated that effect of butanol on SB1 is probably static and not cidal as viable cells have been isolated after prolonged incubation in even 99%butanol , though growth is not seen. An electron micrograph of cells taken after prolonged incubation in 99% butanol shows the presence of long filamentous cells, some of which appear swollen and distorted. The presence of filamentous cells indicates that cell division has been halted. Swelling and distortion can be attributed to the damage induced by butanol

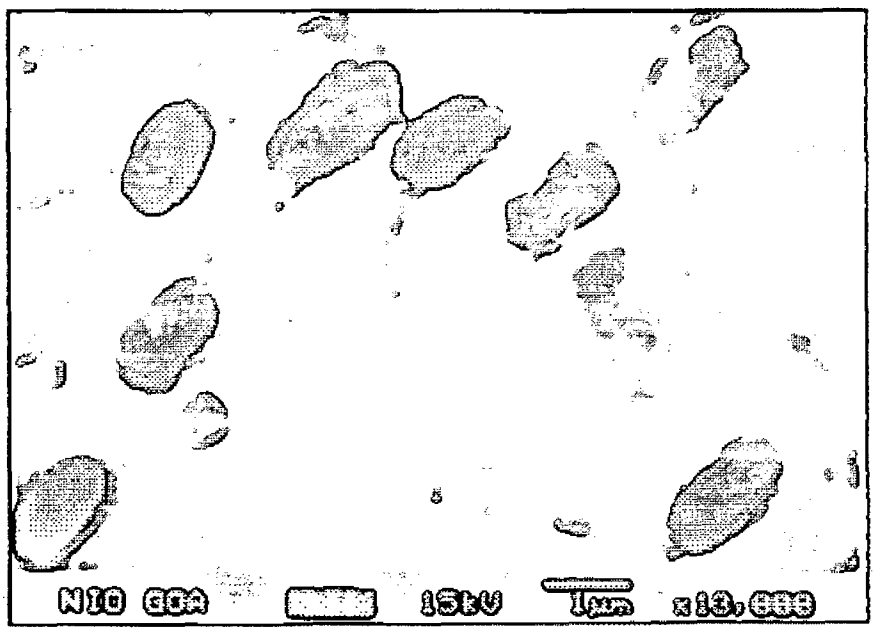
With BC1, there is no significant decrease in cell size when grown in presence or absence of chloroform. However, on vigorous agitation with chloroform, it was seen that a fibre like material oozes out of the cells of BC1. Since fatty acids/ lipids are known to be released by chloroform treatment, the fibres could be membrane fatty acids along with other components. However, despite this treatment high counts of viable cells persist in presence of 50% chloroform. **[fig.4.9 A-H].**

Fig 4A.6: Electron Micrographs of the Organic Solvent Tolerant Cholesterol transforming *Bacillus* Sp. SB1 & BC1

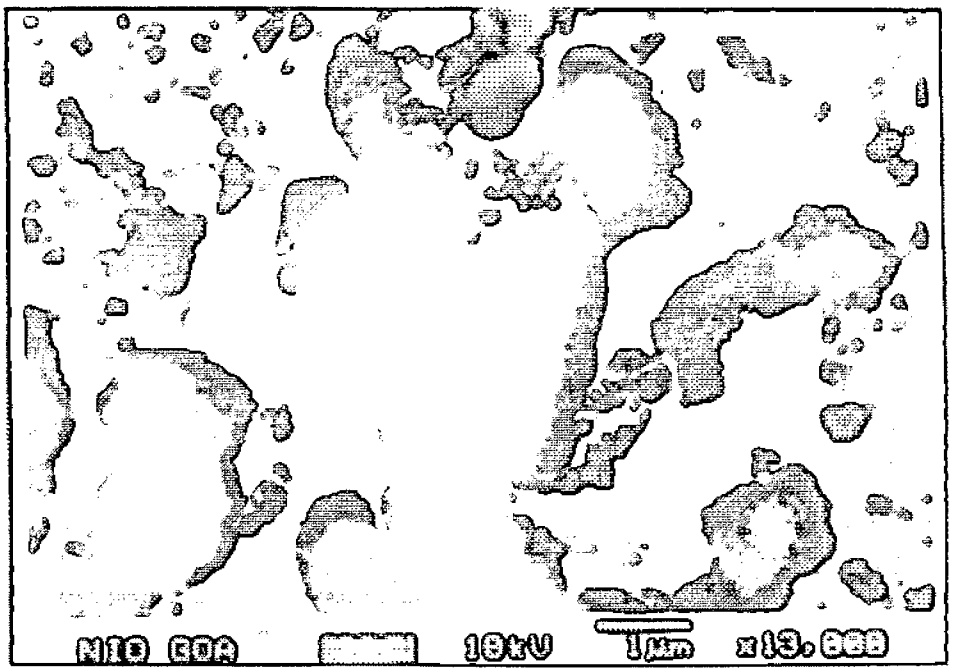
(A) SB1- Cells grown in absence of Organic Solvents.



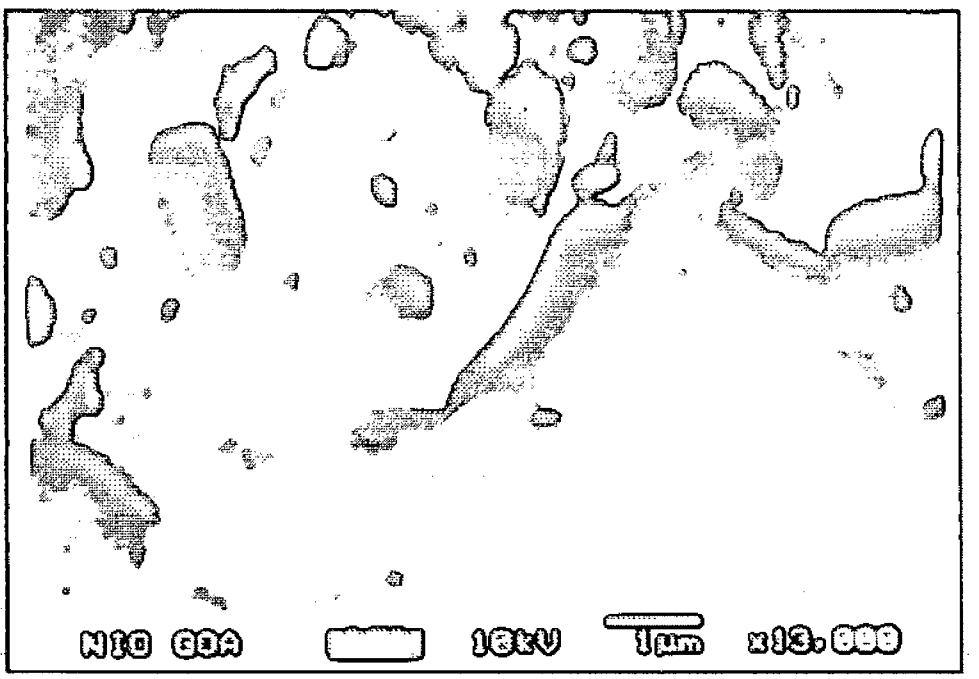
(B) SB1- Cells grown on LBMG Agar overlaid with n-butanol (Index Solvent)



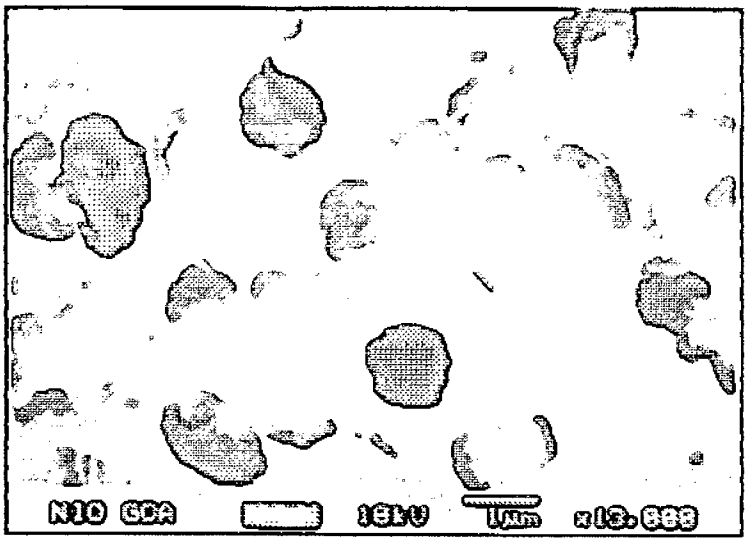
(C) SB1- Cells taken from a flask containing 90% v/v butanol after prolonged incubation



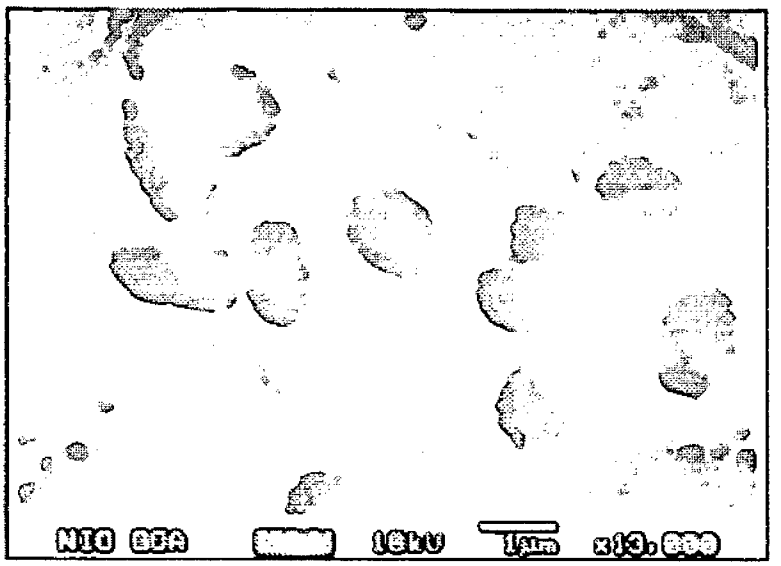
(D) SB1- Resting Cells In Saline after strong agitation in 50% v/v chloroform for 10 minutes



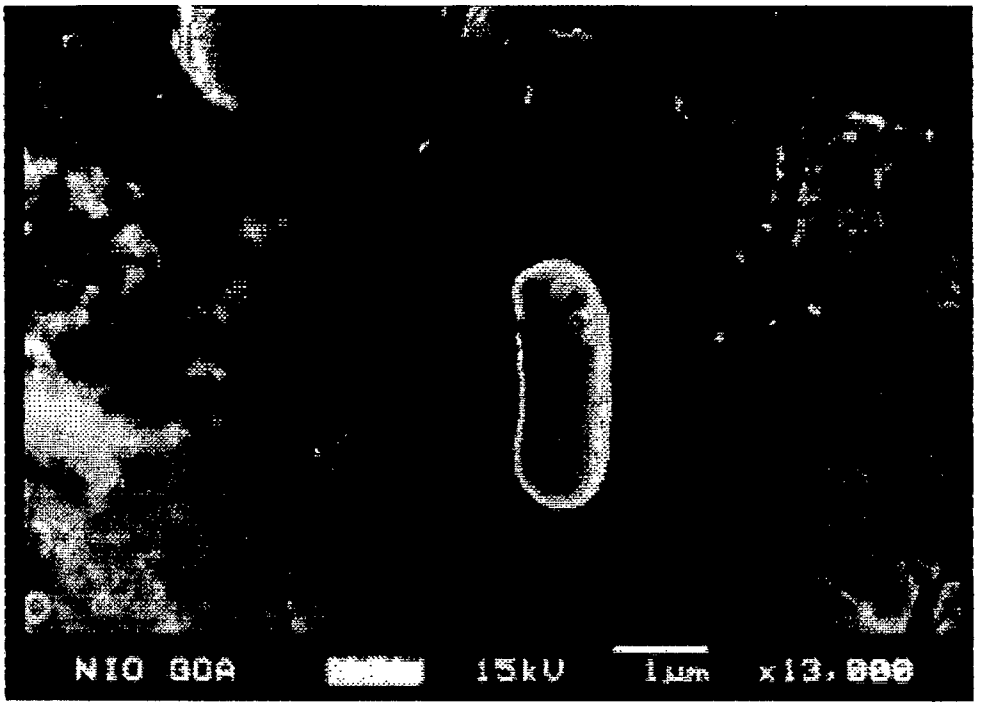
(E) BC1- Cells grown In absence of Organic Solvent



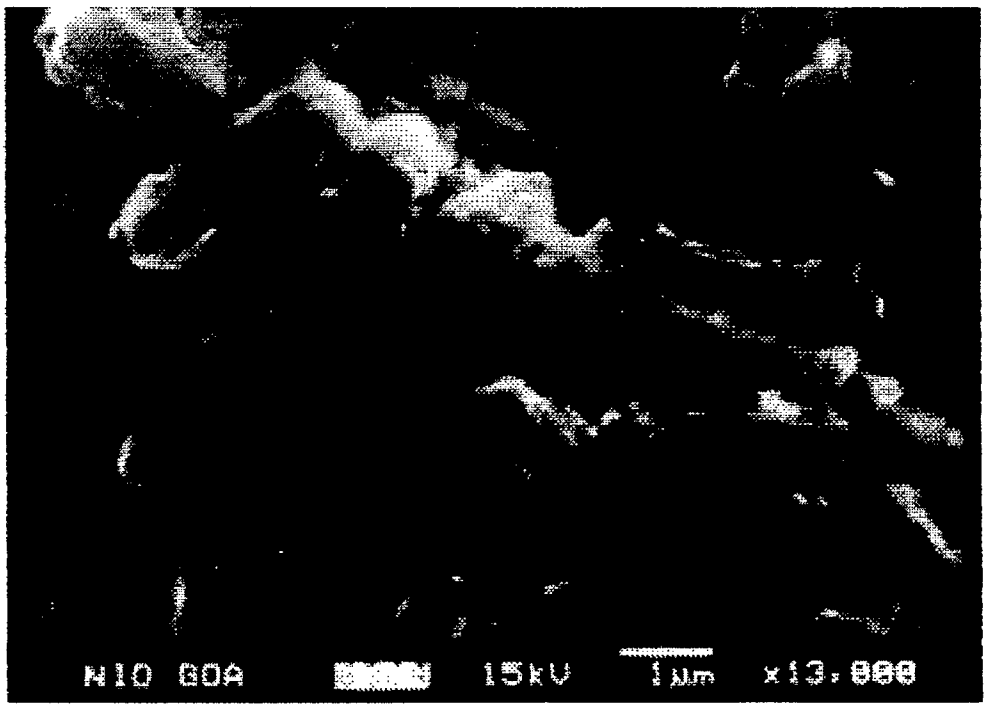
(F) Cells grown on LBMG Agar overlaid with chloroform
(Index Solvent)



(G) BC1- Resting cell before agitation with chloroform



(H) BC1- Resting Cells in Saline after strong agitation with 50% v/v chloroform for 10 minutes.



During the BATH assay, it was found that cells of SB1 as well as the cell-free broth contain a butanol emulsifying substance, since the absorbance increases on shaking the sample with butanol. Many hydrocarbon degrading bacteria are known to produce hydrocarbon emulsifying substances. SB1 is a butanol degrader as well as a solvent tolerant culture. It is possible that the emulsification of butanol may play a role in butanol tolerance. One of the mechanisms to increase solvent tolerance as found in *E.coli* and *Pseudomonas* strains is to decrease cell surface hydrophobicity, so that solvent molecules can not adhere to the cells [12]. The more the number of solvent molecules adhering to the cell, the greater the damage inflicted thus increasing cell fragility. Solvent tolerant bacteria bring about subtle changes in the chemical composition of the cell surface by changing the type and amount of certain proteins, carbohydrates or lipids which decreases cell surface hydrophobicity. In case of SB1, both log phase as well as stationery phase cells do not adhere to butanol, probably on account of changes in the cell surface or even due to production of capsule. Instead, they produce a butanol emulsifying substance [**table 4A.10**]

Table 4A.10: Bacterial Adherance to Hydrocarbon Assay and Detection of Emulsification activity in cells and cell free broth:

O.D.600	Bh	Bb	Ch	Cb
mid- log phase culture grown in 1% butanol:				
A1	0.19	0.23	0.31	0.31
A2	0.23	0.26	0.30	0.30
A3	0.21	0.22	0.30	0.26
Stationery phase culture grown in 1% butanol				
A1	0.00	0.00	0.94	0.16
A2	0.06	0.52	0.97	0.79
A3	0.00	0.16	0.96	0.23
Stationery phase culture grown without n- butanol (control)				
A1	0.30	0.29	0.23	0.23
A2	0.36	0.86	0.26	0.30
A3	0.33	0.54	0.21	0.24

Bh- O.D. of cell free broth when treated with hexadecane

Bb- O.D. of cell free broth when treated with n butanol

Ch-O.D. of cells when treated with hexadecane

Cb- O.D. of cells when treated with n butanol

Section B: Transformation of cholesterol by resting cells of Bacillus SB1 and BC1 in a biphasic system with 50% chloroform.

Materials and Methods :

4B.1 Determination of growth profiles of SB1 and BC1 in mineral medium with cholesterol as the sole carbon source and in Luria broth:

Cultures grown in mineral medium with cholesterol for 8 h were harvested by centrifugation at 10,000 r.p.m. for 20 minutes. The cells were washed and resuspended in saline. 10% (v/v) of this inoculum was added to 100 ml side- arm flasks having 20 % (v/v) of mineral medium with 0.5 mg/ml cholesterol. The flasks were incubated on a rotary shaker (120 r.p.m.) at 30 degrees C. Viable counts were taken on nutrient agar at 0 minute and at periodic time intervals, thereafter. Growth profiles were plotted and growth rate and generation time of the cultures in cholesterol medium was determined.

4B.2 Development of an biphasic organic-aqueous bio-conversion system for cholesterol transformation:

To determine whether resting cells of the organic solvent tolerant cholesterol degrading bacteria SB1 and BC1 could be used to transform cholesterol in a biphasic organic-aqueous bio-conversion system, the following experiments were undertaken.

4A.2 A) Preparation of resting cells: The cultures were inoculated in Luria broth and the flasks incubated overnight at 30

degrees C on a rotary shaker (120 r.p.m.). The cells were harvested by centrifugation at 10,000 r.p.m. for 20 minutes, washed with sterile phosphate buffer (pH 7) and suspended in 50 ml of sterile phosphate buffer (pH 7) and supplemented with 2 % (v/v) mineral salts medium (prepared without a nitrogen source) in 250 ml flasks. The flasks were overlaid with 50 ml chloroform (50% v/v). The chloroform contained dissolved cholesterol such that the concentration of cholesterol in each flask was 1 mg/ml. The flasks were incubated on a rotary shaker (120 rpm) for a period of 2 days. The cellular viability in presence of 50% (v/v) chloroform was continuously monitored by spread plating 0.1 ml of the medium layer from each flask on Luria agar at periodic time intervals.

4A.2 B) Monitoring transformation of cholesterol by SB1 and BC1:

Detection of intermediates by Thin Layer Chromatography:

For detection of intermediates produced due to degradation of cholesterol, aliquots were taken from the chloroform layer at periodic time intervals and used to perform T.L.C. The plates were run in the following solvent systems: hexane-diethyl ether (3:10) [14], ethyl acetate -petroleum ether (2:8), chloroform –acetone (9:1) [194]. The preferred developing agent used was vanillin-phosphoric acid as it gives a violet colour only with steroids [400].

Appropriate controls were maintained to rule out lipids of cellular origin appearing in the TLC. The resting cells of SB1 and BC1 were washed and suspended in phosphate buffer with minerals. Chloroform (50% v/v) without cholesterol was added to the medium and the flasks incubated on a rotary shaker. This flask was devoid of cholesterol. TLC of the chloroform layer was performed as described above to detect any impurities of cellular origin.

To check for auto-oxidation of cholesterol, a flask was maintained with phosphate buffer in contact with 50% (v/v) chloroform containing dissolved cholesterol in absence of cells. This flask was also monitored at periodic intervals.

4A.3 Extraction and purification of the cholesterol degradation intermediates:

After bio-conversion by resting cells as described above, the chloroform layer was removed by transferring the contents of the flask in a separating funnel and allowing to settle for a few minutes. The chloroform layer was concentrated by evaporation. Separation of the intermediates was done by preparative thin layer chromatography. The chloroform layer was developed on 2 mm thick silica gel plates in the ethyl acetate-petroleum ether (2:8) and chloroform-acetone (9:1) systems and spots were detected on the silica gel plate using iodine vapours. Silica gel was scraped from the layers showing brown spots and the compounds were

recovered by filtration with chloroform which was evaporated to give a fine powder.

4A.4 Structural analysis of the cholesterol degradation intermediates:

Structural analysis of the compound was done by performing proton N.M.R. (nuclear magnetic resonance) spectroscopy and ultraviolet (u.v) spectroscopy .

Results and Discussion : Section B

Development of a biphasic organic-aqueous bio-conversion system:

Both BC1 and SB1 were found to utilise cholesterol as the sole source of carbon and energy when grown in mineral salts medium. *Bacillus* BC1 was found to have a shorter doubling time (162 min) as compared to *Bacillus* SB1 (236 min) in cholesterol medium **[tables 4B.1 A and B, fig.4B.1]**

Cholesterol being a steroid is practically insoluble in water. This imposes severe limitations on the rate of transformation in aqueous systems [14]. Hence, a biphasic system consisting of various organic and aqueous phases were tried out in combination such as distilled water-butanol, saline-butanol, phosphate buffer-butanol, mineral medium-butanol, saline-benzene etc for SB1. However, although SB1 tolerates butanol, the cholesterol transformation process does not seem to occur in presence of butanol. This is probably because cholesterol transforming enzymes are inactivated in presence of butanol. Hence, a less toxic solvent was chosen. After several trials, it was found that 50 % (v/v) as the organic phase was ideal for both cultures SB1 and BC1. The ideal aqueous phase was found to be phosphate buffer (pH 7) supplemented with 2% mineral salts medium without a nitrogen source. The latter is added to provide mineral salts if required as co-factors/ stimulators for the required enzymes.

Fig 4B.1 : Growth profiles of *Bacillus* Sp. SB1 & BC1 in mineral medium with cholesterol

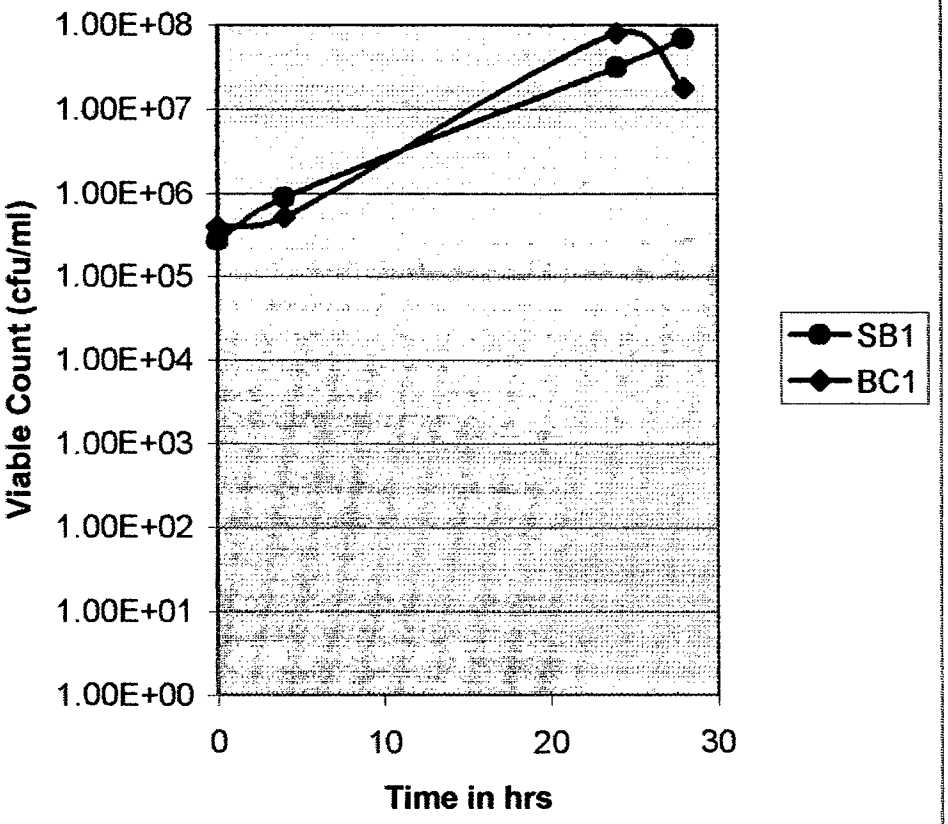


Table 4B.1A):Viable counts of SB1 and BC1 in mineral medium with cholesterol at periodic time intervals:

Time	Viable count of SB1 in cfu/ml	Viable count of BC1 in cfu/ml
0 minute	2.7×10^5	4×10^5
4 h	9×10^5	5.2×10^5
24h	3.12×10^7	8×10^7
28 h	6.9×10^7	1.8×10^7
4d	6×10^5	1×10^5

Table 4B.1 B): Growth rate and generation time of SB1and BC1 in mineral medium with cholesterol (0.5 mg/ml) as a sole source of carbon and energy:

Culture	Growth rate (h^{-1})	Generation time
SB1	0.25	3.93 h (236 min.)
BC1	0.37	2.7 h (162 min.)

Cellular viability in presence of 50% chloroform:

Viability assays were done to monitor the survival of the resting cells in presence of 50% (v/v) chloroform. It was found that the decline in cell number in case of SB1 was from 10^7 to 10^5, whereas in case of BC1 , it was from 10^6 to 10^5 within 4h of incubation. The first step of the conversion occurs within 3 h and the cell number is substantially high during that time. A large number of viable cells are found to be present even on 24 h incubation, during which the second intermediate appears, indicating chloroform tolerance of the cultures [**table 4B.2**].

The bio-transformation of cholesterol by SB1 and BC1 in the chosen biphasic system:

The bio-transformation of cholesterol by SB1 and BC1 in the chosen biphasic system was monitored by thin layer chromatography using the solvent systems hexane-diethyl ether (3:10), chloroform –acetone (9:1), ethyl acetate –petroleum ether (2:8). The samples used for TLC were aliquots of the chloroform layer. The developing agents used were ferric chloride solution (blue spots), iodine vapours (brown spots), vanillin solution prepared in phosphoric acid (intense purple spots with sterolds), sulphuric acid spray in ethanol (pink spots). Of these reagents,

Table 4B.2: Viability assay of SB1 and BC1 resting cells in presence of 50% (v/v) chloroform

Time of exposure to chloroform	*SB1*	*BC1*
0 min.	2.4×10^{7}	7.3×10^{6}
10 min.	3.2×10^{6}	4×10^{6}
4h	2.1×10^{5}	1×10^{5}
5h	7.5×10^{4}	1×10^{4}
24h	1.2×10^{4}	4.8×10^{3}

vanillin-phosphoric acid (VP) spray was found to be most specific as it gave a reaction only with steroids [414]

TLCs were performed in all the three solvent systems at every 30 minute interval for 24 h. Both BC1 and SB1 being *Bacillus* species show the same pattern of transformation as outlined in **table 4B.3 and fig.4B.3.**

The more polar steroids are known to bind more tightly to the silica gel layer and hence have a slower rate of migration (lower Rf values) as compared to the less polar steroids. It is observed that a transformed product is formed which appears as a spot below cholesterol (more polar intermediate-p1) in the chloroform-acetone solvent system in the early stages of transformation (30min-5h). Further, this appears to be a transient intermediate, which is not detected if the flask is incubated overnight. However, after overnight incubation (even 7-10 h), there is the appearance of a clear spot above cholesterol (less polar product- p2), which persists in the system even when incubated for over a week. The TLC pattern suggests that p1 is probably a hydroxylated derivative of cholesterol . greater polarity. On account of the transient nature of the compound, sufficient quantity could not be extracted for analysis. The same spot was also obtained from growing cells of BC1 after several days incubation and growth in mineral medium with cholesterol as the carbon source. However, the rate of conversion is much faster with resting cells in presence of chloroform.

Table 4B.3: Bio-transformation of cholesterol by resting ells of SB1 and BC1 in presence of 50% V/V chloroform

Product	Rf of chol	Rf of prod	Time of appearance
P1	0.71[ca]	0.55[ca]	30 min-3h
	0.62 [hd]	0.31 [hd]	
P2	0.48 [pe]	0.58 [pe]	6-24h, persists
	0.62 [hd]	0.70 [hd]	
	0.71 [ca]	0.87 [ca]	

The brackets represent the solvent systen used for TLC.

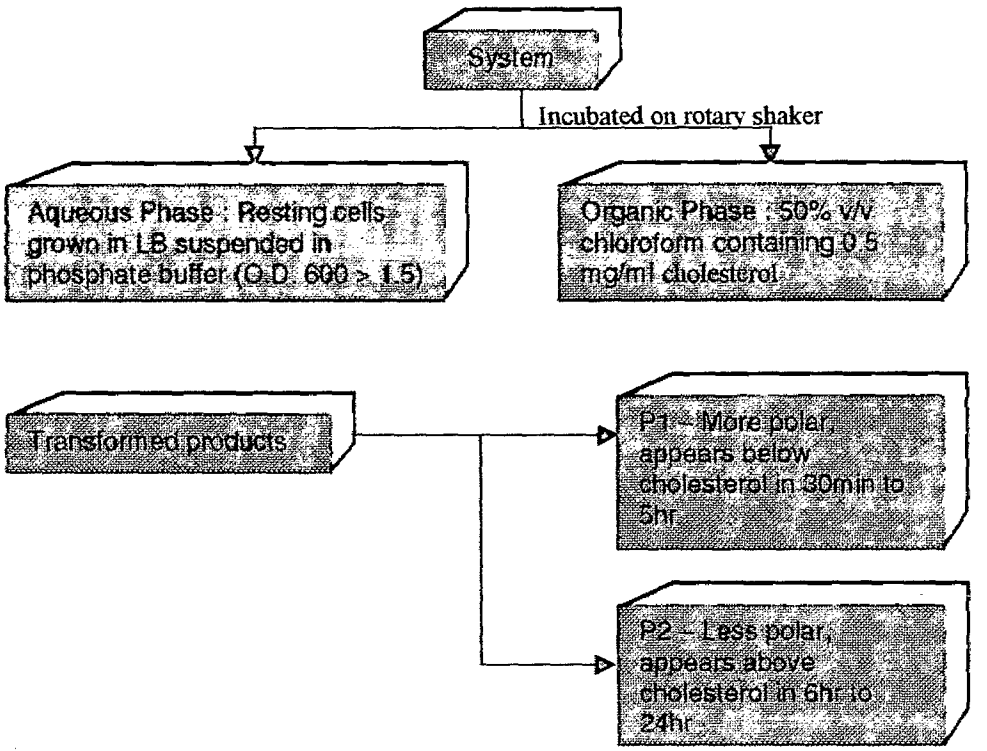
[ca]= chloroform-acetone (9:1)

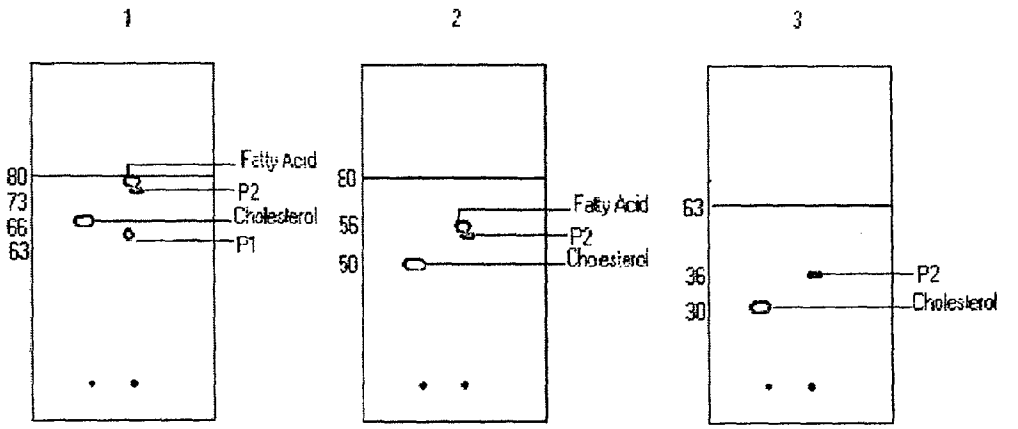
[pe]= Petroleum ether-ethyl acetate (8:2)

[hd]= n-hexane –diethyl ether (3:10)

Fig 4B.3: Bio-transformation of cholesterol by SB1 & BC1.

SB1 & BC1 both being *Bacillus* strains follow the same bio-transformation pattern.

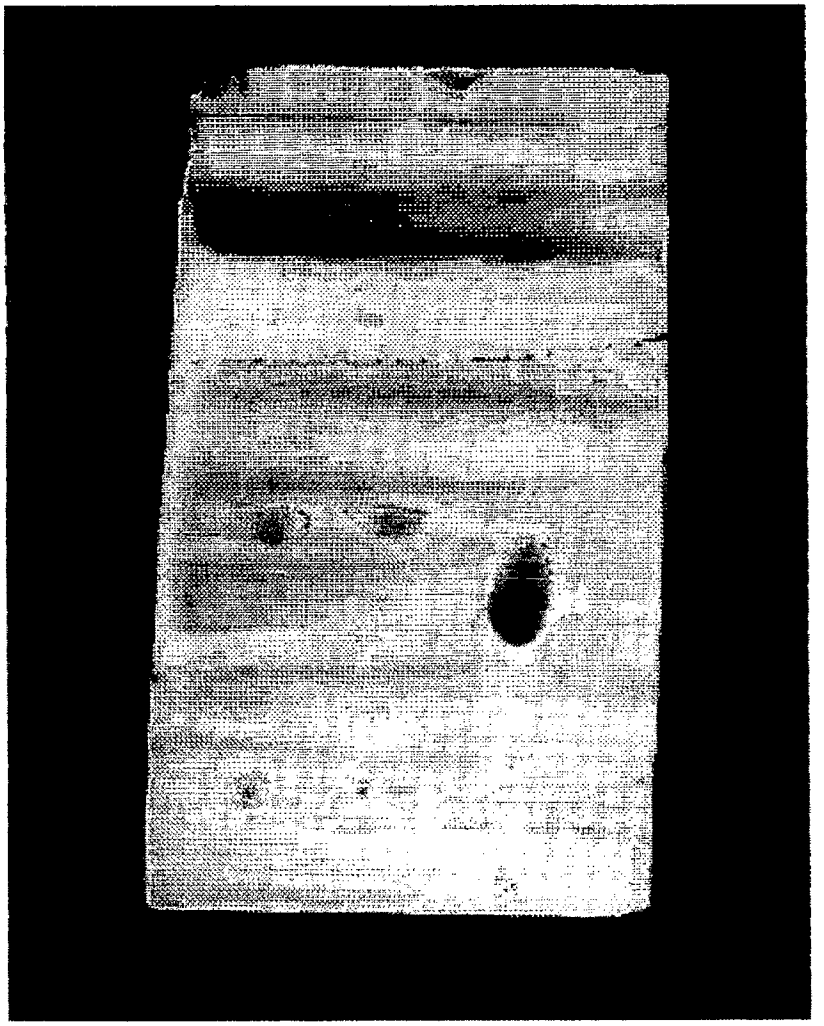




1 – Chloroform-Acetone (9:1)
2 – Hexane-Diethyl-Ether (3:10)
3 – Ethyl-Acetate-Petroleum- Ether (2:8)

Fig 4B.2: TLC Plates showing cholesterol transformatlons

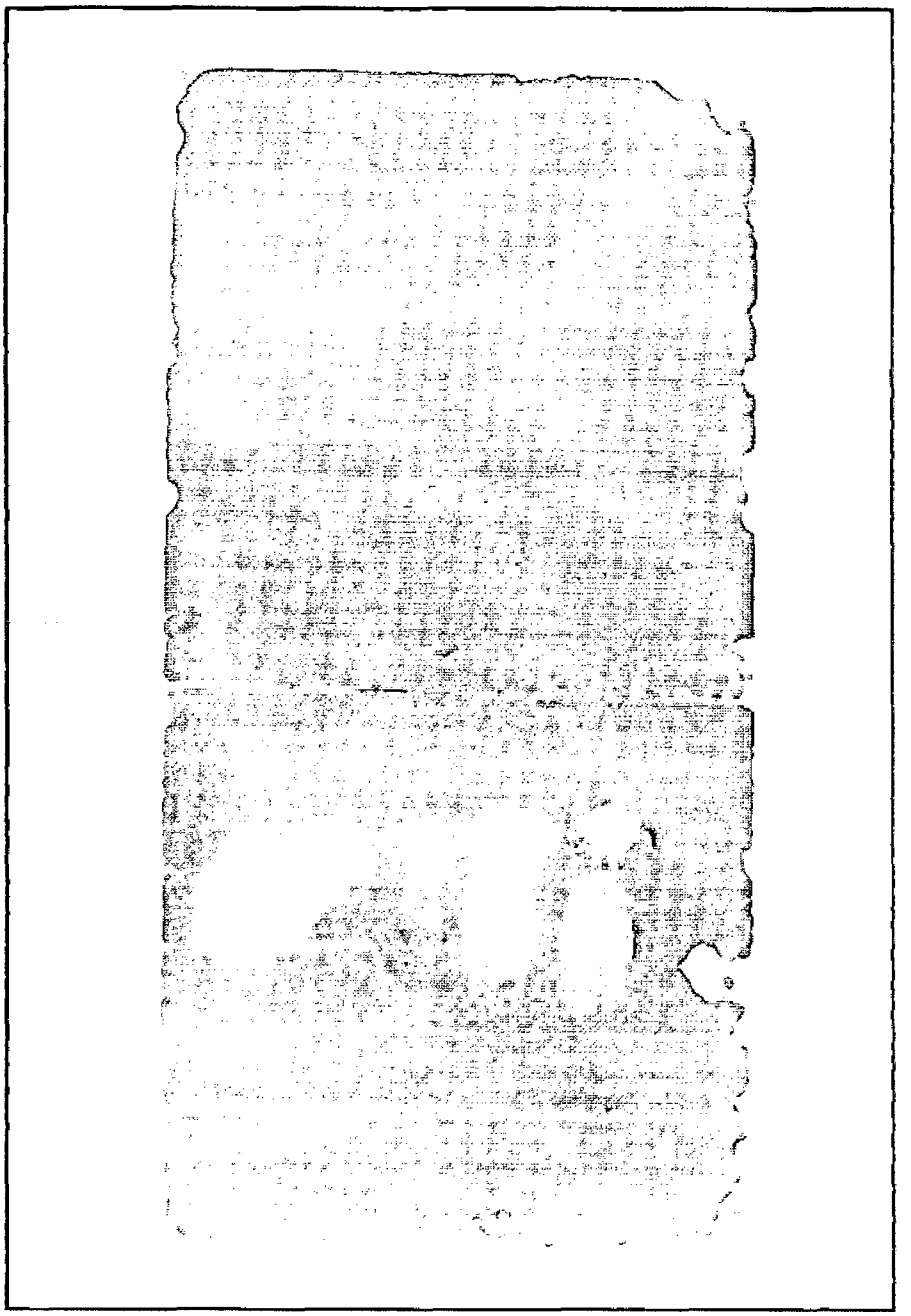
(A) In Hexane-diethyl-ether (3:10)



L to R: spots 1 & 2 show transformed product P2. Spot 3 shows cholesterol.

Developing Agent used was Iodine

(B) In chloroform acetone system (9:1)

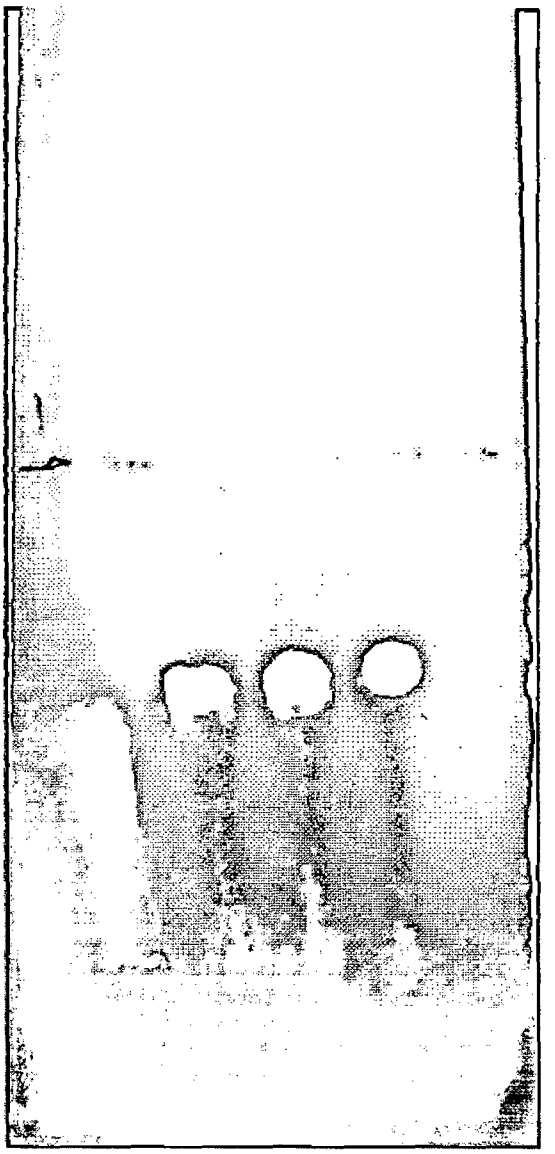


L to R: Spot 1 – cholesterol
Spot 2 – SB1 (30 minutes)
Spot 3 – BC1 (30 minutes)
Spot 4 – SB1 (18 hours)
Spot 5 – BC1 (18 hours)

The upper spots indicate residual cholesterol, the lower spots indicate transformed product P1 which is seen in spots 3,4,5

Developing Agent used was Vanillin in Phosphoric Acid

(C) In Ethyl Acetate Petroleum Ether (2:8)



L to R : Spot 1 – Cholesterol
Spots 2,3,4 – Transformed product P2

Developing Agent used was VanillIn in Phosphoric Acid

(D) In chloroform-acetone (9:1)

L to R : Spot 1 – Cholesterol
Spots 2 & 3 – Transformed product P2

Developing Agent used was VanillIn in Phosphoric Acid

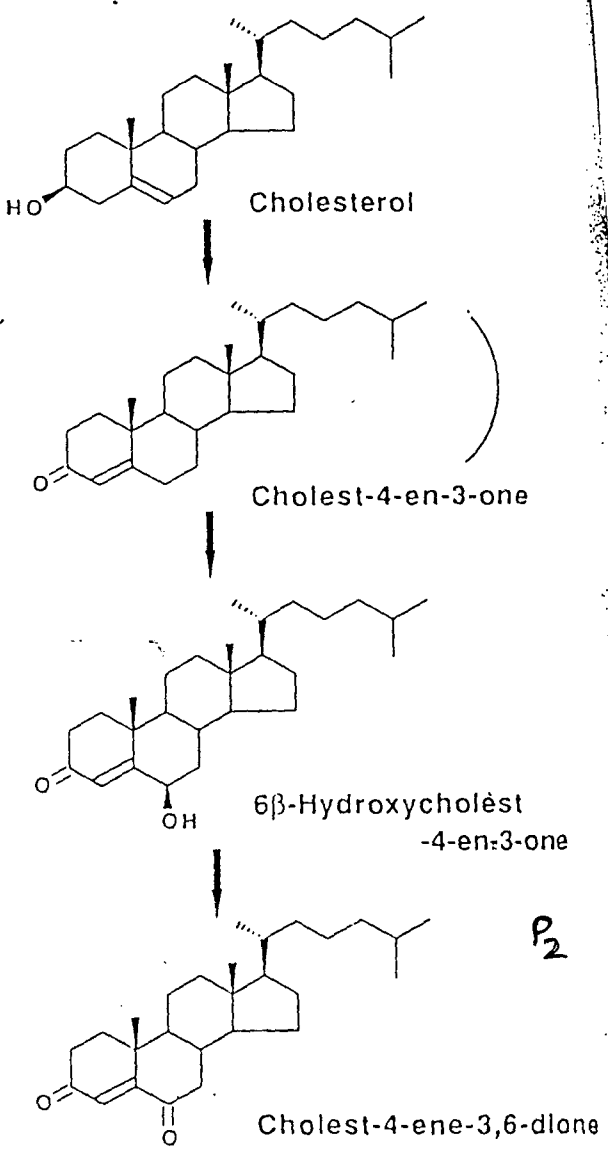
The second product, p2, was detected in 3 different solvent systems. It was purified by preparative chromatography and extracted for analysis. One problem encountered during purification is the presence of interfering cellular origin compounds, probably free fatty acids which have an Rf value similar to p2 and are found just above the p2 spot. Both the impurity as well as p2 are iodine reactive. However, only p2 gives the typical violet-grey colour with the vanillin –phosphoric acid spray, which was used to discriminate between the two. The compound p2 was also detected in growing cells of BC1 and SB1 after several days incubation.

Analysis of the product P2:

Proton NMR of the partially purified product confirms cholesterol transformation. There is splitting of the peaks corresponding to the C18,C19, C21, C25 and C26, which are seen in the range of 0.2 to 1.2 ppm. Also, additional signals are seen in the region between 2.5 to 5.5 ppm, in addition to the signals at 3.5 and 5.5 ppm exhibited by cholesterol (**fig 4B.4).**

u.v scans show the absorbance maxima of this product to be 255 nm, which clearly indicates the presence of conjugated double bonds which are absent in cholesterol (u.v abs= 206nm) **(fig 4B.5).** Based on the Rf values, u.v spectrum and reported data, it is concluded that the product is a ketonic derivative of cholesterol.

Putative Scheme for Cholesterol Transformation by Bacillus SB1 & BC1

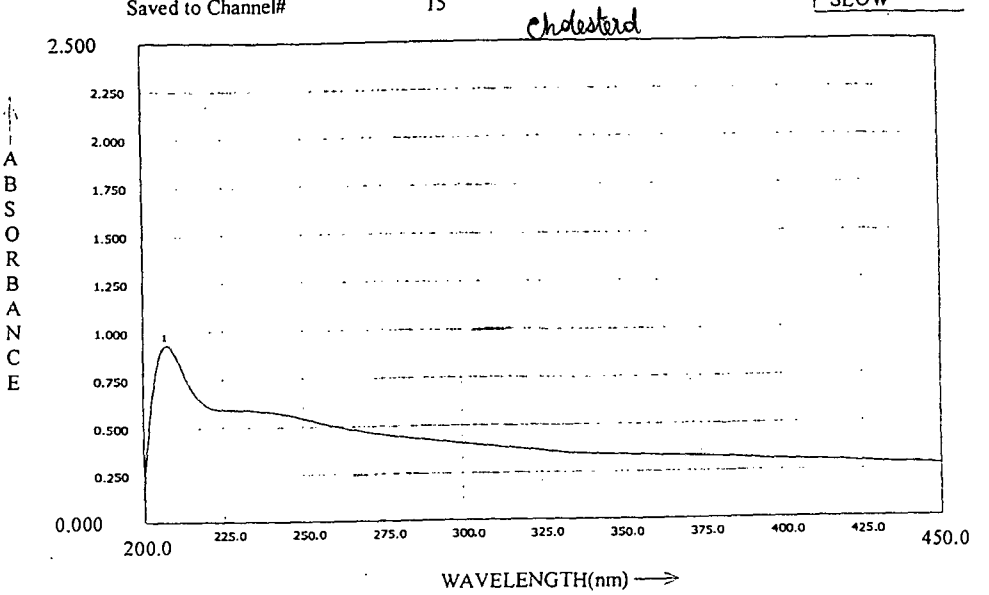


P2

UV 2600 DOUBLE BEAM UV-VIS SPECTROPHOTOMETER Ver5.22 Date : Wed 24-10-2001
Time : 04 : 28 : 18 PM

Spectrum Of cholesterol
Analysed By yns
Dated 24-10-2001
Saved to Channel# 15

SLOW



UV 2600 SPECTRUM PEAKS AND VALLEYS

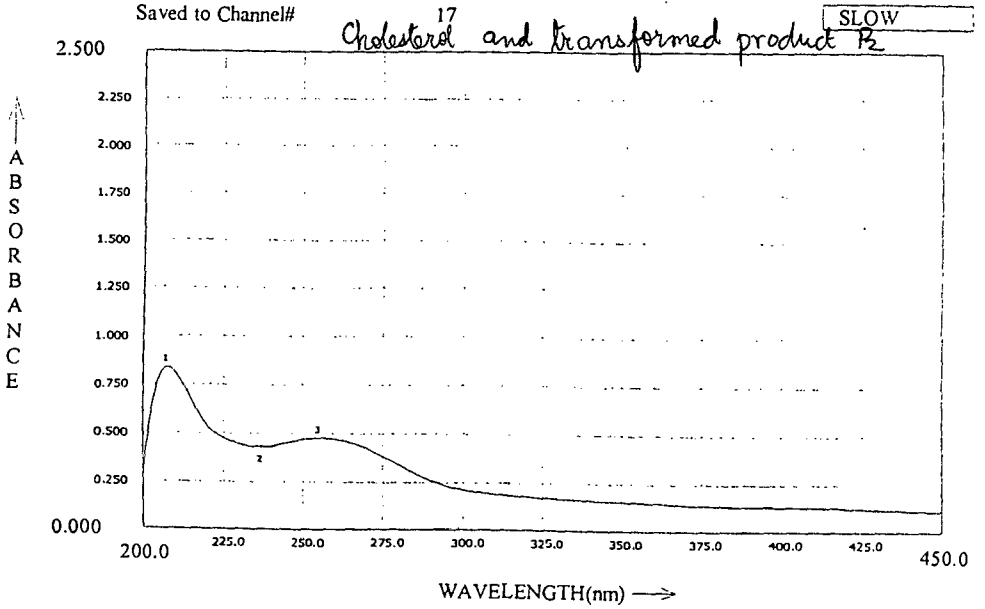
	-- PEAKS --			-- VALLEYS --	
Slno	Wv(nm)	ABS	Slno	Wv(nm)	ABS
1	207.0	0.931			

Fig 4B-5: U.V Spectra of cholesterol and transformed product P_2

UV 2600 DOUBLE BEAM UV-VIS SPECTROPHOTOMETER Ver5.22 Date : Wed 24-10-2001
Time : 04 : 33 : 21 PM

Spectrum Of cholesterol
Analysed By yns
Dated 24-10-2001
Saved to Channel# 17

SLOW



UV 2600 SPECTRUM PEAKS AND VALLEYS

	-- PEAKS --			-- VALLEYS --	
Slno	Wv(nm)	ABS	Slno	Wv(nm)	ABS
1	207.0 (chol)	0.836	2	236.5	0.430
3	255.0 (P_2)	0.476			

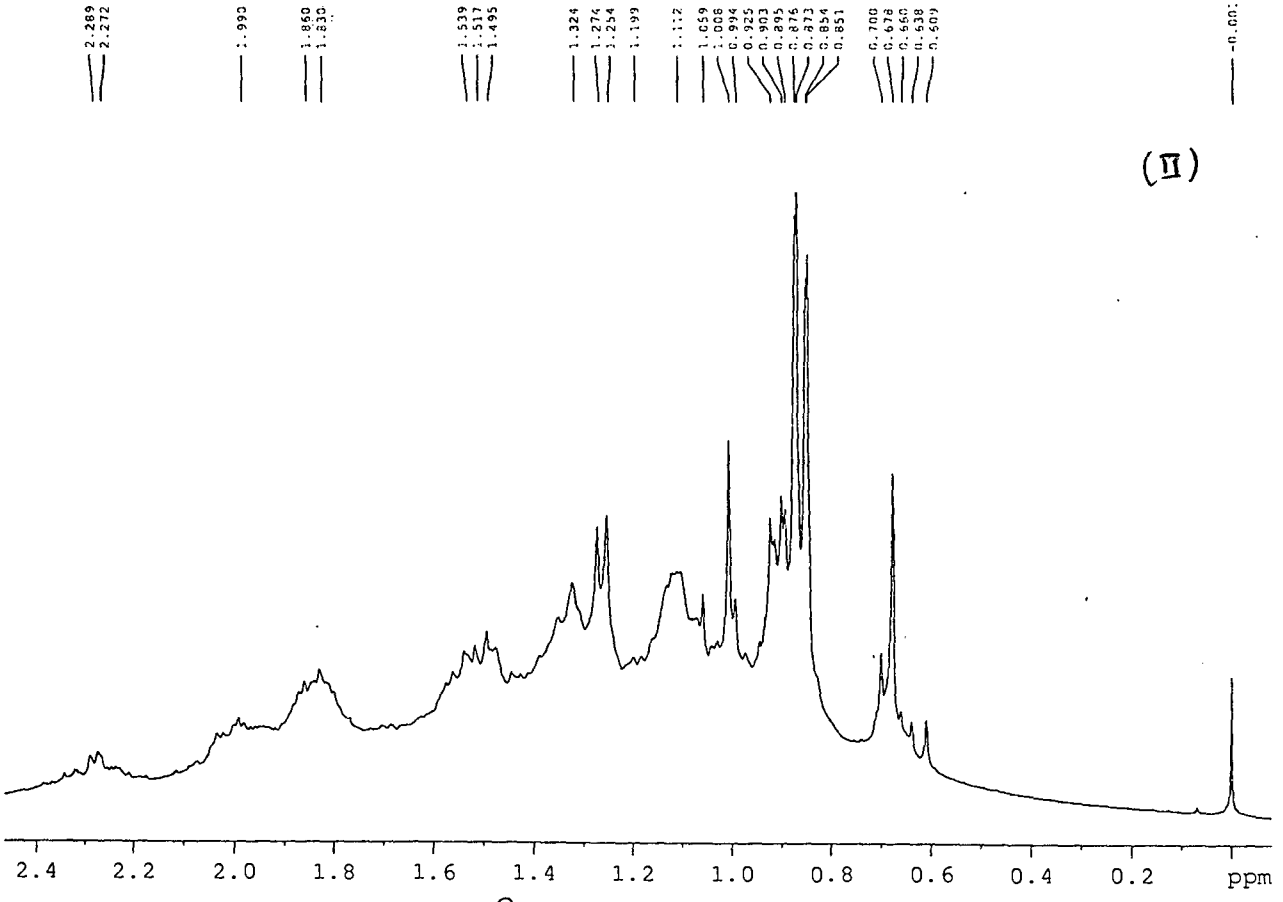
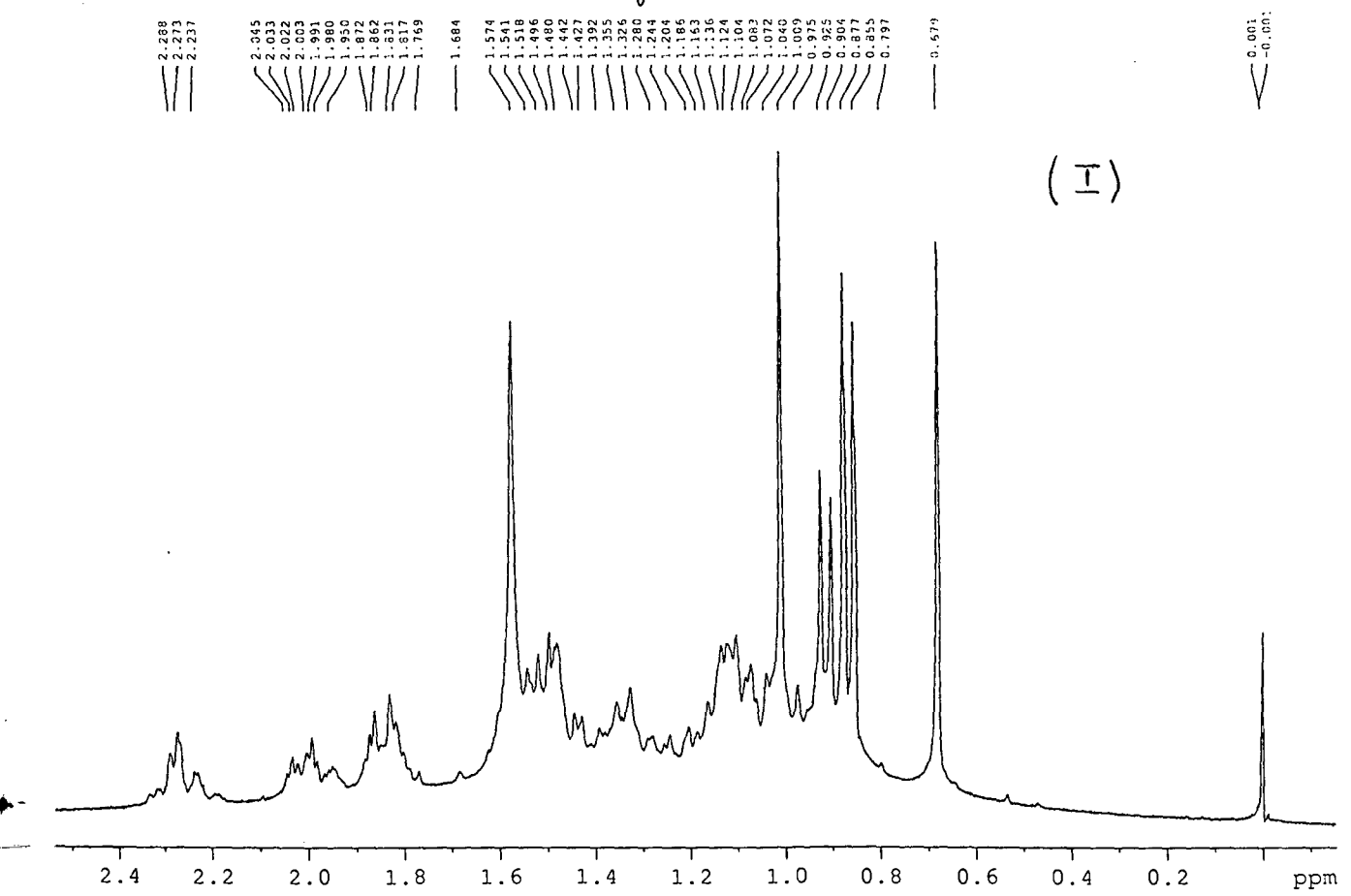
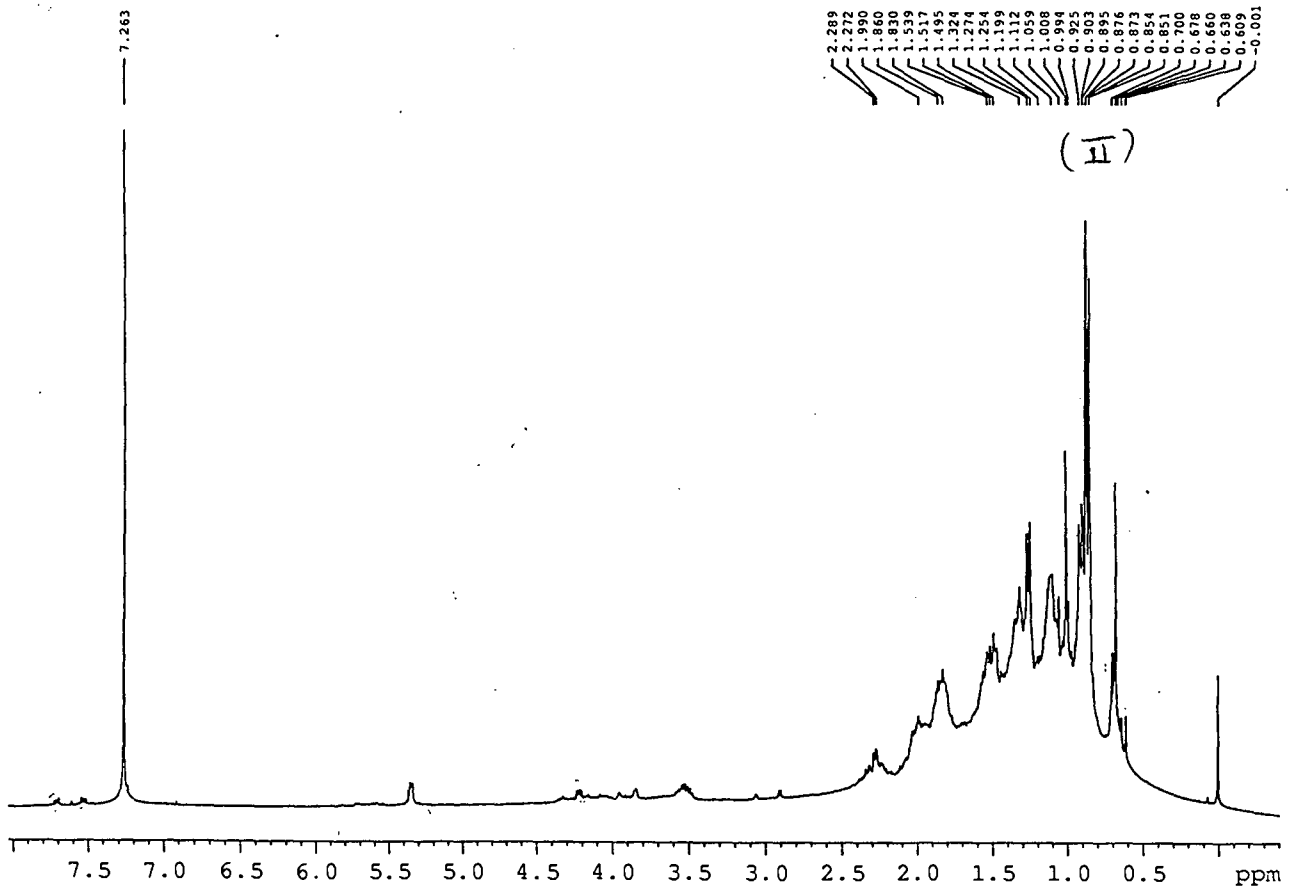
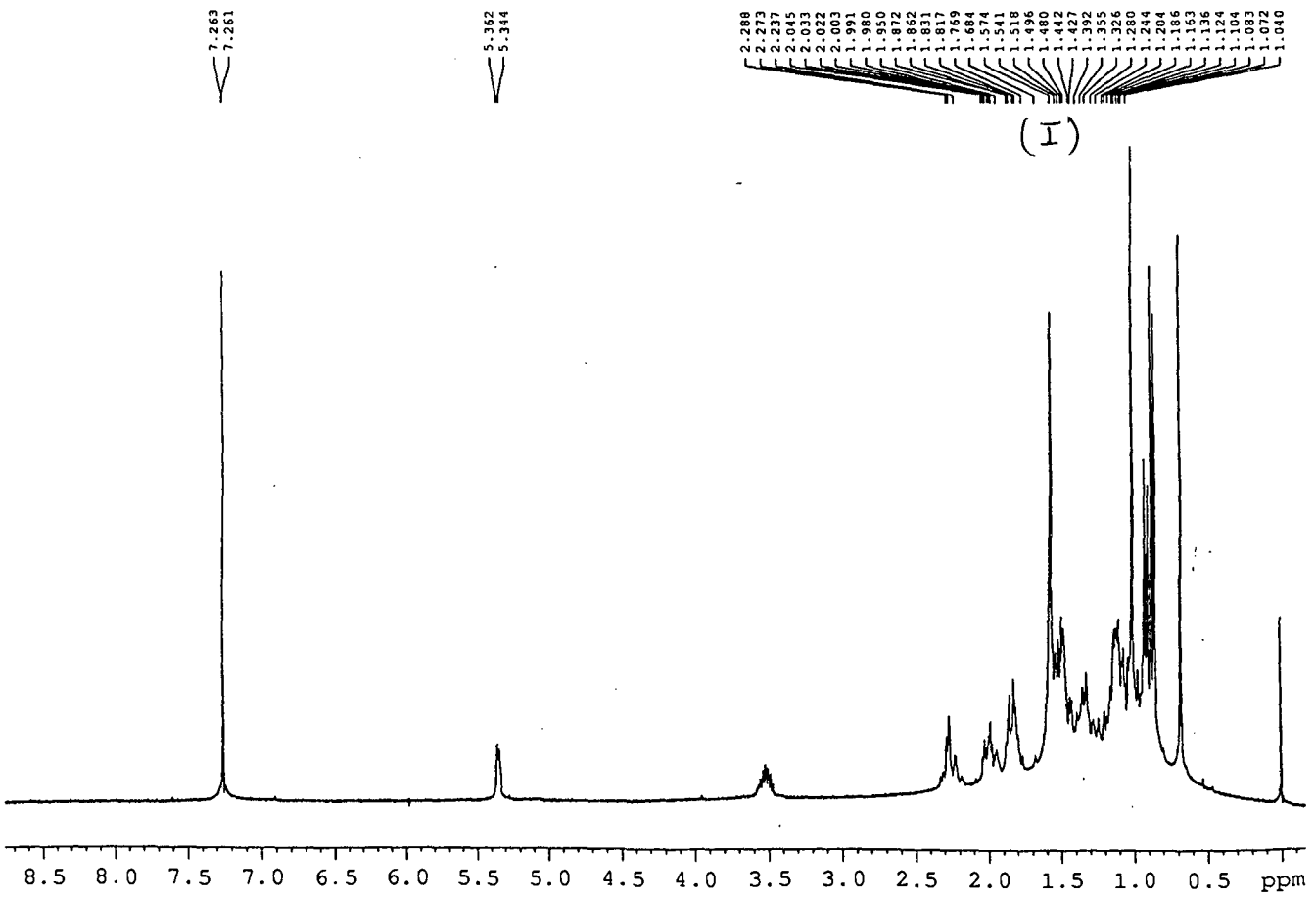


Fig. 4B·4: Proton NMR Spectra of Cholesterol (I) and transformed product P_2 (II)




7.263
2.289
2.272
1.990
1.860
1.830
1.539
1.517
1.495
1.324
1.274
1.254
1.199
1.112
1.059
1.008
0.994
0.925
0.903
0.895
0.876
0.873
0.854
0.851
0.700
0.678
0.660
0.638
0.609
-0.001
(II)
7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 ppm


7.263
7.261
5.362
5.344
2.288
2.273
2.237
2.045
2.033
2.022
2.003
1.991
1.980
1.950
1.872
1.862
1.831
1.817
1.769
1.684
1.574
1.541
1.518
1.496
1.480
1.442
1.427
1.392
1.355
1.326
1.280
1.244
1.204
1.186
1.163
1.136
1.124
1.104
1.083
1.072
1.040
(I)
8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 ppm

The Rf values and u.v absorbance of this compound are similar to the compound **cholest-4-ene-3,6 dione** [4].
However, the transformation is not 100% and residual cholesterol exists in flasks of both SB1 and BC1.

In the recent past, an organic solvent tolerant cholesterol oxidase has been isolated, purified and cloned which is active in presence of benzene, toluene, p-xylene, propylbenzene and diphenylmethane. The cholesterol consumption rate was found to be 3 to 3.5 fold higher than that in absence of cholesterol. The culture, initially known as *Pseudomonas* ST-200 has now been identified as *Burkholderia cepacia*. However, even this enzyme is inactive in presence of chloroform [14, 103,104]. Ogino et al have reported organic solvent stable protease and lipase and stated that it is likely that the extracellular enzymes of OSTB would be active in presence of organic solvents [296,297,298].

This work also conclusively proved that organic solvent tolerant bacteria with the required enzymes can be effectively used to transform water-insoluble substrates in a biphasic system comprising organic solvent as one of the phases. Both SB1 and BC1 are unique cultures in terms of their chloroform tolerance which is a novel trait.
Degradation of steroids requires multi-sub-unit enzymes. Cholesterol, being a C-27 steroid is a difficult molecule to degrade even under routine conditions. In the past, such conversions have

been done with immobilised cells in presence of less toxic organic solvents in order to protect the cells.

The fact that free cells of *Bacillus* SB1 and BC1 not only survive and retain viability in presence of 50% v/v chloroform but also effectively convert the complex substrate is indicative of their extraordinary solvent tolerance and efficiency of enzyme function under such stressful conditions.

Summary

The production of bio-active metabolites by micro-organisms, particularly those of marine origin has gained importance in recent times, on account of their novelty of structure and activity. The ability of microbes to transform complex organic compounds to generate bio-active metabolites is also well-known.

This work is focussed on 2 aspects:

1) *Isolation and study of L-asparaginase (anti-leukemia enzyme) producing bacteria*
2) *Isolation and study of organic solvent tolerant cholesterol transforming bacteria capable of bio-transformation in an organic-aqueous biphasic system*

A total of 23 bacterial cultures were isolated from coastal and estuarine samples collected from the Arabian sea and the Mandovi estuary respectively, 11 of which produced L-asparaginase and 12 of which were organic solvent tolerant cholesterol transforming bacteria.

5 cultures were identified.

The L-asparaginase producing bacteria were isolated by Gulati`s semi-quantitative plate assay and all cultures appeared to produce L-

asparaginase which was active at pH7 and 37 degrees C. However, only a few cultures showed activity at low asparagine concentrations (high affinity for asparagine), which is an essential characteristic for clinical utility. Two cultures A1 and F2 were found to be *Bacillus* strains, whereas A2 was identified as *Alcaligenes* sp.

The L-asparaginase from *Bacillus* A1 was selected for further studies, and appears to be a good candidate for potential clinical use.

BC1 and SB1, cholesterol transforming bacteria tolerant to chloroform and butanol respectively were isolated by enrichment in presence of the respective solvent and identified as *Bacillus* strains. Both the cultures were novel with respect to their tolerance since there are no reports of cultures which can withstand butanol and chloroform, solvents which are extremely toxic to bacterial cells.

The n-butanol tolerance of SB1 was studied in detail. Involvement of spores in butanol and influence of various factors on tolerance to butanol was investigated. Increase in butanol concentration adversely affected both growth rate and yield of SB1, however viable cells were isolated from flasks containing even 99% v/v butanol which indicated the high level of tolerance.

Tolerance of SB1 and BC1 to a wide range of organic solvents was confirmed performing organic solvent tolerance assays.

Since these bacteria utilise cholesterol in addition to tolerating solvents, it was expected that their enzymes would also withstand solvent exposure and function in presence of solvents.
A biphasic bio-transformation system was devised in which cholesterol was dissolved in 50% v/v chloroform and cells were suspended in phosphate buffer. Both the cultures were found to effectively transform cholesterol to cholest-4-ene-3,6-dione, as confirmed by TLC data as well as UV and NMR spectra.

This study demonstrates the tremendous significance of marine /estuarine bacteria in generating compounds with potential bio-activity.

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Appendices

Appendix 1: Media

1) **Luria broth / agar** : tryptone 1%, yeast extract 0.5%, NaCl 1%.

2) **LBMG broth/agar**: To the above Luria medium, add 10mM magnesium sulphate and 0.1 % glucose. [219]

3) **Modified M 9 medium** [144](used for determination of asparaginase activity in Gulati's assay):g/l – Na2HPO4.2H20 –6g, KH2PO4-3g, NaCl-0.5g, 1molar MgSO4- 2ml, 0.1 molar CaCl2-1ml. Phenol red was added from a 2.5% ethanolic stock solution to get a concentration of 0.009 %w/v. Asparagine concentration was maintained at 0.5% and added separately after sterilisation.

4) **Mineral Salts Medium**: 60 mg of ferrous sulfate was dissolved in 250 ml distilled water.Stock solutions were added as follows: K2HPO4 (12.6%)- 50 ml, KH2PO4 (18.2%)- 10 ml, NH4NO3 (10%)- 10 ml, MgSO4 (1%)- 10 ml

5) **Artificial sea-water (half-strength):** LBMG broth and modified M9 medium used in initial stages of isolation were prepared in half-strength artificial sea-water. g/l- NaCl –15g , MgSO4 - 1.5g , MgCl2 -1.2g, KCl-0.38 g, CaCl2- 0.38g.

6) **Saline**- 0.9 % NaCl in distilled water

7) All media / reagents used in identification of bacteria were prepared as per Bergey's Manual of Systematic Bacteriology.

***All the media described above were prepared in distilled water. The pH was adjusted to 7.4 prior to sterilisation. To prepare solid media for plates, 2% agar was added and digested by boiling prior to sterilisation. Sterilisation was done by autoclaving (121 degrees C / 15p.s.i./ 20 min.)

Appendix 2: Buffers and reagents used in L-asparaginase assay [378]

1) **Acetate buffer** pH 5.6 (0.2 M/l)
Place 7 ml of 0.2 M acetic acid in a 100 ml flask. Make up the volume to 100 ml with 0.2 M sodium acetate.

2) **Carbonate buffer** (bicarbonate buffer) (0.1 M)
Place x ml of 0. 1M sodium bicarbonate into a 100 ml volumetric flask and make up the volume to 100 ml with o.1M sodium carbonate.
For pH 9, x=93 ml
For pH 10, x=49 ml

3) **Sodium phosphate buffer** (0.1M)
Add x ml of 0.2 M sodium hydroxide to 50 ml of 0.02 M NaH2PO4and dilute to 100 ml.
For pH 7, x=30 ml
For pH 7.4 , x=40 ml

4) **Nessler`s reagent:** Dissolve 100 g of mercuric iodide and 70 g KI in a small quantity of distilled water. Add the mixture slowly with stirring to a cool solution of 160 g NaOH dissolved in 500 ml distilled water. Dilute to 1 litre and store in a brown bottle.

5) **Trichloroacetic acid** (TCA) (m.w=163.69) To prepare 15% TCA solution used in Wriston`s assay, dissolve 15 grams of TCA in 100 ml of water .

6) **0.04M asparagine solution**: (m.w.=150.14) To prepare 0.04 M asparaginase solution for Wriston`s assay, dissolve 0.6 grams of L-asparagine in 100 ml of pH 7 phosphate buffer. Store in the refrigerator.

7) **0.04M glutamine solution**: (m.w.=146.15) To prepare 0.04 M L-glutamine solution for Wriston`s assay, dissolve 0.584 grams of L-glutamine in 100 ml of pH 7 phosphate buffer. Store in the refrigerator.

Appendix 3: Staining procedures and reagents [129,79]

1) **Gram Stain**: Prepare a smear. Heat-fix. Stain with crystal violet for 30 seconds. Cover the film with Gram`s iodine for 30 seconds. Rinse with water and then decolorise with 95 % ethanol for 30 seconds. Rinse with water. Counter stain with safranine for 30 seconds. Rinse and dry. Examine the slide under the oil immersion objective.

Gram-ve bacteria stain pink and gram+ bacteria stain violet.

Crystal violet:
2g crystal violet, 20 ml ethanol, 0.8 g ammonium oxalate, 80 distilled water. Dissolve crystal violet in alcohol and ammonium oxalate in water . Allow the oxalate solution to stand overnight or heat gently till until in solution. Mix the 2 solutions together and filter.

Gram`s iodine:
1g iodine crystals, 2g KI, 300 ml water, 3g Na2HCO3.

Safranine:
2.5 g safranine, 100 ml ethanol, 900 ml distilled water.

2) **Endospore staining** (Schaeffer & Fulton`s Method): Prepare a smear. Heat-fix. Place slides on a staining rack above boiling water. Cover with small pieces of paper towels. Keep saturated with malachite green (5% aqueous). Continue heating for 5 minutes. Wash with water. Counter stain with safranine for 30 seconds. Wash, dry and observe under oil immersion objective. Endospores stain green and cells stain pink.

3) **Demonstration of capsules by Negative Staining**:
Take a drop of Nigrosine 1% aqueous) on one end of the slide. Mix bacterial culture suspension in the drop. Using another slide, spread the drop acroo to prepare a film. Air dry. Counter stain with 0.5% crystal violet for I minute. Rinse with water , air-dry and observe under oil-immersion objective. Capsules appear as colourless structures on a blue-black background.

Appendix 4: Standard Curves: Colorimetric methods of estimation for 1) protein, 2) ammonia, 3) cholesterol

1) Colorimetric estimation of protein by Folin-Lowry`s method [316]

1. Reagent A: Alkaline Na2CO3 : 20 g/l Na2CO3 in 0.1M NaOH.
2. Reagent B: CuSO4- Na, K tartarate solution (5 g/l CuSO4 in 10 g/l Na, K tartarate
3. Alkaline reagent : 50 ml of reagent A and 1 ml of reagent B.
4. Folinciocalteau (FC) reagent: dilute commercial reagent with 2 parts water before use.
5. Standard protein (albumin solution) :1000 micrograms/ml.

Procedure: Mix 5 ml of alkaline solution with 1 ml of the test solution. Mix and keep for 10 minutes. Add 0.5 ml of FC reagent and shake. Incubate tubes in the dark for 30 minutes. The optical density is determined at 670 nm.

Table: Standard graph for protein by Folin-Lowry`s method

Protein concentration in micrograms/ml	Volume of stock	Volume of water	O.D 670 nm
	100 microgram stock		
10	0.1	0.9	0.05
40	0.4	0.6	0.05
50	0.5	0.5	0.09
60	0.6	0.4	0.10
70	0.7	0.3	0.12
	1000 microgram stock		
100	0.1	0.9	0.26
200	0.2	0.8	0.42
300	0.3	0.7	0.59
400	0.4	0.6	0.80
500	0.5	0.5	0.99
700	0.7	0.3	1.20
800	0.8	0.2	1.34
900	0.9	0.1	1.44
1000	1.0	-	1.58

2)Colorimetric estimation of ammonia by Nessler`s method: [378]

(Using ammonium sulphate) Dissolve 1.179 of ammonium sulphate to get a final volume of 100 ml. Dilute 1.4 ml of this solution to 100 ml to give 1 micromole of ammonia per ml.

Using this 1 micromolar stock, prepare ammonia solutions of various concentrations starting with 0.01 micromoles. Distilled water is added as diluent. The total volume in each tube is made 7.5 ml. 1ml of Nessler`s reagent is added to each tube. Optical density is determined at 470 nm using an Elico-CL 157 colorimeter.

Table: Standard graph for ammonia by Nessler`s method

Concentration of ammonia in micromoles	Amount of stock solution	Volume of water	O.D. 470 nm
0.01	0.075	7.425	0.04
0.04	0.3	7.20	0.07
0.05	0.375	7.125	0.08
0.08	0.60	6.9	0.11
0.10	0.75	6.75	0.14
0.14	1.05	6.45	0.18
0.18	1.35	6.15	0.22
0.20	1.5	6.0	0.25
0.22	1.65	5.85	0.30
0.26	1.95	5.55	0.35
0.32	2.4	5.1	0.42
0.40	3.0	4.5	0.50
0.50	3.75	3.75	0.58
0.58	4.35	3.15	0.70
0.80	6.00	1.5	0.78
0.90	6.75	0.75	0.86
1.0	7.5	0.0	0.88

3) Colorimetric estimation of cholesterol by Lieberman Burchard `s reaction [377]:

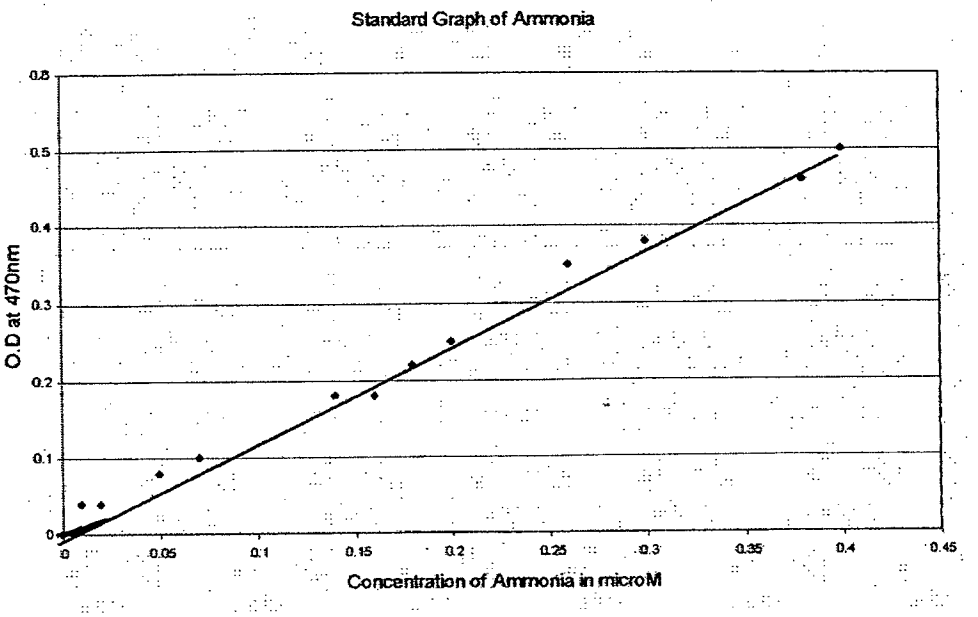
The principle of this method is that acetic anhydride reacts with cholesterol in a chloroform solution to produce a characteristic blue-green colour . The exact nature of the chromophore is not known but the reaction probably includes esterification of the hydroxyl group in the 3 position as well as other rearrangements in the molecule. This method is routinely used to monitor cholesterol in blood and serum.

Requirements: Totally dry glassware, Chloroform, Acetic anhydride – sulphuric acid mixture (30:1, mix just before use very carefully)and stock cholesterol solution (1 mg/ml dissolved in chloroform) to prepare the standard curve.

Method: 1 ml of the cholesterol sample to be estimated present in the chloroform layer is taken in the a clean dry tube , to which1 ml of chloroform is added. Thus the total volume in each tube is 2 ml. A standard curve is prepared using a 1 mg/ml cholesterol standard solution..A blank tube with 2 ml of chloroform only is also maintained. To all the tubes , 2 ml of acetic anhydride –sulfuric acid mixture is added and the tubes are thoroughly mixed. The tubes are left in the dark at room temperature for 20 minutes and the optical density measured at 670 nm using an Elico CL-157colorimete

Table: Standard graph for cholesterol by Lieberman Burchard`s Method:

Cholesterol concentration (mg/ml)	**Volume of 1 mg/ml stock (ml)**	**Volume of diluent chloroform (ml)**	**Total volume (ml)**	**Optical density (670nm)**
0.02	0.04	1.96	2	0.06
0.04	0.08	1.92	2	0.07
0.06	0.12	1.88	2	0.08
0.08	0.16	1.84	2	0.11
0.10	0.20	1.80	2	0.14
0.12	0.24	1.76	2	0.19
0.14	0.28	1.72	2	0.26
0.16	0.32	1.68	2	0.26
0.18	0.36	1.64	2	0.28
0.20	0.40	1.60	2	0.42
0.40	0.80	1.20	2	0.73
0.60	1.20	0.80	2	0.94
0.80	1.60	0.40	2	1.06
1.0	2.0	0.00	2	1.18
	Volume of 2 mg/ml stock (ml)			
1.2	1.2	0.8	2	1.36
1.4	1.4	0.6	2	1.33
1.6	1.6	0.4	2	1.50
1.8	1.8	0.2	2	1.48
Blank	-	2	2	Set to 0


Standard Graph of Ammonia
O.D at 470nm
0.6
0.5
0.4
0.3
0.2
0.1
0
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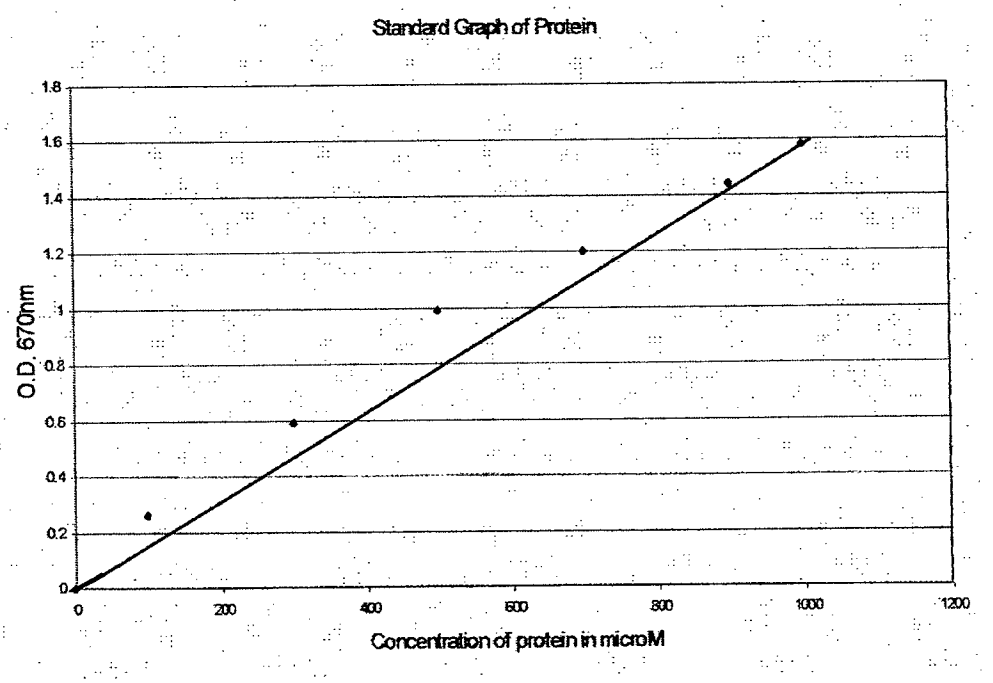
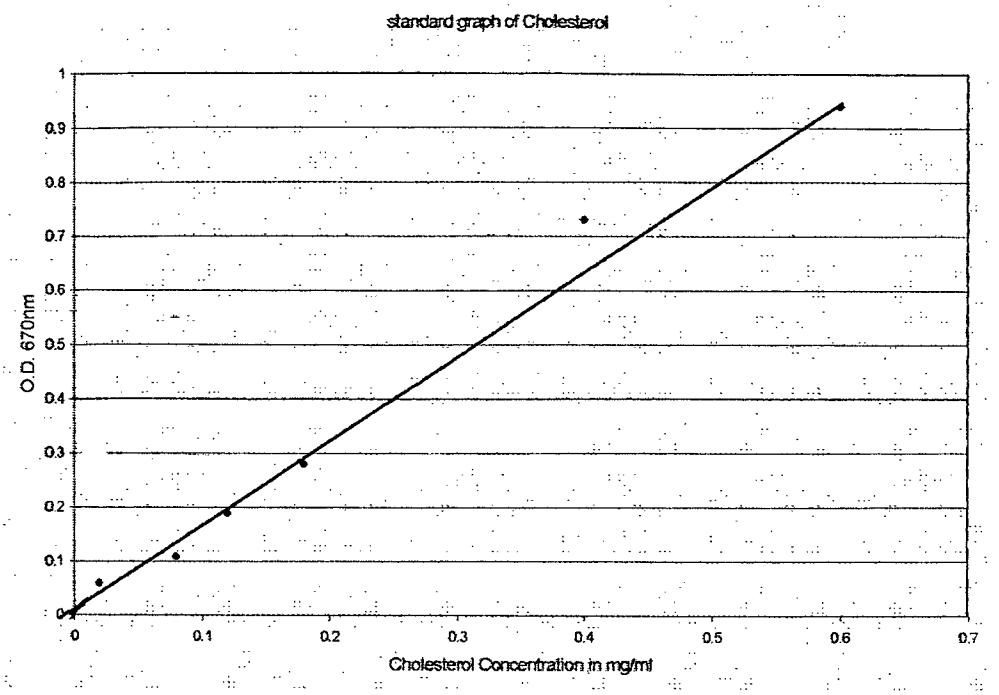
0.05
0.1
0.15
0.2
0.25
0.3
0.35
0.4
0.45
Concentration of Ammonia in microM


Standard Graph of Protein
O.D. 670nm
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
200
400
600
800
1000
1200
Concentration of protein in microM


standard graph of Cholesterol
O.D. 670nm
Cholesterol Concentration in mg/ml
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1

Appendix 5 : Sample calculations

1) Calculations to determine L-asparaginase activity (Wristons` assay)

The optical density (470 nm) is 0.07. The corresponding concentration of ammonia by the standard curve prepared using Nessler`s reagent is 0.04 micromoles.

Only half of the initial reaction mixture is taken and this is diluted 5 times with water. Hence this reading is multiplied by 2 and 5 ie.(X10).

0.04 X 10 =0.40 micromoles released in 30 minutes

Hence, 0.40/30 micromoles released per minute =0.013

0.013 micromoles of ammonia are released from 150 micrograms of protein (determined by Lowry`s method)

Hence, 0.088 micromoles of ammonia would be released from 1000micrograms (1mg) protein.

Hence, the enzyme activity is expressed as 0.088 iu / mg protein.

One iu (international unit) is the number of micromoles of ammonia released per min under standard assay conditions.

2) Calculations to determine the generation time of a given culture:

Generation time- G, No –initial number of cells, Nt- number of cells after a certain time interval t.

No and Nt are determined by finding the viable count

The formula used is:

$G = t / 3.3(\log 10\ Nt - \text{Log } 10\ No)$

After 20 h in cholesterol medium, the viable count of SB1 increases from 9×10^5 to 3.12×10^7.

The log 10 values of these counts are 5.95 and 7.49 respectively.

G=20/3.3 (7.49-5.95)

=3.93h or 236 minutes.

Growth rate (R) is a reciprocal of the generation time

$R = 1/ 3.93 = 0.25\ h^{-1}$

Appendix 6: Thin layer chromatography procedure [316]:

The glass plates were cleaned with ethanol. The aqueous slurry of silica gel was poured on the plates to get a uniform thickness. The plates were activated by heating at 110 degrees C for 1 h and allowed to cool. The sample and the standard (cholesterol) spots were applied on the plate 1.5 cm from the edge. The plates were then run in the respective solvent systems. After the solvent front was marked, the plates were developed by spraying with reagents such as aqueous FeCl3 , 50% ethanolic sulphuric acid solution, vanillin in o-phosphoric acid or by placing in an iodine chamber. After spraying with the reagent sprays the plates have to kept in an oven (110 degrees C) for 15 minutes till appearance of spots. FeCl3 gives blue spots , sulphuric acid gives red spots for steroids and the vanillin-phosphoric acid gives intense purple spots [414].

Publications

1) **Sardessai Y., Bhosle S. (2002)** Tolerance of bacteria to organic solvents. *Research in Microbiology, 153:263-268* [Mini-review]

2) **Sardessai Y., Bhosle S. (2002)** Organic solvent tolerant bacteria in mangrove ecosystem. *Current Science, 82(6):622-623*

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Mini-review

Tolerance of bacteria to organic solvents

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Abstract

Organic-solvent-tolerant bacteria are a relatively novel group of extremophilic microorganisms. They overcome the toxic and destructive effects of organic solvents due to the presence of various adaptive mechanisms. Extensive studies done on the toluene tolerance of certain *Pseudomonas* strains have led to an understanding of the mechanisms of organic solvent tolerance involving novel adaptations such as the toluene efflux pumps, *cis-trans* isomerisation of membrane fatty acids, rapid membrane repair mechanisms, etc. Organic-solvent-tolerant mutants of *Escherichia coli* have been constructed and genes enhancing such tolerance characterised. However, there is practically no information available on the tolerance mechanisms of the reported Gram-positive organic-solvent-tolerant bacterial strains like *Bacillus*, *Rhodococcus* and *Arthrobacter*. This review discusses the general aspects of organic-solvent-tolerant bacteria, their history, biodiversity, mechanisms of tolerance and proposes certain probable adaptations of Gram-positive bacteria in tolerance to organic solvents. © 2002 Éditions scientifiques et médicales Elsevier SAS. All rights reserved.

Keywords: Organic solvents; Toxicity; Tolerance; Efflux; Adaptation; Cell membrane

1. Introduction

Organic solvents are known to be extremely toxic to microbial cells, even at very low concentrations of 0.1% (v/v). Solvents are known to accumulate in and disrupt the bacterial cell membrane thus affecting the structural and functional integrity of the cell [18,37]. Although there are some microorganisms which can assimilate these toxic organic solvents, they do so only when the solvent concentration is very low. Any medium containing large volumes of organic solvents seems an extreme environment for microorganisms and hence for many years it was believed that no microorganism could withstand such a harsh environment [2,18]. The first report of an organic-solvent-tolerant bacterium was by Inoue and Horikoshi in 1989 [18]. They discovered a strain of *Pseudomonas putida* (IH-2000) which could actively grow and multiply in the presence of 50% (v/v) toluene. This surprising observation was confirmed by others [10,25,28,35] and the search to uncover the mechanisms behind this extraordinary characteristic began.

A large number of the reported organic-solvent-tolerant bacteria are *Pseudomonas* strains, especially *P. putida*. Organic-solvent-tolerant mutants, tolerant to p-xylene have been constructed from *E. coli* K-12 [2]. Since Gram-negative bacteria have an additional outer membrane made up of phospholipids and lipopolysaccharides compared to the single cytoplasmic membrane of Gram-positive bacteria, it was assumed that Gram-negative bacteria are better equipped to cope with solvent induced shock [19,32]. But recently, strains of Gram-positive bacteria like *Bacillus*, *Rhodococcus* and *Arthrobacter* tolerant to benzene have been reported [1,23,29,32].

2. Physiological basis of solvent toxicity and the concept of organic solvent tolerance

The primary site of action of organic solvents is the cell membrane. The cytoplasmic membrane of bacterial cells, a phospholipid bilayer, is a matrix in which various enzymes and transport proteins are embedded. It plays a vital role in solute transport, maintaining the energy status of the cell, regulation of the intracellular environment, turgor pressure, signal transduction and energy transducing

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Organic solvent-tolerant bacteria in mangrove ecosystem

Organic solvents are known to be extremely toxic to cells. They dissolve and accumulate in the bacterial cell membrane, resulting in changes in structural and functional integrity and cause cell lysis[1,2]. Organic solvent-tolerant bacteria are a newly discovered group of micro-organisms with novel tolerance mechanisms, which enable them to thrive in solvent-saturated environments[1–3]. These bacteria are significant due to their immense potential in non-aqueous bio-catalysis[3], industrial processes[4,5] involving biphasic organic-aqueous fermentation systems, effluent treatment and bioremediation in hydrocarbon-saturated environments[6–9].

Inoue and Horikoshi[1] used the parameter log P as a measure of solvent toxicity, where P is the partition coefficient of the given solvent in an equimolar mixture of octanol and water. The greater the polarity of a solvent, the lower its log P value and the greater its toxicity. In general, solvents with log P values below 4 are considered extremely toxic. Solvent tolerance is a strain-specific property and every micro-organism has a limiting log P value below which it is unable to grow. This intrinsic tolerance level is determined genetically and is also influenced by environmental factors[10]. The most toxic solvent to which a given microbial strain is tolerant is called the index solvent and the log P value of the index solvent is called the index value of that particular organism[11].

Many micro-organisms are known to degrade organic solvents, but their tolerance to these solvents is less than 0.3% (v/v)[1]. This has been a major limiting factor in biodegradation in natural habitats[6]. Organic solvent-tolerant micro-organisms with the required enzymes could help in degrading pollutants like benzene and toluene which are carcinogenic in ppm amounts[6–8]. Hydrocarbon-oxidizing bacteria and fungi play an important role in bioremediation of oil in the sea and represent one of the primary mechanisms by which petroleum and other hydrocarbon pollutants are eliminated from the environment[12]. It has been demonstrated that the level of organic solvent tolerance of a given strain in soil is related to its level of tolerance to the solvent in liquid medium. The higher the tolerance in liquid media, the faster the recovery of the strain in soil after solvent shock. Therefore, in sites heavily contaminated with aromatic hydrocarbons, solvent-tolerant strains would be expected to establish first, colonize the site and become predominant in the removal of these compounds[7].

The present study was undertaken primarily to determine the existence of bacteria which possess the traits of organic solvent tolerance and hydrocarbon degradation in natural ecosystems. To achieve this goal, mangrove sediment and water samples from various sites of the Mandovi estuary in Goa were collected and analysed. Our studies led to the isolation of a unique strain of *Bacillus* from mangrove sediment which tolerates the organic solvent *n*-butanol having a log P value of 0.8, conferring on it the lowest ever reported index value for any organic solvent tolerant bacterium. In addition to this, the culture also has the potential to degrade aliphatic and aromatic hydrocarbons and hence could play a significant role in waste-water treatment and bioremediation.

This culture was enriched from a mangrove sediment sample by a stepwise process. Initially, the sediment was kept soaked in *n*-butanol for a month. One gram of this sample was transferred to a flask containing artificial sea water supplemented with 20% (v/v) butanol. The flasks were incubated at 30°C on a rotary shaker for one week after which the organic layers from these flasks were transferred to a nutrient-rich medium (LBMG, i.e. modified Luria broth containing 1% tryptone, 0.5% yeast extract, 1% NaCl, 10 mM $MgSO_4$ and 0.1% glucose)[11] and overlaid with 50% (v/v) of the organic solvent. After 2 days of incubation on a shaker, 0.1 ml of the organic layer was plated on LBMG agar and the plates were incubated. A single bacterial culture producing white wrinkled colonies was obtained. This culture, designated as SB-1, was found to be an aerobic endospore forming Gram-positive rod and was identified as a strain of *Bacillus* species[13].

Young culture was inoculated in flasks in a medium containing mineral salts in which the organic solvents (1% v/v) such as *n*-butanol, benzene and toluene served as the only carbon source. SB-1, being a hydrocarbon degrader, was capable of growth under these conditions. It also formed colonies on mineral medium plates overlaid with solvents serving as the sole carbon source.

Organic solvent tolerance assays[1,9] with SB-1 were carried out in solid and liquid LBMG media. In the plate assay, the culture was spread over LBMG agar, overlaid with 2 ml each of the organic solvent and incubated at 30°C. The culture showed growth on direct exposure to a wide range of organic solvents (Table 1). In the liquid medium assay, the culture was inoculated in LBMG broth and mineral medium in flasks overlaid with 1, 10, 50 and 90% (v/v) of benzene, toluene and *n*-butanol, and incubated. Active growth and multiplication occurred in the presence of 1% (v/v) of the solvent; however, growth was inhibited at higher concentrations. Viable cells could be isolated by plating on LBMG agar from all the flasks, including the ones with 90% (v/v) of solvent even after 90 days of incubation. Both the assays indicate that *Bacillus* SB-1 exhibits a high level of tolerance to organic solvents.

Since *n*-butanol tolerance is a novel trait, effects of *n*-butanol concentration on growth rate of SB-1 and the minimum inhibitory concentration of *n*-butanol for its growth were determined. The culture was inoculated in different concentrations of *n*-butanol (0.1 to 10% v/v) and growth was monitored by determining the absorbance and by determining the viable counts on LBMG agar at periodic intervals.

Our results show that SB-1 can grow in up to 2% (v/v) of *n*-butanol, whereas growth is severely retarded at 3% (v/v) in LBMG broth. Growth rate increases with increase in butanol concentration (Table 2). Butanol toxicity is due to its amphiphilic nature. Its water solubility being 8%, the degree of partition into the aqueous phase and from there into the cell membrane of bacterial cells is