PRODUCTION OF POLYHYDROXYALKANOATES USING *BACILLUS* SPP.

Thesis submitted for the degree of

Doctor Of Philosophy

in

Microbiology

to the

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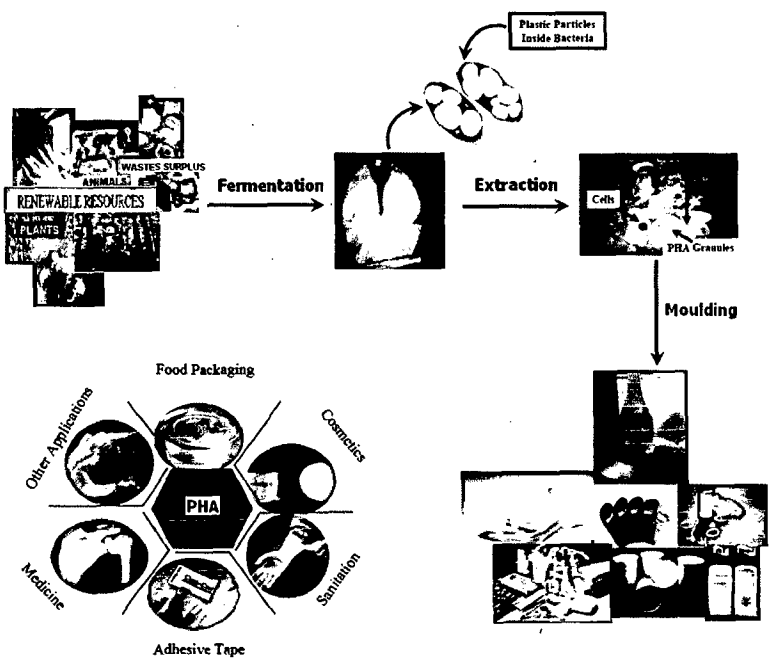

by

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January 2010

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Statement

As required under the University Ordinance 0.19.8 (vi), I state that the present thesis entitled "Production of Polyhydroxyalkanoates using *Bacillus* spp." is my original contribution and the same has not been submitted on any previous occasion. To the best of my knowledge, the present study is the first comprehensive work of its kind from the area mentioned.

The literature related to the problem investigated has been cited. Due acknowledgements have been made whenever facilities and suggestions have been availed of.

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Certificate

This is to certify that the thesis entitled, "***Production of Polyhydroxyalkanoates using Bacillus spp.***" submitted by ***Maria Celisa Santimano*** for the award of the degree of Doctor of Philosophy in Microbiology is based on her original studies carried out by her under my supervision. The thesis or any part thereof has not been previously submitted for any other degree or diploma in any University or Institution.

Place: Goa University

Date: 29/01/2010

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Celisa

Dedicated to my

Mother

LIST OF ABBREVIATIONS

PHAs	polyhydroxyalkanoates	PCI	phenol-chloroform-isoamyl alcohol
PHB	polyhydroxybutyrate	LPS	lipopolysaccharide
spp.	species	EDTA	Ethylene diamine tetra acetic acid
°C	degree centigrade	nm	nanometer
%	percentage	S	Svedberg unit
		PCR	Polymerase chain reaction
w/v	weight/ volume	R^2	coefficient of determination
rpm	revolutions per minute	RSM	Response surface methodology
h	hours	CCD	Central composite design
v/v	volume by volume	N	Normal
min	minutes	FTIR	Fourier-Transform Infrared
µm	micometer	X	The total cellular concentration
g L^{-1}	grams per litre	X_{NPHA}	residual cellular concentration
UV	Ultraviolet	PHA %	PHA purity
DCW	dry cell weight	S_{NPHA}	non-PHA biomass solubilization
DNA	Deoxyribinucleic acid	Δ purity	PHA purity increase
rRNA	Ribosomal Ribonucleic acid	NaOCl	sodium hypochlorite
ml	milliliter	NaOH	sodium hydroxide
µl	microlitre	KOH	potassium hydroxide
M	molar	NH_3	ammonia
mg ml^{-1}	milligrams per milliliter	SDS	sodium dodecyl sulfate
kDa	Kilo Dalton	NPCM	non-PHA cell mass

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Chapter I

Introduction and Literature survey

INTRODUCTION

Polyhydroxyalkanoates (PHAs) are a family of biopolyesters synthesized and accumulated by a wide range of microorganisms as reserve food material. These polymers are produced in response to limitation of any essential nutrient required for cell growth and in the presence of a generous supply of carbon (Anderson and Dawes, 1990). Once the polymer is produced inside the bacterial cell, it serves both, as carbon and energy sources during unfavorable conditions. PHA is an ideal carbon storage material due to its low solubility and high molecular weight thereby causing negligible osmotic stress to the bacterial cells (Anderson and Dawes, 1990; Verlinden *et al.,* 2007). The material properties of PHA resemble those of the conventional thermoplastics used extensively in day to day life. The added advantage of these plastics is that they are completely biodegradable upon disposal under various environments (Lee, 1996). As a consequence of their desired physicochemical properties and biodegradability, PHAs have attracted attention from both academic and industrial research as potential alternatives to the conventional petroleum derived thermoplastics.

1.1 SYNTHETIC POLYMERS AND THEIR IMPACT ON THE ENVIRONMENT

The properties of plastics such as low cost, durability, lightweight, ease in processing and high resistance to chemical and biological degradation have made them an integral part of everyday life. Their chemical structure can be easily manipulated so that they can be molded into almost any desired shape and therefore are used in the manufacture of many durable, disposal goods and as packaging materials (Rivard *et al.*, 1995; Yu, 2007). The four major commodity thermoplastic resins are

polyethylene, polypropylene, polystyrene and polyvinyl chloride. These polymers are produced from inorganic and organic raw materials such as carbon, silicon, hydrogen, nitrogen, oxygen and chloride and these starting materials are extracted from fractions in petroleum or coal cracking (Seymour, 1989).

The very properties of plastics that have contributed immensely towards their popularity are causing grave environmental and societal concerns. Plastics being xenobiotic in nature are recalcitrant to microbial degradation (Flechter, 1993). Their large molecular size is responsible for the resistance of plastics to biodegradation (Reddy *et al.*, 2003). Since their presence in nature is increased enormously during the recent years, new enzyme structures capable of degrading synthetic polymers are yet to be evolved (Mueller, 2006) and nature's in-built mechanisms are unable to degrade these novel unfamiliar pollutants (Reddy *et al.*, 2003). Approximately, 140 millions tonnes per year of synthetic petroleum-based plastics are produced and several thousand tonnes of it is discarded into the environment as industrial waste products (Shimao, 2001). The accumulation of these discarded plastics in the environment is reducing the aesthetic qualities of cities, forests, water bodies as well as endangering the flora and fauna especially in the marine ecosystem (Moore, 2008; Ojumu *et al.*, 2004).

This dramatic increase in production and recalcitrant property of the synthetic polymers is imposing a strain on nature due to their persistence in the environment for centuries (Albertsson *et al.*, 1987). Disposal of these xenobiotics is currently achieved through landfilling, incineration or recycling. Unfortunately, only a small fraction of the discarded plastic wastes reach the disposal sites while the rest litters the landscape or is blown off into the sea posing severe threat to the marine life (Moore, 2008). Incineration of plastic waste results in the generation of large amounts of

carbonaceous material and undesirable pollutants such as carbon dioxide, carbon monoxide, furans, dioxins, hydrogen chloride, hydrogen cyanide, nitrogen oxides and benzopyrene which are highly corrosive and disease inducing (Jayasekara *et al.*, 2005; Johnstone, 1990; Reddy *et al.*, 2003). Recycling of synthetic plastics is disadvantageous due to alteration in the material properties, limiting its further application range and thereby inevitably increasing the cost of the recycled plastics as compared to the virgin plastics (Page, 1992; Hanggi, 1995). The persistence of the discarded plastics is a global threat due to their potential adverse impacts on the environment (Mohanty *et al.*, 2002).

1.2 BIODEGRADABLE POLYMERS

Dwindling crude oil reservoirs and increasing environmental and societal concerns have increased the pressure on development of sustainable and environmentally friendly plastic material independent from petroleum. These materials are termed as biodegradable plastics, green plastics, bioplastics or ecoplastics. For biodegradable polymers to be a part of the solution in plastic disposal, they must be truly degradable, non-polluting and economically priced (Page, 1992). Biopolymers currently of interest include thermoplastic starch (TPS), polylactides (PLA), polymalate (PMA) and polyhydroxyalkanoates (PHAs), each having their advantages and disadvantages. Among these the biodegradable plastics, PHAs are gaining tremendous importance as these are the only plastics produced exclusively by microorganisms and hence are completely degraded to benign compounds (Anderson and Dawes, 1990; Yu, 2007). They are non-polluting as they do not need catalysts or additives to promote their degradation. The common microflora present in soil, water, compost or sewage is capable of degrading this polymer. These polymers completely degrade to carbon

dioxide and water when disposed in aerobic environments (Luzier, 1992; Lee, 1996). In anaerobic conditions, the degradation of PHA can result in methane formation which can be trapped and resold as fuel (Budwill *et al.*, 1992). Biocompatibility of PHA and its manufacture from inexpensive resources is some other key feature which has generated global interest in this polymer (Shah *et al.*, 2008)

1.3 POLYHYDROXYALKANOATES

1.3.1 Occurrence and biosynthesis of PHA

There is widespread occurrence of PHA-producing organisms in the environment. The main candidates for commercial-scale PHA production are transgenic plants and bacteria. Plant cells are able to cope with low yields (<10% of dry weight) of PHA production. Higher levels (10-40% of dry weight) of polymer within the plant cell have negative effect on the growth and development of the plant. At present, a lot of research is focused in overcoming this problem (Verlinden *et al.*, 2007). In contrast, bacterial cells are capable of accumulating PHA to levels as high as 90% (w/w) of the dry cell weight (DCW) (Steinbuchel and Lutke-Eversloh, 2003) thus emphasizing its importance as a potential candidate for PHA production.

A wide variety of bacteria are able to synthesize PHA (Table 1.1). Imbalanced nutrient supply triggers the bacteria to store the excess carbon and energy in the form of PHA. The PHA polymer is deposited in the cell cytoplasm as discrete insoluble granules which vary in number depending on the bacterial strain (Anderson and Dawes, 1990).

Polyhydroxybutyrate (PHB), the first PHA to be discovered, is produced via the classical PHB biosynthetic pathway in three steps catalyzed by the PHA biosynthetic

Table 1.1 PHA accumulating microorganisms*

Acinectobacter	*Ectothiorhodospira*	*Nocardia*
Actinomycetes	*Hyphomicrobium*	*Rhizobium*
Aeromonas[a]	*Klebsiella*[a]	*Oceanospirillum*
Alcaligenes	*Escherichia*[a]	*Paracoccus*
Aphanothece	*Ferrobacillus*	*Pedomicrobium*
Aquaspirillum	*Haemophilus*	*Photobacterium*
Azomonas	*Halobacterium*	*Protomonas*
Azospirillum	*Haloferax*	*Pseudomonas*
Azotobacter	*Halomonas*	*Ralstonia*
Bacillus	*Hydrogenophaga*	*Rhodobacter*
Beggiatoa	*Lamprocystis*	*Rhodococcus*
Beijerinckia	*Lampropedia*	*Rhodopseudomonas*
Beneckea	*Leptothrix*	*Rhodospirillum*
Brachymonas	*Methanomonas*	*Sinorhizobium*
Burkholderia	*Methylobacterium*	*Sphaerotilus*
Caryophanon	*Methylocystis*	*Sphingomonas*
Caulobacter	*Methylomonas*	*Spirillum*
Chloroflexus	*Methylovibrio*	*Spirulina*
Chlorogloea	*Micrococcus*	*Streptomyces*
Chromatium	*Microcoleus*	*Thiobacillus*
Chromobacterium	*Microcystis*	*Thiocystis*
Clostridia	*Moraxella*	*Thiopedia*
Comamonas	*Mycoplana*	*Vibrio*
Corynebacterium	*Nitrobacter*	*Xanthobacter*
Derxia	*Nitrococcus*	*Zoogloea*

*adapted from Singh *et al.*, 2009; Braunegg *et al.*, 2004

[a] Recombinant organisms

enzymes (Fig. 1.1). In the first step, two molecules of acetyl-CoA are condensed together to form acetoacetyl-CoA. This initial reaction is catalyzed by the enzyme 3-ketothiolase (encoded by *phaA*). The next step involving an NADPH/NADH dependent Acetoacetyl-CoA reductase or dehydrogenase (encoded by *phaB*) allows the reduction of acetoacetyl-CoA to 3-hydroxybutyryl-CoA. Finally, the 3-hydroxybutyryl-CoA units are polymerized to PHB by the enzyme PHB synthase (encoded by *phaC*) with the concomitant liberation of coenzyme-A (CoA). This enzyme accepts only the (R)-isomers as substrates for polymerization (Tsuge *et al.*, 2005; Verlinden *et al.*, 2007; Singh *et al.*, 2009).

During normal bacterial growth, the 3-ketothiolase is inhibited by free coenzyme-A released from the Krebs cycle. However, when entry of acetyl-CoA into the Krebs cycle is restricted (during non carbon nutrient limitation), the surplus acetyl-CoA is channeled into PHB biosynthesis (Verlinden *et al.*, 2007).

1.3.2 Chemical structure of polyhydroxyalkanoates

Polyhydroxyalkanotes are hydroxyalkanoic acids linked to each other by an ester linkage. The general chemical structure of polyhydroxyalkanoate is shown in Fig. 1.2. Polyhydroxybutyrate (PHB) is the most commonly occurring and hence most studied PHA. It was discovered in 1926 as a storage material in *Bacillus megaterium* by a French scientist Lemoigne (Lemoigne, 1926). More than 100 hydroxyalkanoic acids have been identified as monomers of bacterial polyesters (Steinbuchel and Valentin, 1995). The composition of the synthesized polymer is influenced by the bacterial strain as well as type and relative quantity and quality of carbon sources supplied to the growth medium (Steinbuchel *et al.*, 1993).

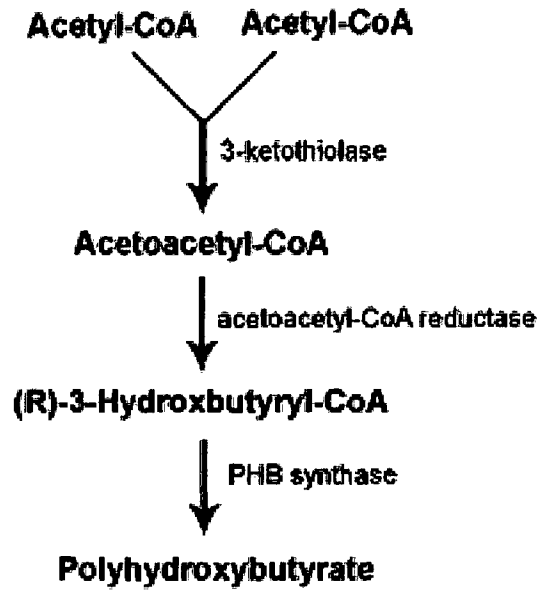
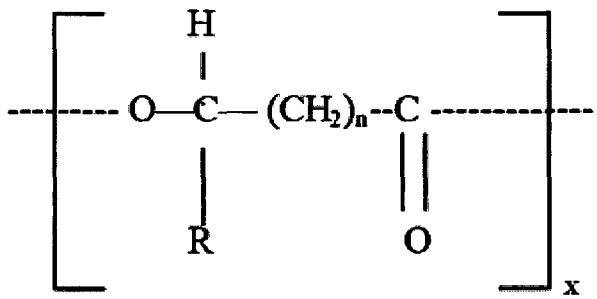


Fig 1.1 PHA biosynthetic pathway



n = 1 R = methyl: polymer = poly(3-hydroxybutyrate)
R = ethyl: polymer = poly(3-hydroxyvalerate)
n = 2 R = hydrogen: polymer = poly(4-hydroxybutyrate)
n = 3 R = hydrogen: polymer = poly(5-hydroxybutyrate)
x = 100-30000

Fig. 1.2 General structure of PHA (Castilho *et al.*, 2009)

The polyhydroxyalkanoates can be divided into three groups depending on the carbon chain length of the monomeric units:

(a) Short-Chain-Length (scl-PHA), which consist of 3 to 5 carbon atoms

(b) Medium-Chain-Length (mcl-PHA), which consist of 6 to 14 carbon atoms

(c) Long-Chain-Length (lcl-PHA), which consist of 17 to 18 carbon atoms (Volova, 2004)

The type of monomer unit present in the polymer greatly influences the material properties of that polymer. The small side chains like methyl and ethyl groups of scl-PHA such as PHB result in a stiff material with high crystallinity, high tensile modulus and low elongation at break. It is brittle and has a relatively high melting temperature (around 170°C) which is close to the temperature at which the polymer decomposes thus limiting the applicability of this homopolymer (Madison and Huisman, 1999). The incorporation of 3-hydroxyvalerate into PHB results in a copolymer (poly-3-hydroxybutyrate-co-3-hydroxyvalerate) that is less stiff and brittle than PHB thereby increasing its application range. As the length of the side chain increases, the polymer produced becomes more elastic with relatively low crystallinity and melting temperature (Kellerhals *et al.*, 2000; Doi *et al.*, 1995).

1.3.3 History and current scenario of industrial PHA production

Polyhydroxybutyrate (PHB), the prototype of the PHA family, commonly synthesized by majority of the bacteria is the most studied polymer. M. Lemoigne in 1927 at the Institut Pasteur demonstrated the production of 3-hydroxybutyric acid polymer in *Bacillus megaterium* (Macrae and Wilkinson, 1958). The production of PHB was explored on a commercial scale only in the late 1950s. During this time, Baptist and Werber at W.R. Grace Co. (USA) conducted the pioneering work of producing PHB

for commercial evaluation (Baptist 1959, 1960). Lack of industrial interest and low production efficiency resulted in the cessation of polymer production (Lee and Chang, 1995). Commercial interest remained dormant until the oil crisis in mid seventies which provided a boost in the quest for alternative plastics. The British chemical company, Imperial Chemical Industry (ICI), in the 1980s began producing PHA copolymers on a commercial scale under the trade name Biopol®. The company used fed-batch cultivation to produce the co-polymer from a mixture of glucose and propionic acid using a mutant strain of *A. eutrophus* H16 (Lee and Chang, 1995).

During the same time, an Austrian company Chemie Linz AG in collaboration with Petrochemia Dunubia (PCD) produced PHB from sucrose as substrate employing a newly isolated strain, *Alcaligenes latus*. In 1993, Dr. Hangii obtained the bacteria as well as the PHA production technology from PCD and a year later started PHB production under the tradename Biomer® (Biomer, 2009).

Meanwhile, Zeneca Bioproducts was created to handle the agricultural and pharmaceutical business of ICI including Biopol® (Philip *et al.*, 2007). In 1996, Monsanto acquired the Biopol® technology from Zeneca. Monsanto produced Biopol® in small volumes and in parallel continued investigations initiated by Zeneca into production of PHA in transgenic plants. Monsanto ceased producing PHA commercially in 1998 and later sold its Biopol® assets to the U.S. biotechnology company Metabolix Inc. (Braunegg, 2003). By 2006, Metabolix was producing 100 tonnes per year and it announced its alliance with Archer Daniels Midland (ADM). Today, Metabolix is a leading producer of PHA polymer. It produces PHAs through fermentation of commercial-grade corn sugar in a 50 cubic meter fermenter (Gilliland, 2006).

Procter and Gamble (P&G), USA is another leading pioneer in the field of PHA polymers and has engaged in R&D efforts to develop and commercialize the Nodax® range of biopolymers. These polymers are based on the copolymer P(3HB-co-3HHx), polyester of 3-hydroxybutyric acid and 3-hydroxyhexanoic acid (Platt, 2006). P&G has patented recovery and processing routes for the polymers and has recently licensed these rights to a Japanese company, Kaneka Corporation. Kaneka is recently developing the commercial process and is expected to be producing bulk volumes (approximately 20,000 tonnes per annum) of P(3HB-co-3HHx) (Bohlmann, 2004; DeMarco, 2005; Crank *et al.*, 2004)

A number of companies worldwide are now producing PHA. The main companies currently involved in the production as well as research and development are summarized in Table 1.2. Some of these companies have plans to increase their production rate in the near future.

Most of the bioplastics cannot compete economically at their present state of technological development with the well established petroleum-based plastics (costing < 1 US per kg) (Mohanty *et al.*, 2002; Choi and Lee, 1997). Jacquel *et al.* (2008) have compiled in their review the cost of various commercially produced PHAs. The commercial sales of Biomer, Biocycle, Biogreen, Biopol and Nodax in 2003 were for a price roughly between 10 and 20 € per kg and in 2010 the price is expected to reduce to 2.20-5.0 € per kg. This high price can be attributed to the high raw material costs, processing costs and the small production quantities. The prohibitive high prices can be reduced by the (a) use of renewable feedstock as raw materials, (b) production using high-yielding bacterial strains and better fermentation strategies and (c) efficient extraction procedures (Lee, 1996; Verlinden *et al.*, 2007).

Table 1.2 Companies currently engaged in PHA research and development worldwide

Company	Trade name	Country	Organism used	Substrate used	Type of PHA	Scale (tons year^{-1})	Period	Reference
Chemie Linz (Petrochemia Danubia (PCD) btF,		Austria	*Alcaligenes latus*	sucrose	PHB	20-100	1980s-2007	Chen, 2009
Metabolix Inc.	Biopol	USA	*E. coli/Ralstonia eutropha*		Unknown	Unknown	1980s to present	Metabolix, 2009
Kaneka Corporation (with Procter & Gamble)	Nodax	Japan/ USA	*E. coli*	Oils (lipids, saccharides)	PHBHHx	Unknown	1990s to present	Crank *et al.*, 2004
Mitsubishi Gas Chemicals (MGC)	Biogreen	Japan		Methanol	PHB	10	1990s to present	MGC, 2009; Chen, 2009
PHB Industrial Biocycle,	Biocycle	Brazil	*Bhurkolderia* sp.	Sugarcane molasses	PHB	100	1990s to present	Biocycle, 2009, Chen, 2010
Biomer	Biomer	Germany			PHB	Unknown	1994 to present	Biomer, 2009
Tepha Inc.	Tepha	USA	Recombinant organism		P(4-HA)	PHA as Bio-Implants	1998 to present	Tepha, 2009
Ningbo Tianan Biologic Material Co. Ltd,	Enmat	China	Natural organisms	Sugars	PHBV	1000	2000 to present	Tianan-enmat, 2009
Archer Daniels Midland (with Metabolix)	Mirel	USA	*E. coli* K12	Corn sugars/ vegetable oils	Unknown	50,000	2005 to present	Mirel, 2009
BioMatera Inc. (formerly known as Bio Venturea Inc.)		Canada	Natural organism	Simple sugars				Biomatera, 2009
Bio-on	Minerv-PHA™	Italy					2007- present	Bio-on, 2009
Meredian Inc.)		USA			PHBHHx	10,000	2007 to present	Chen, 2009
Ningbo Tianan Biologic Material Co. Ltd,	Enmat	China	Natural organisms	Sugars	PHBV	1000	2000 to present	Tianan-enmat, 2009

1.3.4 STRATEGIES FOR IMPROVED PHA PRODUCTION

A. Use of alternative substrates for microbial PHA production

The most important factors affecting the overall economics of PHA production are PHA productivity, PHA content and PHA yield which depend mainly upon the carbon source used, the raw material cost and the recovery methods. In the manufacturing process, the raw material cost, especially the carbon source greatly influences the overall cost of the final product. Synthesis of PHA using expensive carbon sources such as glucose renders the process economically non-viable. Alternatively, inexpensive and easily available raw materials such as wastes generated during agricultural and industrial processes are now being considered as potential carbon substrates (Castilho *et al.*, 2009). These wastes contain large amounts of organic matter which can be utilized as carbon feedstock for PHA production. This will not only ensure the reduction in the raw material cost but also simultaneously save energy and decrease the costs associated with its disposal. The synthesis of value-added products such as PHA from waste carbon sources coupled with their biodegradability makes them increasingly attractive in the pursuit of sustainable development. Various low-cost carbon substrates evaluated for PHA production in the recent years have been summarized in Table 1.3.

A.1 Agricultural wastes and Forest biomass as feedstock for PHA production

Wastes generated in the agricultural sector are being reviewed as potential feedstock for polymer production. An archaeon, *Haloferax mediterraneii* has been reported to utilize extruded rice bran (ERB) and extruded corn starch (ECS) for the production of PHA (Huang *et al.*, 2006). In a wheat-based biorefinery strategy for PHB production using *Cupriavidus necator*, accumulation of PHB occurred when wheat hydrolysate and fungal extract were provided as carbon and nitrogen sources (Koutinas *et al.*,

Table 1.3 PHA production studies conducted during the recent years using various wastes and impure substrates

Organism	Carbon source	Culture time (h)	Cell conc ($g\ L^{-1}$)	PHA conc ($g\ L^{-1}$)	PHA content (%)	Reference
Osmophilic organism	Whey	170		5.5	49.6	Koller *et al.*, 2005
	Waste glycerol			16.2	76	
Recombinant *Aeromonas caviae*	Palm kernel oil		4.3		87	Loo *et al.*, 2005
	Palm olein		3.2		64	
	Crude palm oil		3.1		54	
	Palm acid oil		3.8		40	
A. eutrophus MTCC 1285	Soya waste	72			0.38	Arun *et al.*, 2006
	Malt waste	72			0.26	
	Sesame waste	72			0.44	
	Molasses	72			0.42	
	Bagasses	72			0.42	
	Pharmaceutical waste	72			0.19	
E. coli K24K	Whey	24	70.1	51.1	72.9	Nikel *et al.*, 2006
Haloferax mediterranei	Extruded corn starch (ECS)		62.6	24.2	38.7	Huang *et al.*, 2006
	Extruded rice bran + ECS		140	77.8	55.6	
	Extruded wheat + ECS		131	52.7	40.2	
	Native wheat bran + ECS		68.4	28.0	40.9	
P. Corrugata	Soy molasses		3.6		5-17	Solaiman *et al.*, 2006
Haloferax mediterranei	Enzymatic extruded starch		39.4	20	50.8	Chen *et al.*, 2006
Pseudomonas aeruginosa 47T2	Waste cooking oil	48	7.6		36	Haba *et al.*, 2007
	Technical grade oleic acid	48	9.7		41.2	
	Waste free fatty acid	48	7.9		28.9	
Activated sludge	Rice grain spentwash	96			36.5	Khardenavis *et al.*, 2007
	Jowar grain spentwash	96			34.7	

Table 1.3 contd. PHA production studies conducted during the recent years using various wastes and impure substrates

Organism	Carbon source	Culture time (h)	Cell conc ($g\ L^{-1}$)	PHA conc ($g\ L^{-1}$)	PHA content (%)	Reference
Activated sludge	Food processing waste wash	96			28.3	Khardenavis *et al.*, 2007
Mixed bacteria	Sugarcane molasses				30	Albuquerque *et al.*, 2007
P. putida CA-3	Petrochemical plastic waste				43	Goff *et al.*, 2007
Cupriavidus necator JMP 134	Crude glycerol		50		48	Mothes *et al.*, 2007
C. necator	Wheat hydrolysate + fungal extract	168			70	Koutinas *et al.*, 2007
Curiavidus necator H16	Crude palm kernel oil	72	5.00	3.40	67.00	
	Crude palm oil	72	4.60	3.10	75.00	
	Palm kernel oil	72	5.6	4.3	77	
	Palm olein	72	5.20	3.60	70.00	Lee *et al.*, 2008
	Cooking oil	72	5.40	4.20	78.00	
	Olive oil	72	4.90	3.90	80.00	
	Sunflower oil	72	4.7	3.4	72	
	Coconut oil	72	4.44	3.3	76	
Methylobacteruim sp. ZP24	Cheese whey	30		3.54	64	Nath *et al.*, 2008
Halomonas boliviensis LC1	Wheat bran hydrolysate	30	3.19	1.08	33.8	Van-Thuoc *et al.*, 2008
Pseudomonas fluorescens A2a5	Sugarcane liquor	96	32	22	70	Jiang *et al.*, 2008
P. guezennei biovar. *Tikehau*	Coprah oil	36			63	Simon-Colin *et al.*, 2008
P. putida	Hydrolyzed corn oil		103	28	27.2	Shang *et al.*, 2008
P. hydrogenovora	Whey		5	1.27	25.4	Koller *et al.*, 2008a
Haloferax mediterranei	Hydrolysed whey lactose			12.2	72.8	Koller *et al.*, 2008b
Hydrogenophaga pseudoflava	Hydrolysed whey lactose		12	4.1	30	
Activated sludge	Paper mill wastewater				48.2	Bengtsson *et al.*, 2008

Table 1.3 contd. PHA production studies conducted during the recent years using various wastes and impure substrates

Organism	Carbon source	Culture time (h)	Cell conc ($g\ L^{-1}$)	PHA conc ($g\ L^{-1}$)	PHA content (%)	Reference
R. eutropha NCIMB 11599	Waste potato starch		179	94	55	Haas *et al.*, 2008
Cupriavidus necator PHB-4	Palm kernel oil		7.9		79	Bhubalan *et al.*, 2008
C. necator	Bagasse hydrolysate	48			65	Yu and Stahl, 2008
Activated sludge	Molasses spentwash	48	8.23	2.58	31.3	Khardenavis *et al.*, 2009
Pseudomonas aeruginosa MTCC 7925	Whey	48	0.22		13.2	Singh and Mallick, 2009
	Palm oil	48	1.684		33.2	
	Mustard oil	48	1.576		27.3	
	Soybean oil	48	1.678		30.1	
	Coconut oil	48	1.547		25.3	
	Rice bran	48	0.161		11.6	
	Palm oil cake	48	0.760		23.5	
	Wheat bran	48	0.168		12.5	
	Mustard oil cake	48	0.533		22.6	
P. putida IPT046	Rice oil	72	5.54		61.8	Silva-Queiroz *et al.*, 2009
	Canola oil		5.63		51.9	
	Sunflower oil		5.89		25.6	
	Corn oil		6.42		43.8	
	Soybean oil		6.11		35.6	
P. aeruginosa IPT171	Rice oil	72	2.19		19.6	
	Canola oil		1.92		15.0	
	Sunflower oil		2.16		14.2	
	Corn oil		1.78		15.2	
	Soybean oil		2.47		8.0	
Methylobacterium extorquens DSMZ 1340	Methanol	52	15.40		9.5	Mokhtari-Hosseini *et al.*, 2009
Wautersia eutropha	Wheat derivatives	69	63	41.5	65.87	Xu, *et al.*, 2009

Table 1.3 contd. PHA production studies conducted during the recent years using various wastes and impure substrates

Organism	Carbon source	Culture time (h)	Cell conc ($g\ L^{-1}$)	PHA conc ($g\ L^{-1}$)	PHA content (%)	Reference
Thermus thermophilus HB8	Whey	24	1.60	0.57	35.6	Pantazaki *et al.*, 2009
C. necator DSM 545	Waste glycerol	33.5		38.1	50	Cavalheiro *et al.*, 2009
P. aeruginosa L2-1	Cassava wastewater (CW)	120	3.3		17.6	Costa *et al.*, 2009
	Waste cooking oil (WCO)	120	6.2		43	
	CW + WCO	120	4.2		39	
Comamonas sp. EB172	Treated palm oil mill effluent	60	9.8		59	Zakaria *et al.*, 2010

2007). In medium containing wheat bran hydrolyzed with enzyme from *Aspergillus oryzae* NM1, the moderately halophilic bacterium, *Halomonas boliviensis* LC1 was capable of accumulating 34 wt% of PHB (Van-Thuoc *et al.*, 2008).

Lignocellulosic wastes are abundantly available worldwide. These wastes require pretreatment so that the fermentable substrates are easily available for microbial fermentation. *Burkholderia* species efficiently produced PHA using hydrolyzed or hydrolysates of hemicellulose (Keenan *et al.*, 2006; Silva *et al.*, 2004).

A.2 Industrial wastes as feedstock for PHA production

A large number of industrial processes generate organic wastes such as molasses and corn steep liquor which are rich in carbon. These wastes are being used for the synthesis of value-added products namely; lactic acid, ethanol, enzymes and antibiotics. A number of industrial wastes suitable as feedstock for PHA fermentation are discussed below. Pretreatment of these waste streams may be necessary before they can be utilized by PHA producing microorganisms.

i) Molasses

Research on the use of molasses for PHA production is of interest to scientists and of relevance to the industries eager to utilize this material. It is a by-product generated during sugar production and is the most widely used carbon substrate for the synthesis of polymer using a variety of microorganisms for e.g., the bacterium *Azotobacter vinelandii* UWD synthesizes a co-polymer grown on beet molasses supplemented with valerate (Page *et al.,* 1992). *Bacillus* sp. JMa5 also synthesizes a polymer using cane molasses in conjunction with sucrose (Wu *et al.*, 2001). In a medium containing molasses, strains of *Pseudomonas cepacia* G13, *Rhizobium melliloti* and *Bacillus cereus* M5 accumulate PHA (Celik and Beyatli, 2005; Mercan and Beyatli, 2005;

Yilmaz and Beyatli, 2005). *Bacillus mycoides* RLJ B-017 accumulates PHB in minimal medium containing molasses with a yield of approximately 85% of the DCW (Bordoloi *et al.*, 2007). Soy molasses has also been used as a carbon substrate for PHA production (Yu *et al.*, 1999; Solaiman *et al.*, 2006).

ii) Whey

Whey, by-product of the dairy industry has also been evaluated as a carbon source for PHA production (Yellore and Desai, 1995). *Methylobacterium* sp. has the capability of utilizing lactose to produce PHA. This ability of the isolate has therefore been further exploited for growth and accumulation of PHA on whey supplied as sole source of carbon. Supplementation of the media containing whey with complex organic materials such as meat extract and inorganic nitrogen source such as ammonium sulphate increases the polymer yield further (Yellore and Desai, 1998). Accumulation of PHB in medium containing whey as a fermentable substrate has been observed using recombinant *Escherichia coli* GCSC4401 and GCSC6576 strains expressing the PHB biosynthesis genes of *R. eutropha* (Lee *et al.*, 1997). In a fed-batch culture using whey as feeding solution, recombinant *E. coli* harbouring *R. eutropha* PHA synthase genes was capable of utilizing the organic matter and producing 69 g L^{-1} of PHB (Wong and Lee, 1998). Improvement of the yield as well as productivity of PHB was achieved by employing a high density cultivation process with recombinant *E. coli* expressing the *Alcaligenes latus* PHA biosynthesis genes (Ahn *et al.*, 2001). Subsequently, the yields and productivity of PHB from whey fermentation were also improved by employing a cell-recycle fed-batch culture approach. The approach helped to overcome the culture volume constraint brought on by the continual periodic addition of whey feed stream. With this approach, higher

PHB concentration and productivity were obtained (Ahn *et al.*, 2001). Cells of *R. eutropha* could synthesize a copolymer, poly (3-HB-co-3HV) when grown on whey-containing basal medium supplemented with invert sugar and periodic co-feeding of propionic acid (Marangoni *et al.*, 2002). Strains of *Sinorhizobium melliloti* 41 and *Hydrogenophaga psuedoflava* DSM 1034 were found to use cheese whey for cell growth and polymer production (Povolo and Casella, 2003).

iii) Wastes containing starch

Wastes generated by the instant or fast food manufacturing units can serve as ideal feedstock due to their organic content. Starch and its derivatives are present in wastewaters from industries such as textile, paper, fermentation, beverages and food processing. These wastewaters can be converted into PHA through hydrolysis and polymerization. *Alcaligenes eutrophus* DSM 545 can utilize potato waste hydrolyzed by barley-malt and produce 76.9 wt% of DCW as PHA (Rusendi and Sheppard, 1995). A two step approach has been developed to convert such organic wastes into PHA. These organic wastes are first converted to volatile fatty acids under anaerobic conditions (Yu, 2001) and the acid effluent so generated is subjected to micro filtration and then introduced into a second reactor where the organism, *A. eutrophus* utilizes these acids as carbon substrate for PHA synthesis. Copolymer production (PHB-co-PHV) has been observed using other wastes such as hydrolyzed potato starch by *Azotobacter salinestris* ATCC 49674. In this case, the waste is first hydrolyzed with the help of enzymes and the resultant hydrolysate containing glucose used as a carbon source (Lapointe *et al.*, 2002). Some researchers have tried to exploit the amylase producing ability of some organisms to produce PHA directly from starch

for e.g., *Azotobacter chroococcum* 23 can accumulate PHA when grown in mineral medium supplemented with soluble starch (Kim, 2000).

iv)Glycerol liquid phase from biofuel production

Strains of *Methylobacterium rhodesianum* and *R. eutropha* can synthesize PHB using glycerol and casein peptone as carbon and nitrogen sources, respectively (Borman and Roth, 1999). *Pseudomonas oleovorans* strain NRRL B-14682 synthesizes scl-PHA from glycerol in a chemically defined medium (Solaiman and Ashby, 2005). Higher concentration of glycerol in the medium results in the synthesis of low molecular-weight PHB due to glycerol end-capping, a finding that is certain to have an important implication when using biodiesel-derived glycerol co-product streams to produce PHA. The use of co-product stream generated from soybean oil-based biodiesel production led to synthesis of mcl-PHA by *Pseudomonas corrugata* (Ashby *et al.*, 2004) and unlike the case with scl-PHA synthesis by *P. oleovorans* NRRL B-14682, the molecular weight of the mcl-PHA produced by *P. corrugata* grown on glycerol biodiesel co-product stream does not decrease with increasing concentration of the glycerol substrate. An unidentified osmophilic organism was also reported to produce a P(3HB-co-3HV) using glycerol rich biodiesel co-product stream (Koller *et al.*, 2005). Other organisms such as *Paracoccus denitrificans* and *Cupriavidus necator* JMP 134 also possess the ability to synthesize PHB up to 70% of DCW from pure glycerol as well as crude glycerol obtained from biodiesel production using rapeseed as carbon substrate (Mothes *et al.*, 2007).

v) Fatty acid wastes

Fatty acids have been the preferred substrates for the microbial synthesis of mcl-PHA. Use of triacylglycerols directly as fermentation feedstock can prove to be economically beneficial as the costs inherent to the saponification process can be avoided. The bacterium *Pseudomonas resinovorans* can utilize intact triacylglycerol (tallow) for the synthesis of mcl-PHA (Cromwick *et al.*, 1996). *Pseudomonas stutzeri* 1317 can use soybean oil as a substrate for growth and synthesis of mcl-PHA (He *et al.*, 1998). Fatty acid mixtures produced using steam distillation and saponofication of oily wastes (plant and animal origin) could also be utilized as carbon substrates for mcl-PHA production in *P. putida* (Kellerhals *et al.*, 2000).

R. eutropha could synthesize PHA even from waste sesame oil (Taniguchi *et al.*, 2003). *P. aeruginosa* 47T2 cultivated in mineral medium containing waste cooking oil was found to accumulate up to 36% DCW as PHA (Haba *et al.*, 2007). The mutant of *Wausteria eutropha* could efficiently synthesize the copolymer [P(3HB-co-3HHx)] from palm oil products, the composition of which remained constant regardless of the type, concentration and source of palm oil used (Loo *et al.*, 2005). This copolymer has been reported to possess superior material properties (Doi *et al.*, 1995, Matsusaki *et al.*, 2000). PHA production was also exhibited by *P. putida* PG A1 using saponified palm kernel oil (Annuar *et al.*, 2008). In an attempt to produce novel polyunsaturated long-chain-PHA, long chain polyunsaturated fatty acids such as linseed oil was used. Using hydrolyzed linseed oil, *P. putida* KT2442 was capable of synthesizing polymers made up of a series of novel monomers with multiple unsaturations up to 20% DCW (Casini *et al.*, 1997). *A. eutrophus* and *P. oleovorans* were also capable of producing PHA from carbon substrates such as linseed oil acid and corn oil acid (Kocer *et al.*, 2003). *P oleovorans* was capable of utilizing substrates like linoleic

acid, corn oil acid and laurel seed oil acid to produce mcl-copolymer. The copolymer consisted of hydroxyoctanoate and hydroxydecanoate monomer units. The carboxylic acids derived from the hydrolysis of natural oils such as olive, hazelnut, sesame and anchovy oils were evaluated as substrates for cell growth and production of reserve polyesters by *P. oleovorans*. Poly-3-hydroxyalkanoates containing both saturated (mainly 3-hydroxyoctanoate and 3-hydroxydecanoate) and unsaturated repeating units with 8 to 20 carbon atoms or more were produced using the above substrates (Hazer *et al.*, 1998). *P. saccharophila* NRRL B-628 was capable of utilizing triacylglycerol substrates that contained saturated fatty acyl moieties such as coconut oil and tallow. The organism grown in coconut oil containing medium could produce 0.2 g of mcl-PHA (Solaiman *et al.*, 1999).

B. Fermentation strategies used in PHA production

In PHA production process, the final quality and quantity of the product greatly depends on the strain, metabolic pathway involved, fermentation parameters, PHA synthesis phase (either stationary or throughout growth), carbon source as well as nutrient limitation conditions necessary for PHA production (Somashekara *et al.*, 2009).

Selection of an ideal bacterial strain is very crucial for PHA production. There are several factors that determine the suitability of the organism for large scale production of the polymer such as cell's ability to utilize an inexpensive carbon source, growth rate, polymer synthesis rate and the maximum extent of polymer accumulation (Khanna and Srivastava, 2005a). The factors that directly affect the productivity include growth rate, polymer synthesis rate and the maximum extent of polymer accumulation and should be as high as possible. Further, the cost of PHA can be

significantly reduced using cheap carbon sources. In addition, the microorganisms that produce extracellular polysaccharides (EPS) besides PHA should be avoided since the cells not only use up the carbon source for their synthesis but they also make the recovery process inefficient (Lee and Chang, 1995). Fermentation strategies targeting high productivity of PHA are absolutely essential in reducing the fermentation and purification costs of the process. Since PHA is synthesized and accumulated under unfavorable growth conditions, cultivation strategies that stimulate these conditions and allow efficient production of PHA should be employed (Lee and Chang, 1995). Higher PHA content in the cells will allow more fermentation runs to be carried out for the given total annual operating time thereby resulting in the reduction of the fermenter size and fixed cost (Choi and Lee, 1997). Equipment related costs are also reduced with increasing PHA content as smaller amount of cells need to be produced to obtain the same amount of PHA.

High PHA content also reduces the amount of carbon substrate needed to produce PHA and hence the feedstock will not be wasted on other cellular materials and/or metabolites. This will help in lowering the cost of the carbon substrate, a major contributor to the overall total raw material cost (Kim, 2000). Higher productivity has a profound influence on the purity and yield of the PHA produced. High PHA yield not only reduces the recovery costs by allowing the processing of less amount of non-PHA cellular material to obtain the same amount of PHA, but also reduces the costs associated with waste disposal (Choi and Lee, 1999a).

PHA synthesis has been described in a large number of bacteria but only a few bacteria have been employed for production of PHA at an industrial scale. Based on the culture conditions required for PHA synthesis, these bacteria can be divided into two groups. The first group which includes bacteria such as *Cupriavidus necator*

(formerly known as *Ralstonia eutropha*), *Pseudomonas oleovorans* and several methylotrophs require unbalanced growth conditions for PHA synthesis. The presence of excess carbon with the simultaneous limitation of an essential nutrient triggers the synthesis and accumulation of the polymer. PHA production is growth-associated in bacteria belonging to the second group (e.g., *A. latus*, a mutant of *Azotobacter vinelandii* and recombinant *E. coli*) and does not depend on nutrient limitation (Khanna and Srivastava, 2005a; Lee, 1996). These characteristics have to be taken into consideration during strain selection for PHA production (Khanna and Srivastava, 2005a). In addition, the cyclic nature of PHA metabolism, synthesis and degradation also needs to be considered since prolonged metabolism inside the cells can result in PHA degradation by depolymerases (Jendrossek, 2001).

Various fermentation strategies have been employed to obtain high productivity of PHA including fed-batch fermentation. In this type of fermentation, the intermittent feeding of the nutrients into the reactor is carried out by monitoring one of the several parameters such as dissolved oxygen or pH (Yamane and Shimizu, 1984). Usually the bacteria belonging to the first group is employed for fed-batch fermentation using a two-step cultivation method (Khanna and Srivastava, 2005a). Bacteria are allowed to grow under ambient conditions without any nutrient limitation. In the second phase, the essential nutrient is kept in limiting concentrations to enhance the polymer production. *Cupriavidus necator*, belonging to this group has been extensively studied because of its ability to accumulate large amounts of PHA from simple carbon sources like glucose, fructose and acetic acid. It can accumulate polymer up to 80% DCW when nitrogen or phosphorus is completely depleted (Kim *et al.*, 1994). However, some of the bacteria belonging to this group accumulate PHA more efficiently under nutrient limiting rather than depletion conditions. Therefore a

suitable fed batch strategy has to be developed when using these organisms. Usually in such PHA fermentations, a mixture of carbon source and nutrient to be limited is fed at an optimal ratio to obtain high PHA productivity (Khanna and Srivastava, 2005a).

A different nutrient feeding strategy is applied to obtain high PHA yield using bacteria belonging to the second group. Here, balance between cell growth and PHA accumulation is required in order to avoid incomplete accumulation of PHA or premature termination of fermentation due to low cell concentration. The bacterium belonging to this group, *A. latus* is an ideal candidate for PHA production because of its fast growth rate, growth-associated PHA accumulation and capability to utilize inexpensive raw sugar and molasses (Lee *et al.,* 1999). The PHA concentration in this strain has been increased from 50 to 87% in fed-batch cultivation by induction of nitrogen limitation (Wang and Lee, 1997).

C. Isolation, Purification and Recovery of PHA

In addition to the proper selection of carbon source, the bacterial strain to be employed and ideal fermentation strategies used, the successful large-scale production of PHA also depends upon other factors such as suitable downstream processing (Jacquel *et al.*, 2008). A downstream process for the isolation and purification of PHA is an integral part of PHA production and can account for almost 50% of the overall production cost.

Depending on the bacterial species, carbon source, nutrients and culture conditions, microbial cells accumulate PHA polymers ranging from 20 to 80% of their DCW. The average molecular weight of the microbially produced PHA can range from 1,000 to 2,000 KDa.

Separation and purification of the PHA polymer from non-PHA cell mass presents a technical challenge due to the solid phase of both, the PHA granules and the non-PHA cell mass. Two strategies are usually employed in the downstream processing, PHA solubilization and non PHA cell mass dissolution. In the former, only PHA is dissolved in an appropriate organic solvent and in the latter, the non-PHA cell components are digested and dissolved by chemical agents. The purity, yield and molecular size are the three major factors considered in PHA recovery. PHA recovery has been extensively studied in *Alcaligenes* and recombinant *E. coli* (Hahn *et al.*, 1998; Choi and Lee, 1999b; Kapritchkoff *et al.*, 2006). Due to the difference in the cell wall structure and composition of different bacteria and the conditions for PHA formation, the methods of PHA recovery from these bacteria also differs. Different isolation methods used for recovery of PHA from bacterial cells have been summarized by Jacquel *et al.*, 2008 and have been presented in Table 1.4.

Solvent extraction of PHA has been widely used in the laboratories to prepare a small quantity of high molecular weight PHA. This technique is also proposed in a number of patents for PHA recovery and purification (Noda, 1998; Liddell, 1999; Horowitz, 2002). It has been used for PHA recovery at pilot scale (Gorenflo *et al.*, 2001) and large scale (Chen *et al.*, 2001) with limited success. Extraction using solvents such as chloroform, methylene chloride, propylene carbonate, dichloroethane, tetrachloroethane and dichloroacetate have been tested (Horowitz, 2002). Other solvents which may have been used to extract PHAs from microbial sources include alkyl carbonates such as propylene and ethylene carbonate, trifluoroethanol, acetic anhydride, dimethylformamide, ethylacetoacetate, triolein, toluene, dioxane,

Table 1.4 Summary of polyhydroxyalkanoates isolation methods (Jacquel *et al.*, 2008)

Extraction method	Comments	Bacteria strain	Advantages	Disadvantages
Solvent extraction	Chlorinated hydrocarbon, cyclic carbonates, solvents mixtures	*Bacillus megaterium*	Elimination of endotoxin/high purity, no polymer degradation	Break PHA granule morphology, Harzards connected with halogenated solvents, High price/low recovery
	Chloroethanes, chloropropanes, 1,2-Propane diol lycerol formal diethyl, succinate butyrolactone	*Rhodospirillum rubrum* *Ralstonia eutropha*		
	Tetrahydrofuran methyl cyanide, tetrahydrofuran ethyl cyanide Acetic anhydride Ethylene carbonate 1,2-propylene carbonate,			
	Methylene chloride	*Ralstonia eutropha*		
	Long chain alcohols, esters, amides, ketones Solvent mixture High temperature process Two temperature process			
Digestion method				
Digestion by surfactants	Palmitoyl carnitine	*A. latus A. eutrophus*	Treatment of high cell densities, No polymer degradation	Low purity/ waste water treatment needed
	High cell density digestion by SDS	*Ralstonia eutropha*		
Digestion by hypochlorite	Sodium hypochlorite	*R. eutropha Recombinant E. coli*	High purity	Degradation of the polymer
	Sodium hypochlorite	*Cupriavidus taiwanensis184*		
	Sodium hypochlorite	*Pseudomonas putidia KT2442*		

Table 1.4 contd. Summary of polyhydroxyalkanoates isolation methods (Jacquel *et al.*, 2008)

Extraction method	Comments	Bacteria strain	Advantages	Disadvantages
Dispersion of sodium hypochlorite in chloroform	Sodium hypochlorite and chloroform	*R. eutropha*	Low polymer degradation high purity	High quantity of solvent needed
	Sodium hypochlorite and chloroform with Al- and Fe-based coagulants	*R. eutropha*		
Surfactant-hypochlorite treatment	SDS-hypochlorite	*Azotobacter chroococcum G-3*	Limited degradation/ low operating cost	
Chelate-surfactant	Surfactant-EDTA disodium salt	*R. eutropha*	High purity/low environmental pollution	Large volume of wastewater Low degradation of the polymer
	Recycled-wastewater process	*R. eutropha*		
	Chelate-hydrogen peroxide	*R. eutropha*		
	Selective dissolution by protons	*R. eutropha*	High recovery and high purity low operating costs	
Enzymatic digestion			Good recovery	High cost of enzymes
	Lytic enzymes of *Cytophaga* sp	*R. eutropha*		
	Bromelain; pancreatin	*R. eutropha*		
	Papain	*Burkholderia* sp. *PTU9*		
	Enzyme combined with SDS-EDTA	*Pseudomona*		
	Enzyme combined with SDS-EDTA	*P. putidia*		
Mechanical treatment	Bed mill disruption		No chemicals used	Require several passes
	High pressure homogenization-SDS	*Methylobacterium* sp. *V49*		

Table 1.4 contd. Summary of polyhydroxyalkanoates isolation methods (Jacquel *et al.*, 2008)

Extraction method	Comments	Bacteria strain	Advantages	Disadvantages
Mechanical treatment	High pressure homogenization		No chemicals used	Poor disruption rate for low biomass levels Low micronization
	Ultrasonification	*Haloferax mediterranei*		
	Centrifugation and chemical treatment			
	High pressure homogenization-centrifugation and hypochlorite treatment	*E. Coli*		
Super-critical fluid		*R. eutropha*	Low cost, low toxicity	Low recovery
Recovery using cell fragility			Use of weak extracting conditions	
	Alkaline treatment	*Azotobacter vinelandii UWD*		
	Alkaline treatment	*Recombinant E. coli*		
Air classification		*E. coli*	No chemicals used	Require several consecutive flotation steps
Dissolved-air flotation		*Pseudomonas putida*		
Spontaneous liberation		*E. coli*	No extracting chemicals needed	Low recovery (~80% cells secretes PHB granules spontaneously

tetrahydrofuran, diethylether, pyridine, hydroxyacids and alcohols having more than 3 carbon atoms as well as mixtures thereof (Horowitz, 2002).

A method of effective separation of PHA has been described from matter derived from organisms which contains it, dissolving the PHA in a lower ketone, dialkyl ether, lower alcohol or an ester (Liddell, 1999). The solution formed is then separated from the undissolved matter and the PHA recovered from solution.

Solvent extraction is preferably carried out at a temperature above 100 to 120°C. The temperature however should not exceed 150°C in order to avoid depolymerization of PHA. The PHA in solution can be precipitated by the addition of water when a solvent miscible in water is used for extraction. Incase solvents immiscible in water are used, then the PHA can be recovered by evaporation of the solvent or by temperature cycling (the PHA dissolves at a high temperature and the solution is cooled to allow PHA precipitation). Other techniques include solvent stripping or evaporation, steam stripping or solvent precipitation with a non-solvent. The solvent recovered can be reused for further extraction after distillation. One of the major problems is that only a few solvents are able to dissolve the PHA macromolecules, particularly those of large molecular size and short-chain-length hydroxyalkanoic acids. Popular solvents are halogenated hydrocarbons such as chloroform and dichloromethane (Terada and Marchessault, 1999). The wet PHA containing cells can be directly extracted with the water immiscible solvents but pretreatment of cell mass is usually performed that involves water removal at elevated temperature and extraction of lipids/ pigments by acetone. The pretreatment helps the access of solvents to intracellular PHA polymers. The pretreated cell mass is further subjected to extraction in hot chloroform or other appropriate solvents and the dissolved PHA is separated from insoluble cell mass by filtration and/ or centrifugation. A viscous PHA

solution is usually formed even at relatively low PHA concentration (5% w/v), which renders the separation from residual non PHA cell mass difficult. Precipitation of PHA by adding a PHA insoluble solvent such as methanol and hexane into the filtrate completes the purification of PHA.

In general, solvent extraction yields a PHA polymer of high purity and high molecular weight but its major drawbacks include (a) organic solvents used are toxic and environmentally unacceptable (b) the extracted polymer solution containing more than 5% (w/v) PHA is difficult to handle as the solution is very viscous (c) the removal of cell debris from such a viscous solution is difficult (d) a large amount of organic solvent is required to make a dilute polymer solution and for complete extraction of PHA from biomass. This in turn increases the total production costs (e) time consuming extraction of PHA from non PHA cell mass even at elevated temperatures (f) time consuming separation of the viscous PHA solution from non PHA cell mass (g) biomass may need to be dried prior to solvent extraction which is costly and time-consuming (h) high capital and operation costs for solvent recovery and process safety (i) potential loss of a large amount of volatile and possible mutagenic organic solvents into the environment (j) solvents may co-extract impurities along with PHA such as lipids or other hydrophobic biological materials necessitating further processing of the extract to obtain PHA of satisfactory purity. The other methods used for the recovery of PHA involve cell disruption described in detail below (Jacquel *et al.,* 2008; Choi and Lee, 1997; Koning and Witholt, 1997).

i) Cell disruption methods employed for PHA recovery

Cell disruption methods employed for the recovery of PHA include a) mechanical and b) chemical. Mechanical disruption of PHA producing cells can be achieved using the

bead mills and high pressure homogenizers. These disruption methods have been widely used for recovery of intracellular proteins (Chisti and Moo-Young, 1986: Harrison, 1991a; Kula and Schutte, 1987; Middelberg, 1995) but PHA recovery by mechanical means has received little attention (Harrison, 1991b; Tamer *et al.*, 1998a).

High pressure homogenization with or without chemical pretreatment has been examined for PHA recovery from *Alcaligenes eutrophus* (Harrison *et al.*, 1991b). Pretreatment of biomass substantially improved single pass disruption performance for e.g., cells pretreated with sodium dodecyl sulphate (SDS) could be completely ruptured in a single homogenizer pass at a relatively low operating pressure. With alkaline pretreatment, a minimum of two passes were necessary for complete protein release. Pretreatments with sodium chloride or potassium chloride were less effective whereas that with lysozyme, EDTA or combination of the two also improved single pass disruption relative to untreated material.

Homogenizers are susceptible to blockages (Tamer *et al.*, 1998b) and only relatively dilute biomass slurries can be satisfactorily processed because of viscosity constraints. Bead mills can process much higher concentration (Chisti and Moo-Young, 1986). Bead mill disruption alone or in combination with more benign treatments such as heat shock, salt or hydroxide is likely to be less expensive overall as well as superior to homogenization (Tamer *et al.*, 1998a). Pretreatments improved bead mill disruption to various degrees relative to untreated biomass. However, complete disruption of bacteria in the mill require at least eight passes through the machine, leading to overexposure of cells to severe disruption conditions which causes unwanted reduction of PHA particles (Tamer *et al.*, 1998a).

Chemical cell dissolution methods are in turn of three types: alkaline treatment, acid dissolution of cell material and detergent treatment. In the alkaline treatment method,

sodium hydroxide, potassium hydroxide and ammonium hydroxide have been tested for the dissolution of the non-PHA cell material. Such alkalis efficiently digested the non-PHA cell material resulting in PHB having high purity and recovery yield greater than 91% (Choi and Lee, 1999b). Ammonia has also been used to dissolve the non-PHA cell material and the ammonia extraction waste so generated was recycled in the PHA fermentation as a nitrogen source (Page and Cornish, 1993).

Mineral acids such as hydrochloric and sulphuric were inefficient for the recovery of PHB from *E. coli* cells (Choi and Lee, 1999b). The native granules were also found to be highly resistant to acid hydrolysis (Yu and Chen, 2006). In contrast, the non-PHA cell mass including peptidoglycan of cell wall was vulnerable to acidic dissolution releasing proteins and other biological macromolecules into the aqueous solution. However, the protein release rate largely depends on the acid strength as well as the temperature used and in turn determines the final extent of protein removal. The temperature effect on protein release can be attributed to the increased release of hydrophobic proteins located in cellular and PHA granule membranes since the membranes become more flexible at high temperatures. This indicates that cheap mineral acids at appropriate concentrations selectively solubilize the non-PHA cell mass into small cellular debris with little decomposition of the polyesters.

Surfactants such as sodium dodecyl sulphate (SDS), dioctylsulfosuccinate sodium salt (AOT), hexadecyltrimethylammonium bromide (CTAB), polyoxyethylene (20) sorbitan monolaurate (Tween 20) and polyoxyethylene-p-*tert*-octylphenol (Triton X-100) have also been tested. Although high purity can be obtained using some of these surfactants, the PHA yield in terms of recovery is comparatively low (Choi and Lee, 1999b).

1.3.5 The genus *Bacillus* and PHA production

PHA was discovered first in *B. megaterium* by Lemoigne in 1926 and thereafter research on PHA was conducted using different species from the genus *Bacillus*. These studies indicated the importance of PHA as a carbon and energy reserve which is used to fuel the sporulation process (Macrae and Wilkinson, 1958; Slepecky and Law, 1961; Kominek and Halvorson, 1965). A number of *Bacillus* species are reported to accumulate PHA with yields varying from 4.8 to 69% DCW (Chen *et al.*, 1991; Shamala *et al.*, 2002; Aslim *et al.*, 2002). Recently, *Bacillus* sp. CL1 capable of accumulating 81.2 to 90.9% PHA using different carbon sources ranging from lactose to glucose was reported (Full *et al.*, 2006).

Studies have shown the genus *Bacillus* to be one of the most versatile PHA producers since it can accumulate PHA from a variety of substrates (Table 1.5). The inherent ability to produce various hydrolytic enzymes has been exploited by various research groups for PHA production using complex substrates such as date syrup and beet molasses as sole carbon source (Omar *et al.*, 2001). Cane molasses and corn steep liquor were also used as cheap carbon and energy sources for PHB production in *B. megaterium* (Gouda *et al.*, 2001). *Bacillus* sp. JMa5 isolated from molasses contaminated soil was found to display growth associated PHA production and accumulated 25 to 35% of PHB during fermentation (Wu *et al.*, 2001). *B. mycoides* RLJ B-017 was also found to utilize molasses and pineapple waste for the production of PHA (Bordoloi *et al.*, 2007). PHB accumulation has been reported using *B. thuringiensis* R1 in the presence of table sugar and molasses (Rohini *et al.*, 2006), *B. cereus* CFR06 using starch (Halami, 2008) and *Bacillus* sp. 256 cultivated in Mahua (*Madhuca* sp.) flower extract medium (Anil Kumar *et al.*, 2007).

Table 1.5 Studies on PHA production carried out using various *Bacillus* spp.

Organism	Carbon source	PHA content (%)	Reference
B. amyloliquefaciens DSM 7	Glucose	11.0	Chen *et al.*, 1991
	Acetate	17.0	
	3-hydroxybutyrate	16.5	
B. brevis	Sucrose	32.1	Shamala *et al.*, 2003
B. cereus strain T	Glucose	10.0	Kominek and Halvorson 1965
B. cereus DSM 31	Glucose	32.0	Chen *et al.*, 1991
	Acetate	42.0	
	3-hydroxybutyrate	41.0	
B. cereus UW85	Glucose	24.6	Labuzek and Radecka 2001
B. cereus M5	Beet molasses	73.8	Yilmaz and Beyatli, 2005
B. cereus SPV	Glucose	38.0	Valappil *et al.*, 2007a
	Fructose	40.3	
	Sucrose	38.4	
	Gluconate	41.9	
	Acetate	2.4	
	Propionate	3.3	
	Butanoate	2.6	
	Hexanoate	8.9	
	Heptanoate	1.9	
	0ctanoate	10.5	
	Nonanoate	47.4	
	Decanoate	80.1	
	Do-decanoate	61.8	
	Glucose + ε-caprolactone	5.9	
	ε-caprolactone	8.9	
B. cereus CFR06	Glucose	50.0	Halami, 2008
	Lactose	60.0	
	Sucrose	46.0	
	Maltose	42.0	
	Xylose	38.0	

Table 1.5 contd. Studies on PHA production carried out using various *Bacillus* spp.

Organism	Carbon source	PHA content (%)	Reference
B. cereus (5 strains)	Glucose	31.0 - 62.0	Kumar *et al.*, 2009
	Fructose	2.0- 75.0	
	Maltose	13.0- 70.0	
	Sucrose	12.0- 35.0	
	Pea shell waste	47.0- 72.0	
B. circulans DSM 1529	Glucose	34.8	Chen *et al.*, 1991
	Acetate	36.8	
	3-hydroxybutyrate	43.7	
B. circulans (3 strains)	Sucrose	0- 36.8	Shamala *et al.*, 2003
B. firmus (5 strains)	Nutrient broth	15.6- 33.9	Aslim *et al.*, 2002
B. flexus	Sucrose	50	Somashekara et al., 2009
B. laterosporus DSM 335	Glucose	34.8	Chen *et al.*, 1991
	Acetate	24.4	
	3-hydroxybutyrate	29.5	
B. licheniformis DSM 394	Glucose	21.4	Chen *et al.*, 1991
	Acetate	23.6	
	3-hydroxybutyrate	25.8	
B. licheniformis	Sucrose	24.0	Shamala et al., 2003
B. macerans DSM 7068	Glucose	28.2	Chen *et al.*, 1991
	Acetate	40.5	
	3-hydroxybutyrate	38.6	
B. megaterium DSM 90	Glucose	44.6	Chen *et al.*, 1991
	Acetate	47.2	
	3-hydroxybutyrate	46.5	
B. megaterium	Glucose	33.0	Omar *et al.*, 2001
	Lactose	26.0	
	Gluconate	24.0	
	Maltose	2.0	
	Fructose	17.0	
	Sucrose	5.0	
	Xylose	5.0	
B. megaterium	Molasses	46.2	Gouda *et al.*, 2001
B. megaterium	Malt waste	19.22	Law *et al.*, 2001
B. megaterium (6 strains)	Nutrient broth	11.8- 48.1	Aslim *et al.*, 2002

Table 1.5 contd. Studies on PHA production carried out using various *Bacillus* spp.

Organism	Carbon source	PHA content (%)	Reference
B. megaterium	Sucrose	22.0	Shamala *et al.*. 2003
	Sucrose	4.29	
	Arabinose	2.17	
	Mannitol	6.25	
B. megaterium 12	Glucose	19.5	Yuksekdag *et al.*, 2004
B. megaterium ATCC 6748	Molasses	35.0	Chaijamrus and Udpuay, 2008
	Sucrose	30.2	Kulpreecha *et al.*, 2009
	Molasses	55.5	
B. megaterium strain OU303A	Glycerol	62.4	Reddy *et al.*, 2009
	Glucose	58.6	
	Sodium acetate	48.8	
B. megaterium	Sucrose	34.0	Faccin *et al.*, 2009
B. megaterium	Dairy waste + sea water	11.3	Pandian *et al.*, 2010
B. megaterium NQ-11/A2	Glucose	61	Prabhu *et al.*, 2010
B. mycoides DSM 2048	Glucose	25.3	Chen *et al.* 1991
	Acetate	44.7	
	3-hydroxybutyrate	45.0	
B. mycoides RLJ B-017	Sucrose	69.4	Borah *et al.*, 2002
B. pumilis BI	Nutrient broth	22.5	Aslim *et al.*, 2002
B. sacchari IPT101	Glucose (Glu)	63.1	Lopes *et al.*, 2009
	Xylose (Xyl)	58.1	
	Glucose + xylose	53.4	
	Glu + Xyl + arabinose	47.5	
B. sphaericus	Glucose	12.4	Chen *et al.*, 1991
	Acetate	16.8	
	3-hydroxybutyrate	18.5	
B. sphaericus (5 strains)	Nutrient broth	12.6- 36.4	Aslim *et al.*, 2002
B. sphaericus (5 strains)	Sucrose	15.7- 30.2	Shamala *et al.*, 2003
B. sphaericus	Cornflour	3.3	Ramadas *et al.*, 2009
	Wheat bran	6.8	
	Cassava bagasse	6.4	
	Jackfriut seed powder	46.0	
	Potato starch	47.0	

Table 1.5 contd. Studies on PHA production carried out using various *Bacillus* spp.

Organism	Carbon source	PHA content (%)	Reference
B. sphaericus	Sesame oil cake	14.6	Ramadas *et al.*, 2009
	Groundnut oil cake	18.7	
B. subtilis (8 strains)	Nutrient broth	6.5-32.5	Aslim *et al.*, 2002
B. thuringiensis	Glucose	39.1	Chen *et al.*, 1991
	Acetate	41.3	
	3-hydroxybutyrate	47.6	Chen *et al.*, 1991
B. thuringiensis (3 strains)	Nutrient broth	7.5- 29.4	Aslim *et al.*, 2002
B. thuringiensis R1	Glycerol	34.2	Rohini *et al.*, 2006
	Molasses	23.1	
	Table sugar	28.2	
	Fructose	19.8	
	Lactose	12.7	
	Lactic acid	4.6	
	Glucose	9.2	
	Maltose	2.1	
	3-hydroxybutyrate	52.5	
B. thuringiensis EGU45	Glucose	50.0	Kumar *et al.*, 2009
	Fructose	40.0	
	Maltose	35.0	
	Sucrose	29.0	
	Pea shell waste	55.0	
Bacillus sp.JMa5	Molasses	35.0	Wu *et al.*, 2001
Bacillus sp. INT005	Glucose	32.9	Tajima *et al.*, 2003
	Butyrate	31.5	
	Valerate	18.8	
	Hexanoate	13.0	
	Octanoate	64.5	
	Decanoate	23.5	
	4- hydroxybutyrate	54.9	
	ε-caprolactone	23.2	
Bacillus sp.	Soytone	25.4	Full *et al.*, 2006
	Raffinose	89.7	
	Glucose	90.9	
	Fructose	86.3	

Table 1.5 contd. Studies on PHA production carried out using various *Bacillus* spp.

Organism	Carbon source	PHA content (%)	Reference
Bacillus sp.	Lactose	81.7	Full *et al.*, 2006
	Sucrose	87.0	
	Glycerol	52.1	
Bacillus sp. 256	Mahua flower extract	51.0	Anil Kumar *et al.*. 2007
	Sucrose	54.0	
Bacillus sp. MA3.3	Glucose (Glu)	62.2	Lopes *et al.*, 2009
	Xylose (Xyl)	64.4	
	Glucose + xylose	38.2	
	Glu + Xyl + arabinose	39.9	
Bacillus sp. COL1/A6	Hydrolyzed coconut oil cake	41.92	Santimano *et al.*, 2009a
Bacillus sp. COL1/A6	Hydrolyzed wafer residue	62.4	Santimano *et al.*. 2009b
	Cane molasses	54.7	
	Hydrolyzed citrus pulp	47.5	
Bacillus sp.	Glucose	20.1	Tay *et al.*, 2010
	Fructose	18.2	
	Acetate	39.2	
	Valerate	14.9	

Various agro-industrial residues have also been evaluated for polymer production using *B. sphaericus* 5149 (Ramadas *et al.*, 2009). This organism was able to utilize hydrolysates of diverse residues such as corn flour, wheat bran, cassava bagasse, jackfruit seed powder, potato starch, sesame oil cake and groundnut oil cake. *B. megaterium* BA-019 was could also utilize renewable and inexpensive bioresources such as molasses and urea as carbon substrate for polymer accumulation (Kulpreecha *et al.*, 2009). In addition, the ability of various *Bacillus* sp. to produce bioplastic from pea shell slurry has also been demonstrated recently (Kumar *et al.*, 2009).

Members of this genus are capable of accumulating PHA with a wide range of compositions varying from homopolymers to a variety of copolymers depending on the carbon substrate used for e.g., poly-3-hydroxybutyrate-co-poly-3-hydroxyvalerate [P(3HB-co-3HV)] when the cultures are fed with odd-chain-length *n*-alkanoic acids such as propionic, valeric and heptanoic acid (Chen *et al.*, 1991). Growth of *Bacillus cereus* UW85 using ε-caprolactone as sole carbon source resulted in the formation of a tercopolymer containing 3-hydroxybutyrate (3HB), 3-hydroxyvalerate (3HV) and 3-hydroxyhexanoate (3-HHx) units (Labuzek and Radecka, 2001). Production of copolymers containing 3HB and 3HHx on octanoate and decanoate, copolymers of 3HB-4HB-4HHx on 4-hydroxybutanoate and 3-HB-3-HHx-6-HHx on ε-caprolactone in presence of very low glucose concentration have also been reported (Tajima *et al.*, 2003). Recently, the synthesis of various PHA with 3HB, 3HV and 4HB like monomer units from structurally unrelated carbon sources such as fructose, sucrose and gluconate have been demonstrated (Valappil *et al.*, 2007a). Copolymer production from a single carbon source such as glucose has also been reported by Reddy *et al.* (2009).

1.3.6 APPLICATIONS OF PHA

The current scenario and the increasing awareness of environmental pollution caused by the non-biodegradable petro-plastics have generated a resurgence of interest in producing the bioplastics. PHAs are the most potential candidates for replacing the conventional synthetic plastics since both are thermoplastics, moldable and can be tailor made for several applications (Arun *et al.*, 2009; Azehar *et al.*, 2003; Fusun and Zeynep, 2000). The first commercial PHA product in the form of shampoo bottles made with Biopol (ICI, UK) came into market as early as in 1990 (Weiner, 1997). However, in the recent years, PHAs have found a wide range of applications (Table 1.6) other than in packaging such as PHA based biofuel production (Zhang *et al.*, 2009), synthesis of novel chiral polyesters (de Roo *et al.*, 2002), production of PHA blends and nanocomposites (Pandey *et al.*, 2005) however, the major area of PHA applications still remains to be 1) medical and pharmaceutical, 2) industrial and 3) agricultural (Holmes, 1985; Lee, 1996). Applications of PHAs in these major areas are described in detail below.

A) Medical and pharmaceutical applications

The PHA polymer is of interest and importance in the biomedical arena due to its non-toxicity and the benign nature of its degradation products including monomers and oligomers (Cheng *et al.*, 2005, 2006; Sun *et al.*, 2007). The degradation product of P(3HB), D(-)-3-hydroxybutyric acid is a common intermediate metabolic compound found in all higher organisms (Sevastianov *et al.*, 2003). Therefore this polymer can be implanted without any adverse reactions in the recipient. However, commercial scale production of PHA is currently dominated by the Gram negative organisms such as *Cuprividus necator, Alcaligenes latus* and recombinant *E. coli.*

These organisms contain lipopolysaccharides (LPS) in their outer membrane. These LPS act as endotoxins and are purified along with the polymer during the recovery processes. The LPS-contaminated polymers can elicit adverse immunological reactions in the recipient curtailing their potential for biomedical applications (Chen and Wu, 2005; Valappil *et al.*, 2007b). Therefore the PHAs to be used for medical purposes have to meet stringent requirements. Gram positive bacteria are devoid of such LPS and hence better sources of biomedical-grade PHA (Sevastianov *et al.*, 2003; Valappil *et al.*, 2007b).

A variety of PHAs are now being investigated for use as sutures, repair devices, repair patches, orthopedic applications, adhesion barriers, guided tissue repair or regeneration devices, articular cartilage repair devices, nerve guides, tendon repair devices, bone plates, osteosynthetic materials, cardiological stents and wound dressings (Steinbuchel and Fuchtenbusch, 1998; Babel *et al.*, 1990; Williams and Martin, 2002; Chen and Wu, 2005; Zinn *et al.*, 2001). The degradation rates of the polymer can be controlled but modulating the copolymer composition and therefore these polymers can be used for tissue engineered heart valve scaffolds and viable ovine blood vessels (Chen and Wu, 2005; Abe and Doi, 2002; Williams and Martin, 2002). Further, since these materials are biodegraded to harmless nontoxic compounds within the animal tissues, additional surgery for the removal of these implants is not necessary.

PHAs are also ideal candidates as drug carriers due to their biodegradability, biocompatibility and their degradation by surface erosion. Therefore these polymers (especially PHB and PHBV) are being used for controlled drug release and synthesis of hormones as well as a biodegradable carrier for long term dosage of drugs inside

the body (Steinbuchel and Valentin, 1995; Pouton and Akhtar, 1996; Gould *et al.*, 1987; Koosha *et al.*, 1989).

B) Industrial applications

PHAs are also being developed as packaging films mainly for use as shopping bags, containers and paper coatings, disposable items such as razors, utensils, diapers, feminine hygiene products, cosmetic containers and cups as well as medical and surgical garments, upholstery, carpets, packaging, compostable bags and lids or tubs for thermoformed articles, foils, films etc. (Lauzier *et al.*, 1993; Chen, 2005; Babel *et al.*, 1990; Philip *et al.*, 2007; Clarinval and Halleux, 2005; Mikova and Chodak, 2006).

The polymer PHB is being used to make articles such as combs, pens and bullets. Since this polymer has low viscosity, it can be melted and used for injection molding (Chen, 2005). The copolymer polyhydroxybutyrate-co-polyhydroxyvalerate, (PHB-co-PHV) finds application in food packaging due to its gas barrier property (Hocking and Marchessault, 1994). The copolymer polyhydroxybutyrate-co-polyhydroxyhexanoate, (PHB-co-PHHx) has been used industrially to manufacture flushables, non-wovens, binders, flexible packaging, thermoformed articles, synthetic paper and medical devices (Chen *et al.*, 2001). The copolymer polyhydroxybutyrate-co-polyhydroxyoctanoate, (PHB-co-PHO) marketed as Metabolix PHA, has been approved by Food and Drug Administation (FDA) for production of food additives (Clarinval and Halleux, 2005). PHAs are also used to make other articles due to their piezoelectric nature such as pressure sensors for keyboards, gas lighters, microphones, headphones, loudspeakers, ultrasonic therapy, atomization of liquids etc. (Babel *et al.*, 1990).

C) Agricultural applications

As PHAs are biodegraded in soil under both, aerobic as well as anaerobic environments, their use in agriculture is very promising (Philip *et al.*, 2007). They have been used as mulch films (Hocking and Marchessault, 1994). A brand of PHA called Nodax can be used to manufacture biodegradable agricultural film, as coating for urea fertilizers or as herbicides and insecticides (Hocking and Marchessault, 1994; Philip *et al.*, 2007). The copolymer (PHB-co-PHV) has been used for the controlled release of insecticides. They can be used as seedling containers and plastic sheaths for protecting saplings, biodegradable matrix for drug release in veterinary medicine and tubing for crop irrigation (Jendrossek, 2001). PHA producing organisms have also been used in agriculture to formulate bacterial inoculants for enhancement of nitrogen fixation in plants. Here, the accumulated PHA plays an important role in improving the shelf life, efficiency and reliability of commercial inoculants (Kadouri *et al.*, 2003, 2005).

Table 1.6 Applications of PHA in various fields*

Applications	Examples
Packaging industry	All packaging materials that are used for a short period of time, including food utensils, films, daily consumables, electronic applications *et al.*
Printing and photographic industry	PHA are polyesters that can be easily stained
Other bulk chemicals	Heat sensitive adhesives, latex, smart gels, PHA nonwoven matrices can be used to remove facial oils
Block copolymerization	PHA can be changed into PHA diols for block copolymerization with other polymers
Plastic processing	PHA can be used as processing aids for plastic processing
Textile industry	Like nylons, PHA can be processed into fibers
Fine chemical industry	PHA monomers are all chiral R-forms, and can be used as chiral starting materials for the synthesis of antibiotics and other fine chemicals
Medical implant biomaterial	PHAs have biodegradability and biocompatibility, and can be developed into medical implant materials. PHA can also be turned into drug controlled release matrices
Medical	PHA monomers, especially 3HB, have therapeutic effects on Azheimer's and Parkinson's disease, osteoporosis and even memory improvement *et al.*
Healthy food additives	PHA oligomers can be used as food supplements for obtaining ketone bodies
Industrial microbiology	The PHA synthesis operon can be used as a metabolic regulator or resistance enhancer to improve the performances of industrial microbial strains
Biofuels or fuel additives	PHA can be hydrolyzed to form hydroxyalkanoate methyl esters that are combustible
Protein purification	PHA granule binding proteins phasin or PhaP are used to purify recombinant proteins
Specific drug delivery	Coexpression of PhaP and specific ligands can help achieve specific targeting to diseased tissues

*Chen (2009)

Aims and objectives of the research work

Biodegradable, aliphatic copolyesters collectively termed polyhydroxyalkanoates or PHAs, are polymers synthesized by bacteria when exposed to adverse environmental conditions and excess carbon source. Unlike other petrochemical-based polymers that take centuries to degrade after disposal, these polyesters break down rapidly in both aerobic and anaerobic conditions. Because of its "green" feature combined with its benign degradation behavior, PHB has the potential to replace petroleum-based plastics in packaging, agricultural, and biomedical applications.

Production of the polymer on a commercial scale is presently through use of Gram negative microorganisms such as *Cupriavidus necator*, *Alcaligenes latus* and recombinant *Escherichia coli* (Valappil *et al.,* 2007b). The polymers extracted from these bacteria require additional purification procedures since the lipopolysaccharide (LPS) co-purifies along with the polymer. This additional process thereby increases the overall production costs (Chen and Wu, 2005). Gram positive bacteria such as *Bacillus* spp. are known to possess an added advantage as potential candidates for industrial scale PHA production due to the lack of LPS layer. Members of this genus are also known to grow rapidly, possess various hydrolytic enzymes and produce copolymers from structurally unrelated carbon sources (Halami, 2008; Valappil *et al.,* 2007a).

The widespread use of this biodegradable polymer is restricted due to the high production costs associated with their synthesis on an industrial scale. Due to this reason the applications of the polymer is currently limited to the medical field (Valappil *et al.,* 2007b). A major bulk of the production cost is attributed to the raw material cost, especially the carbon source used. The use of inexpensive and readily available carbon substrates viz., agro-industrial wastes and byproducts as PHA

feedstock can reduce as much as 40 to 50% of the overall cost (Kim, 2000). The total production costs are also influenced by parameters such as fermentation strategies and recovery processes besides the carbon feedstock employed.

The present work was therefore focused on the evaluation of the PHA producing potential of *Bacillus* spp. isolated from various econiches. The innate ability of *Bacillus* spp. to grow and accumulate PHA on various substrates was exploited by cultivation on various agro-industrial by-products. Maximization of the PHA yield can be achieved by optimization of fermentation medium and employing different cultivation strategies. Efficient recovery of the polymer from the cells is another critical parameter in determining the cost and final applicability of the polymer.

In view of this, the following objectives were undertaken for study:

- Screening of carbon rich wastes for PHA accumulation by *Bacillus* spp.
- Optimization of batch fermentation process for growth and maximum PHA accumulation.
- Development of a suitable fed-batch process for growth and PHA accumulation.
- Screening of low-cost downstream processing methods for high yield of PHA.

Chapter II

PHA production from low-cost feedstock using Bacillus spp.

INTRODUCTION

Gram positive bacteria such as *Bacillus* spp. are potential contenders for industrial scale PHA production due to their rapid growth, ability to produce various hydrolytic enzymes, accumulate copolymers from structurally unrelated carbon sources and lack of the lipopolysaccharide (LPS) layer (Halami, 2008; Valappil *et al.,* 2007b; Singh *et al.*, 2009).

The widespread use of this polymer is restricted due to its high production cost. The use of inexpensive and renewable carbon substrates such as agro-industrial wastes and by-products as PHA feedstock can contribute to as much as 40 to 50% reduction in the overall PHA production cost (Kim, 2000). Therefore there is a need to screen for bacteria belonging to the genus *Bacillus* capable of producing appreciable amounts of PHA from complex carbon sources.

This chapter reports the polymer production from sixteen *Bacillus* spp. previously isolated and identified in the laboratory (Prabhu, 2010) from various pure carbon substrates. Further, the ability of these isolates to synthesize hydrolytic enzymes was exploited by cultivating them on diverse, complex carbon sources such as industrial and agricultural by-products for PHA production. The response of the isolates towards selected hydrolysates of various carbon feedstocks was also monitored. Quantitative estimation of the PHA accumulated by different *Bacillus* isolates using hydrolysates of the selected agronomic by-products as sole carbon source under submerged cultivation conditions was also carried out.

2.1 MATERIALS AND METHODS

2.1.1 Isolates used for the study

Bacterial strains belonging to the genera *Bacillus* previously isolated from different marine and coastal environment were used for the study.

2.1.2 Maintenance of isolates

The isolates were grown on nutrient agar (Appendix 1) slants. The slants were maintained at 4°C and subcultured after every 30 days.

2.1.3 PHA production under submerged cultivation

A) Medium used

E2 mineral medium containing glucose (2% w/v; Appendix 1) was used for the production of PHA under submerged cultivation.

B) Inoculum preparation

The isolates were streaked on nutrient agar plate and a single isolated colony of the 24 h old culture was inoculated in E2 liquid mineral medium containing glucose (1% w/v). The flasks were incubated at 30°C on an Orbitek environmental shaker (170 rpm) for 24 h. Inoculum preparation was carried out in this manner for all the experiments involving submerged cultivation.

C) Submerged cultivation

E2 mineral medium containing glucose (2% w/v) was inoculated with 5% (v/v) of the inoculum in triplicates and incubated at 30°C on an Orbitek environmetal shaker (170 rpm). The flasks were harvested after an interval of 24 h for a period of three days.

2.1.4 Analytical procedures

A. Determination of biomass

Twenty five ml of fermented culture broth was centrifuged at 5,000 rpm (REMI, C24, INDIA) for 10 min. The cell pellet obtained was washed twice with double distilled water and transferred to preweighed aluminum foil cups. Cells were dried at 60°C till constant weight was obtained.

B. Determination of PHA

The PHA was extracted by sodium hypochlorite method described by Rawte and Mavinkurve (2002). The cell pellet obtained from 25 ml of the fermented culture broth was washed thrice with saline (Appendix 1). The pellet was treated with sodium hypochlorite solution containing 2% (v/v) of active chlorine (Appendix 1) for 20 min under shaking conditions. The treated biomass was then centrifuged at 8,000 rpm for 20 min. The polymer pellet obtained was thoroughly washed with chilled diethyl ether and collected in a preweighed glass tube. The polymer content was determined gravimetrically.

The data presented are a mean of triplicate experiments.

2.1.5 PHA production using agronomic and industrial by-products

A. Procurement of agricultural and industrial by-products

Citrus pulp waste was procured from a local commercial food joint, Sanyog (Goa, India). Cane molasses and bagasse were obtained from Sanjeevani sugar mill (Goa, India). Rice chaff, coconut oil cake and cotton seed cake were procured from a local market in Margao (Goa, India) and starch based wastes from a Fast Moving Consumer Goods (FMCG) industry at Ponda (Goa, India). Bakery wastes were collected from a local bakery in Bardez (Goa, India).

B. Processing of the agricultural and industrial by-products

The by-products except molasses were air-dried at room temperature (30°C). Citrus pulp waste was dried at 50°C. The wastes were ground in an electric grinder and sieved using a mesh with a pore size of 500 μm. These by-products were used as sole source of carbon.

C. PHA production by plate assay method

Qualitative analysis was performed by spot inoculating freshly grown isolates on E2 mineral medium agar plates containing pure carbon substrates (2% w/v). Further, the ability of the isolates to utilize agronomic and industrial by-products as sole carbon source as well as the response of the isolates towards the hydrolysates (Appendix 2) of these by-products (except molasses) was also tested. The by-products or their hydrolysates were supplied as sole carbon source at a concentration equivalent to 20 g L^{-1} of reducing sugars. All the plates were incubated at 30°C for three days. PHA accumulation was visualized after every 24 h using the method described by Kitamura and Doi (1994).

D. Visualization of intracellular PHA accumulation (Kitamura and Doi, 1994)

PHA accumulating ability was visualized by staining the plates using the method described by Kitamura and Doi (1994). The plates were flooded with an ethanolic solution of Nile Blue A (Appendix 1) and kept in the dark for 20 min with intermittent shaking. After the stain was discarded, the plates were dried and illuminated with UV light. The isolates exhibiting bright orange fluorescence were scored as PHA producers. The intensity of fluorescence exhibited by the isolates was noted.

2.1.6 Selection of the agro-industrial by-products for further study

Based on the response of the isolate towards PHA production using the different agro-industrial by-products, following by-products were selected for further studies: citrus pulp waste, coconut oil cake, wafer residue, rice chaff, sugarcane bagasse and molasses.

2.1.7 Estimation of physical and chemical parameters of the selected agro-industrial by-products

Various physico-chemical properties of the agro-industrial by-products were determined. The total sugar content was estimated by phenol sulphuric acid method (Dubois *et al.*, 1956), reducing sugar content by DNSA method (Miller, 1959), inorganic phosphate using Fiske and Subbarao method (Buchanan, 1975) and ammonia nitrogen by phenate method (Franson, 1985; Appendix 3). The carbon and nitrogen content was determined using the CNS-analyzer (NCS 2500, CE instruments, Italy) at National Institute of Oceanography (NIO), Dona Paula.

2.1.8 Submerged fermentation for PHA production using various hydrolysates of agro-industrial by-products

A. Pretreatment of the agro-industrial by-products

PHA accumulation was tested using hydrolysates of agronomic and industrial by-products. Except molasses, all the carbon wastes were subjected to dilute acid hydrolysis. Starch based wastes as well as citrus pulp wastes were subjected to Treatment II (Appendix 2). Cane baggase and rice chaff were treated as per Treatment III (Appendix 2) and coconut oil cake using Treatment IV (Appendix 2). Reducing

sugar content in all the hydrolysates was quantified using DNSA method (Miller, 1959). The hydrolysates were then added in the medium at a final concentration of reducing sugar equivalent to 20 g L^{-1} of glucose.

B. Quantitative estimation of PHA using agro-industrial by-products as sole carbon source

PHA accumulation by the isolate grown in production medium (Appendix 1) containing respective carbon substrates such as molasses, hydrolysates of wafer residue, citrus pulp waste and coconut oil cake were monitored by individually growing the culture in a 250 ml Erlenmeyer flask containing sterile 100 ml of the medium. Hydrolyzed carbon wastes were autoclaved separately and added to the medium prior to inoculation. Flasks were incubated at 30°C for 48 h at 170 rpm on Orbitek environmental shaker.

C. Analytical procedures

The processing of cell biomass obtained on cultivation of the isolates using various hydrolysates, except coconut oil cake was achieved in the following manner.

i. Determination of biomass content

Twenty five ml of the culture broth was centrifuged at 8,000 rpm for 15 min and washed thoroughly with double distilled water. The cell pellet obtained was transferred to a pre-weighed aluminum foil cup and dried at 60°C till constant weight was obtained.

In case of coconut oil cake hydrolysate, processing of the cell pellet was similar to the procedure described above except that the washed cell pellet was rinsed using hexane, transferred to a preweighed aluminum foil cup and dried until constant weight was obtained.

ii. Determination of polymer content

To extract the polymer from the cells, the pellet obtained from 25 ml of fermented broth was washed thoroughly with saline. The polymer was then extracted by sodium hypochlorite method described in section 2.1.4.

To determine the polymer content accumulated by the isolates when cultivated on hydrolysate of coconut oil cake, the extracted polymer was rinsed with hexane in addition to diethyl ether and dried until constant weight was obtained (Santimano *et al.*, 2009a). Further, the purity of the polymer was analyzed using the method described by Law and Slepecky (1961).

All the experiments were carried out in triplicates and the average values are reported.

2.2 RESULTS AND DISCUSSION

2.2.1 *Bacillus* spp. as PHA producers

Sixteen bacterial strains belonging to the genus *Bacillus* previously isolated and identified in the laboratory were used for the study (Table 2.1). These strains were isolated from sediment samples collected from diverse econiches. Preliminary investigations revealed the isolates to be potential candidates for PHA production. Kumar *et al.* (2009) have observed that among the different bacteria belonging to various genera, *Bacillus* species were found to possess high PHA producing ability.

Submerged cultivation of the different *Bacillus* species used in the present study revealed that these isolates grew rapidly (Fig. 2.1). The content of PHA accumulated by these isolates, which is calculated as the ratio between the polymer extracted and the cell biomass, both in terms of cell dry weight (CDW) ranged between 39.6% (COL2/A2) to 62.3% (COL1/A6). Generally, members of the genus *Bacillus* are known to produce PHA content ranging from 6.53 to 48.2% (Shamala *et al.*, 2003;

Table 2.1 Different *Bacillus* spp. used for the study isolated from various econiches

Ecological site	Isolate designation	Tentative identification
Bombay High Oil Field	BHR-1/A7	*Bacillus licheniformis*
	BLQ-2/A7	*B. megaterium*
	ICP-1/A3	*B. licheniformis*
	NQ-11/A2	*B. megaterium*
	PPA/Z6	*Bacillus sp.*
Coastal beaches	L2/A1	*B. megaterium*
	L4/A3	*B. licheniformis*
	L4/A4	*B. megaterium*
	L5/A1	*B. megaterium*
	L7/A2	*B. megaterium*
Mangrove swamp	MGP/A5	*Bacillus sp.*
Humic soil	COL1/A1	*Bacillus sp.*
	COL1/A6	*B. megaterium*
	COL1/A11	*B. megaterium*
	COL2/A2	*B. megaterium*
	COL2/A6	*B. megaterium*

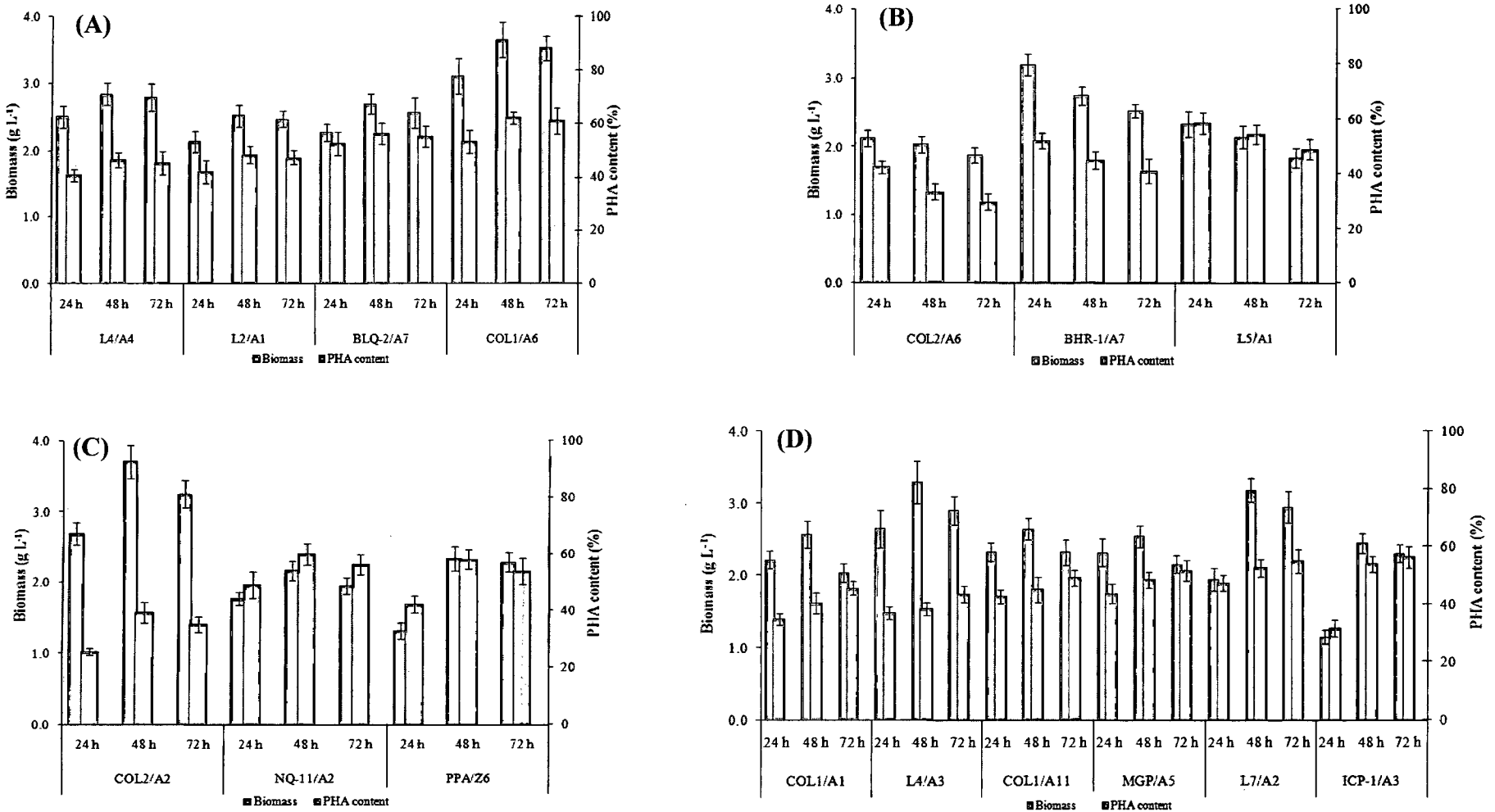


Fig. 2.1 Temporal variation in PHA producing ability among different *Bacillus* spp. **(A)** consistent PHA production, **(B)** maximum PHA production at 24 h, **(C)** maximum PHA production at 48 h, **(D)** maximum PHA production at 72 h

Aslim *et al.*, 2002; Chen *et al.*, 1991). Recently, few researchers have reported higher amounts of accumulated PHA in different *Bacillus* spp. such as *B. megaterium* NQ-11/A2 (61% DCW) *Bacillus thuringiensis* R1 (64.1% DCW), *Bacillus mycoides* RLJ B-017 (69.4% DCW) and *Bacillus* sp. CL1 (90% DCW) (Prabhu *et al.*, 2010; Rohini *et al.*, 2006; Borah *et al.*, 2002; Full *et al.*, 2006).

On comparing the PHA yield produced for three consecutive days of incubation, it was observed that the time of incubation influenced PHA production. It was interesting to note that these *Bacillus* species could be categorized into four groups based on the PHA content produced. Most of the isolates showed a reduction in the accumulated PHA content on prolonged incubation whereas only about one third of the isolates were capable of consistently maintaining the maximally accumulated PHA till the termination of the experiment which included isolates BLQ-2/A7, L2/A1, L4/A4 and COL1/A6 (Fig. 2.1A). With further incubation till 72 h, the intracellular PHA content accumulated by these strains was found to be stable indicating that PHA depolymerase enzyme of this organism remains inactive over a longer period of time. *Bacillus cereus* SPV is also reported to exhibit similar pattern for PHA production (Valappil *et al.*, 2007c; Philip *et al.*, 2009).

The isolates belonging to the second group namely, BHR-1/A7, L5/A1 and COL2/A6 produced maximum PHA at 24 h of incubation (Fig. 2.1B). Further increase in the incubation time reduced the PHA content in the cells suggesting that the accumulated PHA was possibly utilized for growth or spore formation as observed by Wu *et al.* (2001). A slightly different trend was observed with isolates NQ-11/A2, PPA/Z6 and COL2/A2 (belonging to the third group) which exhibited maximum PHA content at 48 h and further incubation resulted in a decrease in the accumulated PHA content (Fig. 2.1C). These findings were consistent with that reported by Shamala *et al.*

(2003). They observed maximal production of PHA at 24 h (or 48 h in case of *B. megaterium*) and the amount of accumulated PHA decreased on further incubation in fermentations employing various *Bacillus* species. The amount of PHA accumulated in six isolates belonging to the fourth group namely, ICP-1/A3, L4/A3, L7/A2, MGP/A5, COL1/A1 and COL1/A11 increased with increase in the incubation time (Fig. 2.1D). Halami (2008) has also reported a similar observation using *B. cereus* CFR06 wherein the PHA content increased on prolonged incubation of 72 h. This trend could be attributed to the decline in the cell biomass consequently resulting in the observed increase in PHA content.

The market price of commercially available PHA (approximately 10 to 12 € per kg) is comparatively higher than that of conventional synthetic plastics (Castilho *et al.*, 2009). The high manufacturing costs involved during the production of these polymers is mainly responsible for hampering the commercialization of biodegradable plastics. The major factors affecting the overall economics of PHA production are the PHA content, PHA yield and productivity, the cost of the raw materials and the recovery methods (Choi and Lee, 1999a). Among these various factors, the raw material costs dominate the manufacturing costs (Van-Thouc *et al.*, 2008). Therefore, for economical PHA production inexpensive substrates that can be used as carbon sources for bacterial strains to synthesize large quantities of intracellular PHA are necessary. By-products such as molasses, straw, bagasse generated in the agricultural sector are available abundantly and are generally used as cattle feed since they have little economic value. These agricultural residues are rich in carbohydrates and the use of such materials for the synthesis of value-added products can be advantageous and also contribute significantly to the reduction of their disposal costs (Thomsen, 2005; Castilho *et al.*, 2009).

Members of the genus *Bacillus* are ubiquitous in nature and possess innate ability to produce various hydrolytic enzymes that metabolize complex residues present in the surrounding environment. Therefore, such native *Bacillus* strains are now being explored industrially for economic PHA production from complex residues for e.g., agro-industrial by-products (Santimano *et al.*, 2009a; 2009b; Gouda *et al.,* 2001; Kumar *et al.*, 2009; Kulpreecha *et al.*, 2009).

The ability of different *Bacillus* spp. used in the present study to utilize diverse carbon substrates generally found as major components in agro-industrial wastes was assessed. The accumulation of PHA by the isolates on various pure polymers showed that the isolates were able to utilize soluble starch for growth as well as PHA accumulation (Table 2.2). Four isolates, ICP-1/A3, PPA/Z6, COL1/A1 and COL2/A6 exhibited weak fluorescence, while the fluorescence exhibited by the other isolates was of moderate intensity. Among the various species of the genus *Bacillus, B. sphaericus* and *B. cereus* have been reported to produce PHA from starch (Halami, 2008; Ramadas *et al.,* 2009).

All the isolates except ICP-1/A3, PPA/Z6 and COL1/A1 were able to grow on pectin as sole source of carbon (Table 2.2). Isolate BLQ-2/A7 was able to utilize pectin for growth but failed to metabolize it for the synthesis of PHA. Isolates BHR-1/A7, NQ-11/A2, L4/A3 and L7/A2 exhibited moderate fluorescence intensity within 24 h of incubation whereas the other isolates exhibited poor fluorescence. Isolates NQ-11/A2, L2/A1, L7/A2 and COL2/A6 were able to accumulate PHA only for 24 h and the fluorescence disappeared on further incubation. Isolates L4/A3 and BHR-1/A7 were able to accumulate PHA for the first three days of incubation. Further, isolates COL1/A11, COL2/A2, COL1/A6, L4/A4 and MGP/A5 were able to consistently

Table 2.2 Duration of maximum PHA production on various polymeric substrates

Isolates	Carbon wastes			
	Starch	**Pectin**	**Chitin**	**Cellulose**
BHR-1/A7	++ $^{24\text{-}72}$	++ ^{24}	-	-
BLQ-2/A7	++ $^{24\text{-}72}$	-	-	-
ICP-1/A3	+ $^{24\text{-}120}$	-	-	-
NQ-11/A2	++ ^{24}	++ ^{24}	-	-
PPA/Z6	+ ^{24}	-	-	-
L2/A1	++ ^{24}	+ ^{24}	-	-
L4/A3	++ ^{24}	++ ^{24}	-	-
L4/A4	++ ^{24}	+ $^{48\text{-}120}$	-	-
L5/A1	++ ^{24}	+ $^{72\text{-}120}$	-	-
L7/A2	++ ^{24}	++ ^{24}	-	-
MGP/A5	++ ^{24}	+ $^{48\text{-}120}$	-	-
COL1/A1	+ $^{48\text{-}120}$	-	-	-
COL1/A6	++ $^{24\text{-}72}$	+ $^{48\text{-}120}$	-	-
COL1/A11	++ ^{72}	+ $^{48\text{-}120}$	-	-
COL2/A2	++ $^{24\text{-}72}$	+ $^{48\text{-}120}$	-	-
COL2/A6	+ $^{24\text{-}120}$	+ ^{24}	-	-

Key: +, ++, +++: degree of PHA accumulation in comparison to glucose (positive control)

-: no PHA accumulation

Superscripted numbers indicate the duration (h) during which maximum fluorescence was observed

maintain the accumulated PHA after its production at 48 h whereas isolate L5/A1 exhibited delayed PHA production (72 h onwards) on pectin as sole carbon source.

Various oils such as palm, coconut and olive, were also tested for their ability to support growth and polymer production. It was observed that all the isolates were able to utilize the different oils tested for growth, with coconut oil supporting the best growth. Unfortunately, PHA production could not be visualized due to the interference caused by the fluorescence of the oil incorporated in the medium. Singh and Mallick (2009) have reported the use of different oils as substrates for PHA production. They found that plant oils displayed stimulatory effects on growth as well as PHA production of *P. aeruginosa* MTCC 7925 with maximum polymer production on palm oil. Use of plant oils has been described by various authors for PHA production with different Gram negative organisms (Fukui and Doi, 1998; Kocer *et al.*, 2003; Hazer *et al.*, 1998).

Among the other polymeric substrates tested as sole carbon source for growth and PHA production the isolates grew poorly on chitin and were unable to accumulate PHA whereas cellulose did not support the growth of any isolate.

2.2.2 Agronomic and industrial by-products as carbon feedstock for PHA production

The capability of the isolates to utilize diverse carbon substrates was further exploited by assessing their efficiency to synthesize PHA on different agricultural and industrial by-products. The results observed using agronomic and industrial by-products as carbon feedstock for PHA accumulation are compiled in Tables 2.3a and 2.3b.

Table 2.3a Duration of maximum PHA production on various agronomic and industrial by-products

Isolates	Agronomic and industrial by-products				
	Wafer	**Rice crispies**	**Biscuit**	**Bread**	**Molasses (cane)**
BHR-1/A7	++ ^{72}	++ $^{24-96}$	++ $^{24-120}$	++ ^{120}	+++ $^{24-96}$
BLQ-2/A7	+++ $^{48-72}$	++ $^{24-96}$	++ $^{24-120}$	++ $^{48-120}$	+++ $^{24-96}$
ICP-1/A3	+ $^{24-120}$	++ ^{24}	++ $^{72-120}$	++ ^{48}	+++ $^{48-72}$
NQ-11/A2	++ $^{24-120}$	++ ^{24}	+ $^{24-48}$	+ $^{24-120}$	+++ $^{24-72}$
PPA/Z6	+ $^{24-48}$	++ ^{48}	+ $^{24-120}$	+ $^{24-72}$	++ $^{48-72}$
L2/A1	+ $^{24-120}$	++ ^{24}	++ $^{24-48}$	+ $^{24-120}$	++ $^{48-96}$
L4/A3	++ $^{72-120}$	++ ^{24}	++ $^{24-120}$	+ $^{24-48}$	++ $^{48-72}$
L4/A4	+ $^{72-96}$	++ $^{24-48}$	++ $^{24-96}$	++ $^{48-96}$	++ $^{48-96}$
L5/A1	++ $^{48-120}$	++ $^{24-48}$	++ $^{24-72}$	++ ^{120}	++ $^{48-72}$
L7/A2	+ $^{24-120}$	++ ^{24}	++ $^{24-120}$	++ $^{24-48}$	++ $^{48-96}$
MGP/A5	++ $^{24-72}$	++ $^{24-48}$	++ $^{24-120}$	++ $^{48-96}$	++ $^{48-96}$
COL1/A1	+ $^{72-120}$	+ $^{72-120}$	+ $^{72-120}$	+ ^{120}	++ $^{72-96}$
COL1/A6	+++ $^{48-120}$	++ $^{24-72}$	++ $^{24-120}$	++ $^{48-120}$	+++ $^{48-96}$
COL1/A11	+ $^{48-120}$	++ $^{24-48}$	++ $^{24-48}$	++ $^{48-96}$	+++ $^{24-96}$
COL2/A2	++ $^{48-72}$	++ $^{24-48}$	++ ^{48}	++ ^{72}	++ $^{48-96}$
COL2/A6	++ ^{96}	++ $^{24-48}$	++ $^{24-48}$	++ $^{24-120}$	++ $^{48-96}$

Key: +, ++, +++: degree of PHA accumulation in comparison to glucose (positive control)

- : no PHA accumulation

Superscripted numbers indicate the duration (h) during which maximum fluorescence was observed

Table 2.3b Duration of maximum PHA production on various agronomic and industrial by-products

Isolates	Agronomic and industrial by-products				
	Coconut oil cake	**Citrus pulp**	**Whey**	**Bagasse (cane)**	**Rice chaff**
BHR-1/A7	++ ^{24}	++ ^{48}	++ ^{72}	-	-
BLQ-2/A7	++ ^{24}	+ ^{48}	+ $^{24-120}$	-	-
ICP-1/A3	+ $^{48-120}$	++ ^{48}	++ $^{48-120}$	-	-
NQ-11/A2	++ ^{24}	++ ^{48}	++ ^{48}	-	-
PPA/Z6	+ ^{48}	+ $^{48-72}$	-	-	-
L2/A1	++ ^{24}	++ ^{48}	+ $^{72-120}$	-	-
L4/A3	++ ^{24}	++ ^{48}	+ $^{24-120}$	-	-
L4/A4	++ ^{24}	+ $^{24-72}$	++ $^{48-120}$	-	-
L5/A1	++ ^{24}	++ ^{48}	+ $^{24-120}$	-	-
L7/A2	++ ^{24}	++ ^{48}	++ $^{48-72}$	-	-
MGP/A5	+ ^{48}	+ $^{24-72}$	++ $^{48-120}$	-	-
COL1/A1	-	+ $^{48-72}$	+ ^{72}	-	-
COL1/A6	++ ^{24}	++ $^{24-48}$	++ $^{72-120}$	-	-
COL1/A11	++ ^{24}	+ $^{24-72}$	++ ^{72}	-	-
COL2/A2	++ ^{24}	+ $^{24-72}$	-	-	-
COL2/A6	++ ^{24}	+ $^{48-72}$	++ $^{48-120}$	-	-

Key: +, ++, +++: degree of PHA accumulation in comparison to glucose (positive control)

- : no PHA accumulation

Superscripted numbers indicate the duration (h) during which maximum fluorescence was observed

A. Starch-based residues

Starch is abundantly found in nature and is a major component of the by-products or residues generated by various sectors in the food industry. For example, the potato processing industry generates large volumes of waste during the production of potato chips, slices and shredded potatoes. These starch-rich wastes can cause environmental problems if discharged in nature due to their high carbohydrate content (Mishra *et al.*, 2004). The potato consumption of MacDonald, India is estimated to reach 12,000 tonnes by the year 2010. A large number of confectionery products contain starch based ingrdients such as wafers, rice puffs, etc. Food manufacturing units producing instant foods (such as noodles, biscuits) also generate residues rich in starch which is sometimes disposed off as animal feed.

Starch has been evaluated as carbon source for PHA production by a number of research groups (Rusendi and Sheppard, 1995; Kim 2000; Halami 2008; Van-Thouc *et al.*, 2008). Since the isolates used in the present study were able to use starch for the production of PHA better than any of the other polymeric substrates tested, a number of starch-based residues were evaluated. It was observed that the isolates were able to grow on all the residues tested. However, the PHA accumulation pattern varied depending on the isolates as well as the type of residue used.

Wafer residue proved to be an ideal carbon source for PHA accumulation in isolates BLQ-2/A7 and COL1/A6 as these isolates exhibited maximum fluorescence intensity for two and four days respectively. Isolates such as NQ-11/A2, COL2/A6, COL1/A11 and COL2/A2 could accumulate PHA only after 48 h of incubation and continued synthesizing the polymer until termination of the experiment. Isolates COL1/A1 and L4/A3 started PHA accumulation after 72 h of incubation whereas isolates PPA/Z6 and MGP/A5 exhibited fluorescence within 24 h of incubation.

On medium supplemented with rice crispies as sole carbon source, majority of the isolates exhibited average fluorescence intensity. Only isolate COL1/A1 exhibited poor fluorescence intensity. This isolate was able to accumulate PHA from 72 h onwards whereas all the others exhibited fluorescence from the first day itself and continued PHA accumulation till the termination of the experiment, however, in most of the isolates the fluorescence intensity decreased with prolonged incubation.

It was observed that PHA accumulation on biscuit trimmings initiated after incubation for 48 and 72 h in the case of isolates ICP-1/A3 and COL1/A1, respectively. The intensity of fluorescence exhibited by the isolates COL1/A1 and PPA/Z6 was found to be weak on this substrate. However, the other isolates exhibited consistent fluorescence intensity for all five days.

Growth of all sixteen isolates was observed on medium containing bread trimmings and most of the isolates exhibited average fluorescence intensity. Isolates PPA/Z6, L4/A3 and COL1/A1 exhibited weak PHA accumulation. Further, variation in the duration of PHA accumulation was observed among these isolates.

B. Molasses (sugarcane)

Molasses is a sucrose-rich by-product of sugar manufacturing industry. It is considered ideal for fermentative processes as an inexpensive carbon source. Molasses consists of approximately 50% of sucrose and some amount of protein (6 to 9%) (Wee *et al.*, 2004). In India, about 19.55 million tonnes of molasses are generated and out of which 14% is exported without any value addition (ISMA, 2009).

Molasses served as an excellent source of carbon since all the isolates were able to grow luxuriantly on media supplemented with it. Maximum fluorescence intensity was observed with isolates BHR-1/A7, BLQ-2/A7, COL1/A11 for four days, NQ-

11/A2 and COL1/A6 for three days and ICP-1/A3 for two days. Interestingly, fifteen of the isolates tested were able to accumulate PHA for all 5 days monitored except COL1/A1 which started to accumulate PHA only after 48 h.

C. Coconut oil cake

Oil cakes are by-products generated during the oil extraction process from various oil seeds. These cakes can be used as a fuel or animal feed due to their rich protein content. However, mostly these wastes are discarded as such in the environment. The global production of oil cakes and meals is estimated to be approximately 99.7 million tonnes (FAO, 2009). These residues have been lately considered as carbon substrates for the production of industrial enzymes, antibiotics, etc. (Joo *et al.*, 2003; Kota and Sridhar, 1999; Shashirekha *et al.*, 2002).

Medium supplemented with coconut oil cake was able to serve as a substrate for growth. Most of the isolates exhibited average fluorescence when stained with Nile Blue A. Isolate COL1/A1 failed to accumulate PHA using this agro-industrial residue. Isolates ICP-1/A3, PPA/Z6 and MGP/A5 exhibited low fluorescence intensity. Most of the isolates were able to accumulate PHA during the initial two days of incubation. Only isolates BHR-1/A7 and ICP-1/A3 were able to exhibit fluorescence for a longer duration.

D. Citrus pulp waste

Industries dealing with the production of fruit based products such as juices, jams, fruit preserves generate large quantities of waste comprising of fruit peels, pulp waste and seeds. Due to its organic nature, these wastes tend to deteriorate rapidly and hence have to be disposed immediately. Among the various fruits used, fruits belonging to the citrus family are most abundantly utilized in the industries. According to the Food and Agriculture Organization (FAO) statistics, the total citrus fruit utilized for

processing are around 0.745 million tonnes and about half the amount is discarded as waste in the form of peels and pulp (FAO, 2009).

Citrus pulp waste when tested served as a good substrate for growth of all the isolates. However, only fifty percent of isolates tested were able to exhibit average fluorescence whereas the remaining exhibited low intensity. Isolates NQ-11/A2 and ICP-1/A3 were able to exhibit fluorescence during the entire duration monitored. Besides these two, the other isolates were able to produce PHA only for two or three days.

E. Whey

Whey, the residual watery portion of milk from dairy industry, represents about 80 to 90% of the total volume of milk transformed. About 130 to 145 million tonnes of whey is generated per year globally and only half of it is used for the production of lactose and lactic acid whereas the remaining is used as animal feed (Yellore and Desai, 1995). In addition to its surplus status, the environmental concern associated with the direct disposal of whey due to its high BOD makes this co-product stream a prime target for developing new uses for example serving as a feedstock for various fermentative processes. The added advantage is that it is inexpensive and easily available (Ahn *et al.*, 2000).

The different *Bacillus* spp. were tested for their ability to utilize whey as a carbon substrate for PHA production. Majority of the isolates tested exhibited average fluorescence whereas isolates L4/A3, BLQ-2/A7, L5/A1, L2/A1 and COL1/A1 exhibited poor fluorescence intensity. Isolates PPA/Z6 and COL2/A2 were unable to produce PHA.

F. Bagasse (sugarcane) and Rice chaff

Sugarcane bagasse is the major by-product of the sugar mills and represents about 30% by weight of the sugarcane processed (Selman-Housein *et al.*, 1999). In India, 355.52 million tonnes of sugarcane was harvested in the year 2008 (FAO, 2009). Sugarcane bagasse contains significant amounts of cellulose (40% of DM) and hemicellulose (21%) (Gong *et al.*, 1993). Despite the use of bagasse in the sugar mills to generate energy, this material is still available in large quantities and could be used for other purposes.

FAO has estimated the global rice production (milled) in the year 2008 to be about 685.013 million tonnes (FAO, 2009). Rice chaff represents about one fifth of the waste generated during the processing of rice. This residue contains 26% cellulose and 13.5% hemicellulose (Schultz *et al.*, 1984).

All the sixteen isolates were capable of growing on bagasse and rice chaff when individually supplied as sole carbon sources. However, the growth of the isolates was poor and none of the isolates were able to accumulate PHA even on prolonged incubation.

2.2.3 Analysis of the physico-chemical parameters of the agro-industrial by-products

Based on the response of the isolates towards growth and PHA production, by-products or residues such as coconut oil cake, bagasse, rice chaff, citrus pulp waste and wafer trimmings were selected for further studies. These by-products were analyzed for their physicochemical composition especially with respect to carbon and nitrogen content (Table 2.4a and 2.4b). High moisture content was observed with citrus pulp waste as compared with the other agricultural by-products. Therefore this

Table 2.4a Physical characteristics of agronomic & industrial byprodu

By-products	Colour	Wet /dry	Smell	Form	Texture	pH	Moisture conten: (%)
Citrus pulp waste	light yellow	wet	citrus	mass	fibrous	3.6-4.0	85.82
Coconut oil cake	brownish grey	dry	coconut oil	mass	granular	6.0-6.4	3.67
Wafer waste	light brown	dry	starchy	thin flat	fibrous	3.6-3.8	4.05
Rice chaff	golden brown	dry	starchy	small shells	fibrous	6.0-6.4	5.34
Baggasse	brown	dry	sweet smell	mass	fibrous	6.6-6.8	0.97
Molasses	dark brown	wet	sweet smell	syrup	viscous	5.1-5.4	20.5

Table 2.4b Chemical composition of selected agronomic and industrial by-products

By-products	Ammonia-nitrogen (mg N g^{-1})	Inorganic phosphate (mg P g^{-1})	Reducing sugar (g g^{-1})	Total sugar (g g^{-1})	C (%)	N (%)
Citrus pulp waste	0.308-0.325	2.48-2.65	0.436-0.456	0.658-0.708	44.4	1.1
Coconut oil cake	0.809-0.871	1.66-2.69	0.156-0.161	0.718-0.794	51.2	5.6
Wafer waste	Nil	Nil	Nil	1.26-1.396	45.2	Nil
Rice chaff	Nil	2.713-3.264	Nil	0.0534-0.056	42.3	1.2
Baggasse	Nil	Nil	Nil	0.0176-0.0235	45.5	Nil
Molasses	0.064-0.0824	Nil	0.292-0.3	0.782-0.844	48.7	0.4

waste needs to be dried before storage to avoid growth of unwanted microorganisms. The pH of all the by-products was below pH 7.0 suggesting the acidic nature of these wastes. As seen in Table 2.4b, all the by-products selected were rich in carbon content, 42.33% in case of rice chaff to 51.24% in case of coconut oil cake. A high percentage of this carbon is in the form of carbohydrates as seen from the total sugar content of these residues. In case of bagasse and rice chaff, negligible amount of total sugars was found even though presence of more than 40% of carbon in these by-products was estimated. The weak growth of the isolates and their inability to utilize these by-products can be attributed to the presence of carbon in a form not metabolizable by the isolates. Hence all the by-products except molasses were subjected to hydrolysis.

2.2.4 Hydrolysis of the agro-industrial by-products

The current method for the hydrolysis of carbon wastes (for PHA production) at an industrial scale employs dilute acid (Yu, 2007). It is cheap and releases 75 to 90% of the substrate as metabolizable sugars. Therefore, the by-products used in this study were also hydrolyzed using this method and the response of the isolates towards these hydrolysates supplied as sole carbon source was monitored. The response of the isolates towards acid hydrolysate of the pure polymeric substrates was also studied (Table 2.5).

The isolates were able to grow and accumulate PHA better on hydrolyzed starch as compared to unhydrolyzed soluble starch. The hydrolyzed products present in the medium being more amenable and of small molecular weight compounds as compared to unhydrolysed starch. Among the various starch-based residues, insoluble wafer trimmings were selected for further studies. All the isolates were able to utilize

Table 2.5 Duration of maximum PHA production on acid hydrolysates of agro-industrial by-products

Isolates	Hydrolysates of agro-industrial by-products						
	Starch*	**Pectin***	**Coconut**	**Bagasse**	**Rice chaff**	**Citrus**	**Wafer**
BHR-1/A7	+++ 24-72	++ 24-48	+ 24-72	+ 24	+ 24	+ 24-72	+++ 24-72
BLQ-2/A7	+++ 24-72	++ 24-48	++ 24-72	+ 24	+ 24	++ 24-72	+++ 48-72
ICP-1/A3	++ 48	-	+ 24-72	+ 24	+ 24	+ 24	++ 48
NQ-11/A2	+++ 24-48	++ 24-72	++ 24	+ 24	+ 24	++ 48-72	+++ 48-72
PPA/Z6	+++ 48	++ 48	+ 72	+ 24	+ 24	+ 24-48	+++ 48
L2/A1	+++ 24-48	++ 24-48	+ 24-72	+ 24	+ 24	+ 24-72	+++ 48-72
L4/A3	+++ 48	++ 24-48	++ 24	+ 24	+ 24	+ 24-72	+++ 48-72
L4/A4	+++ 24-48	++ 48	++ 72	+ 24	+ 24	++ 72	+++ 48-72
L5/A1	+++ 24-72	++ 24-48	+ 24-72	+ 24	+ 24	++ 48-72	+++ 48-72
L7/A2	+++ 24-48	++ 24-72	+ 24-72	+ 24	+ 24	++ 72	+++ 24-72
MGP/A5	+++ 24-72	+ 24-72	++ 72	+ 24	+ 24	++ 48-72	+++ 48-72
COL1/A1	+++ 48	+ 72	+ 72	+ 24	+ 24	+ 24-72	+++ 48
COL1/A6	+++ 24-72	+ 24-72	++ 24-72	+ 24	+ 24	++ 48-72	+++ 48-72
COL1/A11	+++ 24-48	++ 48	++ 24-72	+ 24	+ 24	+ 24-72	+++ 48-72
COL2/A2	+++ 24-72	+ 24-48	++ 24-72	+ 24	+ 24	++ 72	+++ 48-72
COL2/A6	+++ 24-48	+ 24-72	++ 24-72	+ 24	+ 24	++ 48-72	+++ 48-72

Key: +, ++, +++ degree of PHA accumulation in comparison to glucose (positive control); - no PHA accumulation

Superscripted number indicates the duration (h) during which maximum fluorescence was observed
*Analytical grade

and produce PHA on the acid hydrolysate of wafer trimmings. Isolate ICP-1/A3 exhibited average fluorescence at 48 h only. Isolate COL1/A1 started accumulating PHA after 48 h. Most of the isolates exhibited good fluorescence intensity. In comparison with the unhydrolyzed wafer trimmimgs, ICP-1/A3 and COL1/A6 did not show any visual difference in the fluorescence intensity. All the other isolates showed remarkable improvement especially in the case of isolates PPA/Z6, L2/A1, L4/A4, L7/A2, COL1/A1 and COL1/A11 where increased PHA accumulation was observed.

The hydrolysis of pectin was found to have a positive effect on the PHA synthesizing ability of the isolates as seen in Table 2.5. All the isolates except ICP-1/A3 were able to accumulate PHA on pectin hydrolysate.

All the isolates were able to grow and utilize the hydrolysate of citrus pulp waste for PHA production. The isolates exhibited PHA production on all the three days except PPA/Z6 which produced PHA only for an initial duration of two days. Some of the isolates exhibited fluorescence intensities different from that of unhydrolyzed citrus pulp waste. Hydrolysis adversely affected the intensity of four isolates BHR-1/A7, ICP-1/A3, L2/A1 and L4/A3. In contrast, the intensity was higher in the case of L4/A4, MGP/A5, COL2/A2 and COL2/A6 using citrus pulp hydrolysate.

Isolates BHR-1/A7, BLQ-2/A7, ICP-1/A3, L2/A1, L5/A1, L7/A2, COL1/A6, COL1/A11, COL2/A2 and COL2/A6 were able to consistently produce the polymer on coconut oil cake hydrolysate (COCH) for three consecutive days. In comparison with the unhydrolysed oil cake, ten isolates showed no difference in the intensity of fluorescence. However, a variation in the duration of maximum PHA accumulation was definitely observed (Table 2.4b and Table 2.5). PHA accumulating ability was

improved on hydrolysis in case of isolates BHR-1/A7, L2/A1, L5/A1, L7/A2, MGP/A5 and COL1/A1.

In case of bagasse and rice chaff hydrolysates, all the isolates exhibited growth as well as PHA production. However, the fluorescence exhibited was of poor intensity and visible only for one day. In comparison with their unhydrolyzed counterparts, acid hydrolysis improved the ability of these two by-products to serve as carbon feedstock for PHA production.

After the preliminary evaluation of various inexpensive and easily available agro-industrial residues, the polymer producing ability of the selected *Bacillus* isolates on these carbon substrates was quantified by cultivation under submerged conditions. These isolates were specifically selected based on the maximum intensity of fluorescence exhibited by the isolates on the respective substrates.

2.2.5 PHA production using submerged cultivation

A. Molasses

Molasses served as an excellent source for growth as well as polymer production. The growth of the isolates was highest on molasses (3.58 to 6.23 g L^{-1}) as compared with any other substrate. A PHA content as high as 68.56% DCW was achieved with ICP-1/A3 (Fig. 2.2). All the isolates grown under these conditions accumulated more than 50% DCW as PHA.

The enhanced growth and PHA production of the isolates can be attributed to the additional nutrients such as vitamins and minerals found in molasses which function as growth factors (Oliveira *et al.*, 2004; Kulpreecha *et al.*, 2009). The PHA content accumulated by *B. megateruim* BA-019 improved significantly when molasses rather than sucrose was used as a carbon source. This isolate was able to accumulate 55.46%

DCW as PHA when cultivated on molasses and urea (Kulpreecha *et al.*, 2009). Gouda *et al.* (2001) reported maximum PHA production using *B. megaterium* with cane molasses and glucose as sole carbon sources (40.8 and 39.9% DCW, respectively). These authors demonstrated that higher molasses concentration (3% w/v) resulted in increased growth whereas 2% molasses yielded maximum PHA content. *B. thuringensis* R1 cells was found to accumulate 22.95 and 31.36% DCW as PHA in the presence of molasses and table sugar, respectively (Rohini *et al.*, 2006). Wu *et al.* (2001) demonstrated that under fed-batch conditions, *Bacillus* sp. JMa5 could accumulate 25 to 35% PHA during fermentation using molasses as a sole carbon source.

B. Starch-based residue

Even though the isolates were capable of hydrolyzing starch with the enzyme amylase, acid hydrolysis of the wafer residue could not be avoided since the insoluble starch particles interfered during downstream processing. The resulting hydrolysate of wafer residue was hence used as a carbon source for PHA production. Majority of the isolates exhibited bright fluorescence indicating excellent PHA producing ability of these isolates on wafer hydrolysate. Isolates able to produce PHA on all the three days were further selected for submerged cultivation. Luxuriant growth of the isolates was observed with isolate L7/A2 producing maximum biomass of 5.24 g L^{-1} (Fig. 2.3). The PHA content accumulated in the various isolates ranged between 56.4 and 62.4% DCW.

Use of soluble starch without hydrolysis has been reported by Halami (2008) and Kim (2000). *B cereus* strain described by Halami (2008) was able to accumulate a PHA content of 48% DCW using starch based medium, whereas 46% DCW was obtained

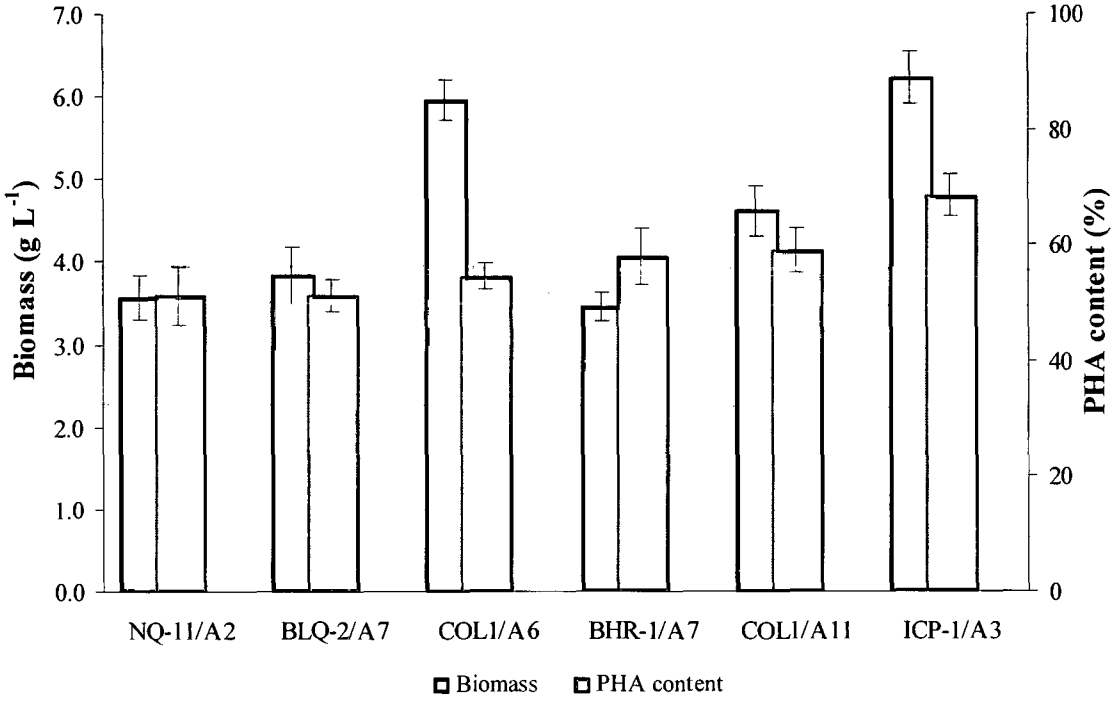


Fig. 2.2 Production of PHA in various *Bacillus* species using sugarcane molasses

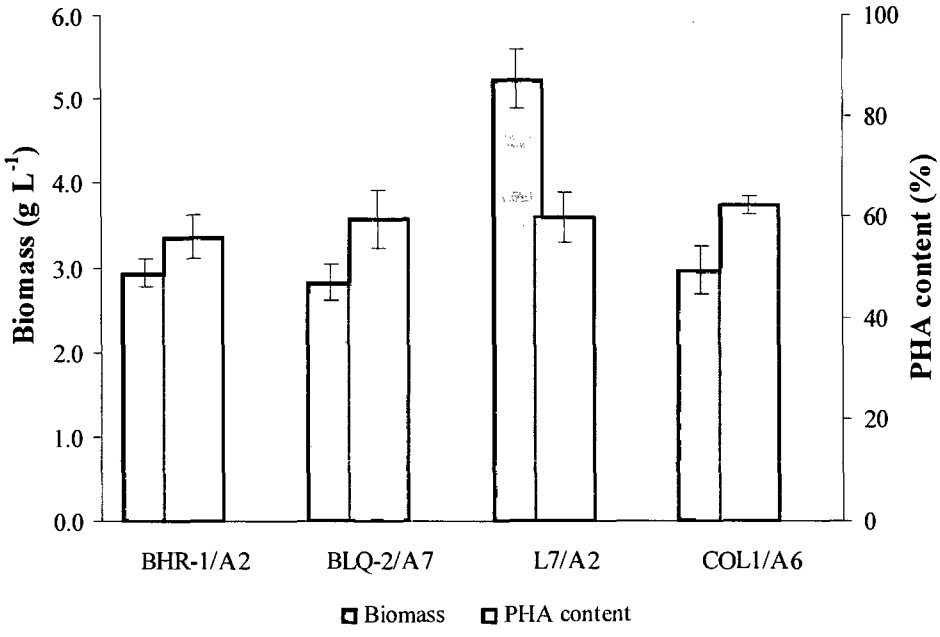


Fig. 2.3 Production of PHA in various isolates *Bacillus* species using wafer residue hydrolysate

using *Azotobacter chroococcum* (Kim, 2000). Lillo and Rodriguez-Valera (1990) have reported soluble starch as the ideal carbon substrate for *H. mediterranei* growth and polymer synthesis. Koutinas *et al.* (2007) have demonstrated the use of wheat hydrolysate and fungal extract (as carbon and nitrogen source, respectively) for PHA production in *C. necator*. Under these conditions, a PHA content of 70% DCW was achieved. Production of PHA from inexpensive extrude rice bran (ERB) and corn starch (ECS) employing *H. mediterranei* was investigated by Huang *et al.* (2006). Repeated fed-batch fermentation with ERB resulted in a PHA content of 38.7% DCW and on using a mixture of ERB and ECS resulted in a PHA content of 55.6% DCW. However, *B. sphaericus* NCIM 5149 grown on hydrolysates of cornflour and wheat bran was able to produce only 3.3 and 6.8% DCW of PHA content, respectively. It was observed that wheat bran hydrolysate favoured cell growth rather than PHA synthesis (Ramadas *et al.*, 2009). Using waste potato starch hydrolysate as the chief carbon source, Rusendi and Sheppard (1995) have reported PHA production with a yield of 77% DCW employing *Ralstonia eutropha. Halomonas boliviensis* LC1 attained a PHA content of 34% DCW when grown on wheat bran hydrolysate (Van-Thouc *et al.*, 2008).

C. Citrus fruit waste

Exploiting the ability of the various *Bacillus* species to grow and produce PHA from citrus pulp waste resulted in a PHA content ranging from 38.87% DCW (BLQ-2/A7) to 48.86% DCW (NQ-11/A2) (Fig. 2.4). Citrus pulp waste promoted biomass production with maximal growth observed in case of isolate BLQ-2/A7 (4.5 g L^{-1}) followed by COL1/A6 (4.13 g L^{-1}).

Till date no studies have been published reporting the use of citrus pulp waste as a carbon source. However, a number of studies are conducted using organic matter from wastes as an alternative to produce the polymer from inexpensive sources. These studies also highlight the necessity of incorporating a hydrolysis step prior to inoculation (Rebah *et al.*, 2009). Polymer production from mahua flower extract employing fermentations with *Bacillus* sp. 256 resulted in a PHA content of 51% DCW (Anil Kumar *et al.*, 2007). Kumar *et al.* (2009) have evaluated PHA production from pea-shell waste with the help of different *Bacillus* strains. They have reported higher yields (22 to 65% DCW) with enzyme hydrolysed substrate as compared to the unhydrolysed waste.

D. Coconut oil cake

Preliminary evaluation of coconut oil cake, an agroindustrial residue suggested it to be a potential carbon source for PHA production. Further, studies involving submerged fermentation were also carried out. Isolates exhibiting good fluorescence intensity on staining with Nile blue A were selected for these studies. Among the five isolates tested, isolate COL1/A6 exhibited maximal biomass and PHA of 3.75 and 1.58 g L^{-1}, respectively (Fig. 2.5) whereas the highest PHA content was observed with isolate COL1/A11 (42.4% DCW). The removal of adherent fatty acids from the coconut oil cake hydrolysate was achieved by a brief hexane wash thereby facilitating the quantification process (Lee *et al.*, 2000; Santimano *et al.*, 2009a).

Recently, a few studies have been conducted using oil cakes such as sesame, groundnut, mustard and palm under submerged fermentation conditions and babassu and soy cake using solid-state fermentation (Ramadas *et al.*, 2009; Singh and Mallick, 2009; Oliveira *et al.*, 2004). The PHA content accumulated using these oil cakes

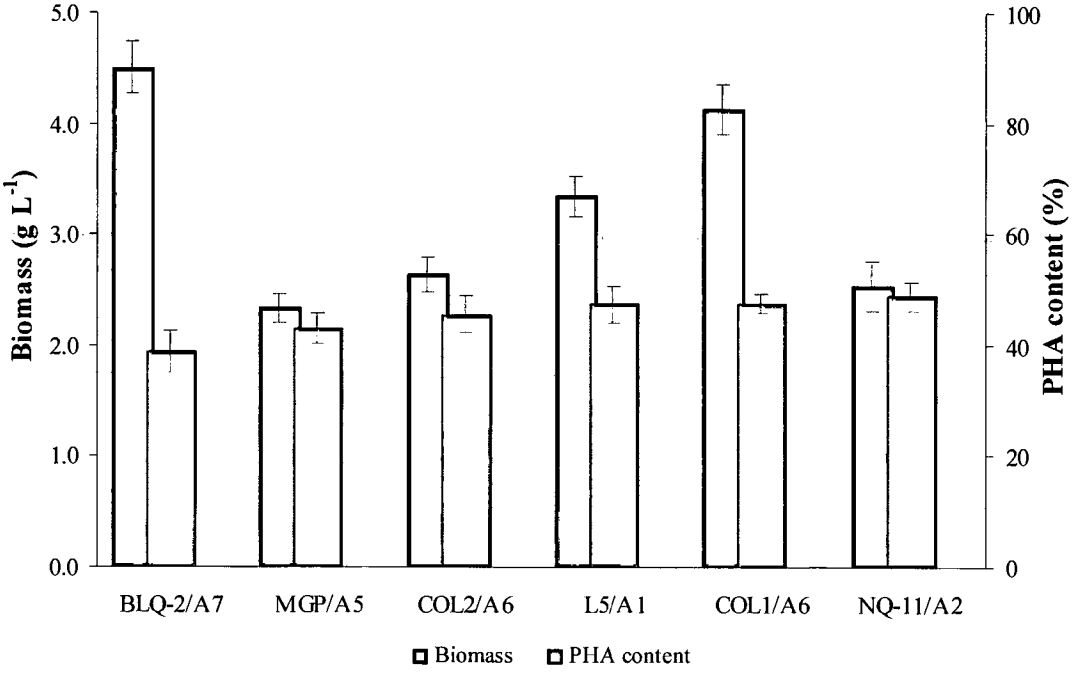


Fig. 2.4 Production of PHA in variuos isolates *Bacillus* species using citrus pulp waste hydrolysate

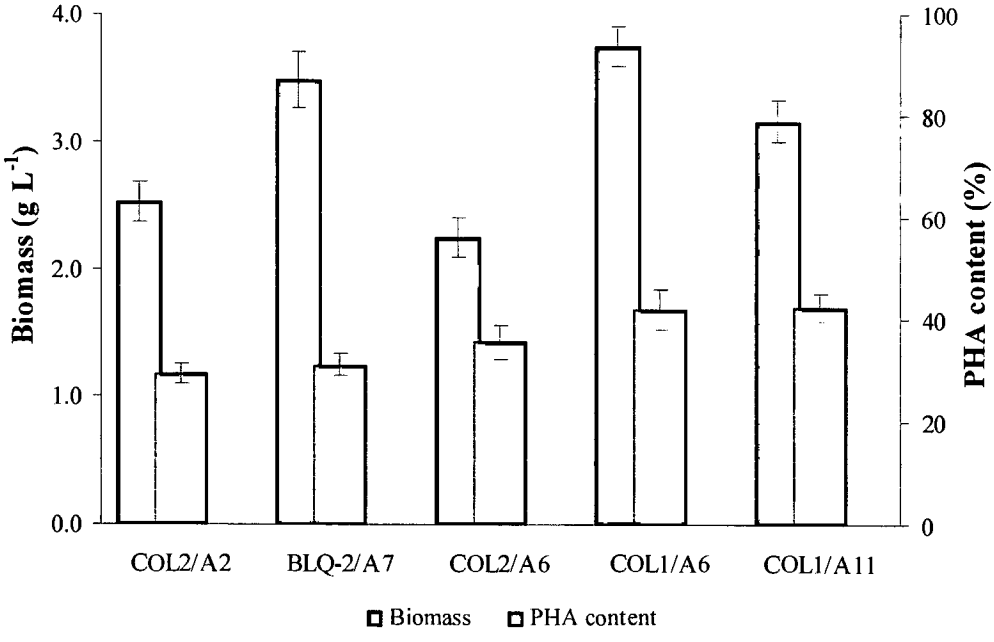


Fig. 2.5 Production of PHA by various isolates *Bacillus* species using coconut oil cake hydrolysate

varied from 14.0 to 39.2% DCW. As seen from these studies, the species belonging to the genus *Bacillus* are able to utilize the oil cakes better for growth as compared to Gram negative organisms.

Bacterial strains belonging to the genus *Bacillus* were evaluated for their PHA producing ability with glucose as sole carbon source. It was observed that the PHA content within the bacterial cells varied with the incubation time. Isolates BLQ-2/A7, COL1/A6, L2/A1 and L4/A4 were able to consistently maintain the maximally produced PHA till the termination of the experiment.

Among the polymeric carbon substrates tested, starch proved to be an excellent source for PHA production. All the *Bacillus* isolates (except isolates PPA/Z6 and COL1/A1) were able to synthesize PHA on majority of the polymers tested. In comparison to the other by-products, molasses served as an ideal carbon feedstock for PHA production since majority of the isolates were able to accumulate the polymer in large amounts. Isolates were unable to utilize bagasse and rice chaff as raw material for polymer production. Many of the isolates were able to produce PHA on the agroindustrial by-products tested with isolates COL1/A6 and BHR-1/A7 proving to be most versatile in their carbon utilization range. As observed earlier with pure substrates, PPA/Z6 and COL1/A1 exhibited poor PHA accumulating ability from agricultural and industrial residues.

Further, hydrolysis of these by-products using dilute acid not only improved the ability of the isolates to assimilate the released fermentable sugars as PHA but also avoided the interference caused by the insolubles present in the wastes during downstream processing when grown under submerged cultivation conditions.

Quantitation of PHA using submerged cultivation of selected isolates on molasses and hydrolysate of wafer residue resulted in high polymer production which ranged from 51.23 to 68.56% DCW in case of molasses and 56.14 to 62.41% DCW in case of wafer hydrolysate.

Based on their PHA producing ability, maintenance of maximally produced polymer and diversity in utilization of agro-industrial by-products as carbon feedstock for PHA production, isolates COL1/A6 and BLQ-2/A7 were selected for further studies.

Chapter III

Fermentation strategies for increased PHA yield

Section I

Optimization studies

INTRODUCTION

In order to make the overall PHA production process economically feasible for industrial applications, high yields of PHA are essential. The PHA yield is largely determined by the cultivation media and the fermentation strategy employed besides the type of strain used. Therefore designing and optimizing the media composition is a crucial step in order to achieve high PHA yields.

Even though, accumulation of PHA in *Bacillus* species was the first to be reported (Lemoigne, 1926), most PHA production and optimization studies were focused on Gram negative bacteria and till date, very few reports on optimization of PHA production using *Bacillus* species are available.

Therefore, the main aim of this chapter was to optimize the fermentation conditions using two potential PHA accumulating *Bacillus* isolates to achieve high PHA yields. These isolates namely, COL1/A6 and BLQ-2/A7 were selected based on their diversity in substrate utilization and PHA accumulation pattern in medium supplemented with glucose. The effect of various parameters on biomass and PHA production was studied. In all these studies, glucose was used as the carbon source since it is the most easily metabolizable sugar.

Growth and PHA production was also monitored under optimized conditions using batch fermentation. Further studies were conducted to gauge the influence of varying concentrations of carbon and nitrogen on biomass and PHA accumulation using response surface methodology. Improvement of PHA content employing fed-batch cultivation system was also carried out.

3.I.1 MATERIALS AND METHODS

3.I.1.1 Isolates selected for study

The two *Bacillus* isolates namely; COL1/A6 and BLQ-2/A7 were selected for the optimization studies due to their ability to utilize a wide variety of agroindustrial by-products for PHA production and the pattern of PHA synthesized using glucose as sole carbon source under submerged cultivation conditions.

3.I.1.2 Identification of bacterial strains

A. Extraction of genomic DNA from bacterial cells

The genomic DNA was extracted from the selected bacterial isolates using the method described by Marmur (1961) and Sambrook *et al.* (1989). Twenty five ml of nutrient broth was inoculated with a loopful of a single isolated colony and incubated at 150 rpm for 24 h. The culture broth was centrifuged at 5,000 rpm for 10 min at 4°C. The pellet was washed with 10 ml saline EDTA (Appendix 4). The washed pellet was resuspended in saline EDTA. One ml of this suspension was incubated with 10 µl of lysozyme solution (5 mg ml^{-1}) at 37°C for 30 min. The suspension was further incubated at 60°C for 10 min after addition of 100 µl sodium dodecyl sulphate (25%), 100 µl of sodium chloride (5M) and 50 µl of proteinase K (20 mg ml^{-1}). The contents were thereafter centrifuged at 8,000 rpm for 5 min under cold conditions. To the supernatant obtained, equal volumes of saturated solution of phenol-chloroform-isoamyl alcohol (PCI) was added and mixed continuously for 10 min. The mixture was then centrifuged at 5,000 rpm for 10 min. The aqueous layer was carefully collected and treated again with saturated PCI solution. This step was repeated thrice. Chilled absolute ethanol was added slowly from the side of the tube and allowed to stand for 10 min. The precipitate obtained was collected in small microfuge tube

using a hooked Pasteur pipette. The ethanol dried precipitate was dissolved in 100 µl of Tris-EDTA (TE) buffer (Appendix 4) and subjected to RNAase treatment (5 µl, 1 mg ml^{-1}) for 30 min at 37°C. The suspension was subjected to PCI treatment again. The genomic DNA in the aqueous phase was precipitated by the addition of chilled ethanol and centrifuged at 10,000 rpm for 10 min under cold conditions. The supernatant was discarded and the precipitate was dried by evaporating the ethanol. The precipitate was finally dissolved using minimum volume of TE buffer and the absorbance was recorded both at 260 and 280 nm. The extracted chromosomal DNA was sent to Jawaharlal Nehru University, New Delhi for amplification (Appendix 4) and sequencing of 16S rRNA gene.

B. Identification of bacterial strains

The sequences of the partial 16S rRNA received were compared with the 16S rRNA sequences available in the public nucleotide databases at the National Center for Biotechnology Information (NCBI) using its world wide web site (http://www.ncbi.nlm.nih.gov), and the BLAST (Basic local alignment search tool) algorithm.

3.1.1.3 Optimization of the culture medium for PHA production

Different environmental and nutritional parameters were varied to optimize the conditions for maximum PHA accumulation. This was achieved by varying one parameter at a time while keeping the others constant. A glucose concentration of 20 g L^{-1} was supplemented in the medium for all the experiments. E2 mineral medium (Appendix 1) was used in all the experiments.

The individual flasks were inoculated with 5% (v/v) of culture inoculum (as described in chapter II) and incubated at 30°C for 48 h under shaking conditions (170 rpm). At

the end of the incubation period, the culture broth was processed in order to estimate the biomass and PHA produced using analytical methods as described in Chapter II.

A. Effect of temperature

The effect of various temperatures on the cell growth and PHA production in the selected isolates was assessed. The range of temperature selected for the study was between 10 and 50°C.

B. Effect of initial pH

The pH optima for growth as well as PHA accumulation were determined by cultivating the isolates in the medium adjusted to desired pH values. The pH values ranging from 5.0 to 8.0 were tested.

C. Effect of different nitrogen sources

The utilization of different nitrogen sources was tested in E2 mineral medium minus microcosmic salt and supplemented with ammonium chloride, ammonium sulphate and ammonium phosphate.

D. Effect of yeast extract

The effect of one of the routinely used complex nutrients i.e. yeast extract on the growth of the organism and PHA accumulation was monitored. The concentration of yeast extract in the culture medium was varied between 0.1 and 5 g L^{-1}.

3.I.1.4 Batch fermentation

A time course study using batch fermentation under the optimized conditions was carried out.

A. Preparation of inoculum

The inoculum was prepared in a 250 ml Erlenmeyer flask containing 100 ml of sterile production medium (Appendix 1) supplemented with 1% (v/v) glucose. The medium was inoculated with a single bacterial colony from overnight grown nutrient agar plate. The inoculum flask was incubated at 30°C for 24 h on Orbitek environmental shaker at 170 rpm.

B. Batch fermentation

The seed culture (5% v/v) was inoculated in the production medium [with glucose (2% w/v) as the carbon source]. In order to maintain reproducibility and comparable conditions of aeration after sample removal, three flasks (set I, II and III) were simultaneously inoculated with the same inoculum. Samples were withdrawn from flask I immediately after inoculation. The next sample was withdrawn at an interval of 6 h from flask II followed by the withdrawal of culture broth at the subsequent interval from flask III. This pattern of sample withdrawal was followed throughout the experiment. Appropriate volumes of culture broth were removed (maximum 50 ml) aseptically from each flask for the analysis of dry cell weight, PHA production, residual glucose and nitrogen concentration and for the determination of pH.

3.I.1.5 Analytical methods

Determination of DCW and PHA was carried out as described in chapter II. Nitrogen and reducing sugar concentrations were estimated using methods described by Franson (1985) and Miller (1959) (Appendix 3). The pH of the fermented culture broth was analyzed using a pH analyzer (LABINDIA).

All the experiments were carried out in triplicate to examine the reproducibility and the average values are reported.

3.I.2 RESULTS AND DISCUSSION

Out of the sixteen *Bacillus* strains, two potential PHA producers COL1/A6 and BLQ-2/A7 were selected further for optimization studies due to their ability to synthesize intracellular polymer using a wide variety of agroindustrial by-products as well as the pattern of PHA production exhibited by these isolates using glucose as sole carbon source. The identification of these isolates by analyses of their 16S rRNA gene sequences has been described below.

3.I.2.1 Identification of the selected isolates

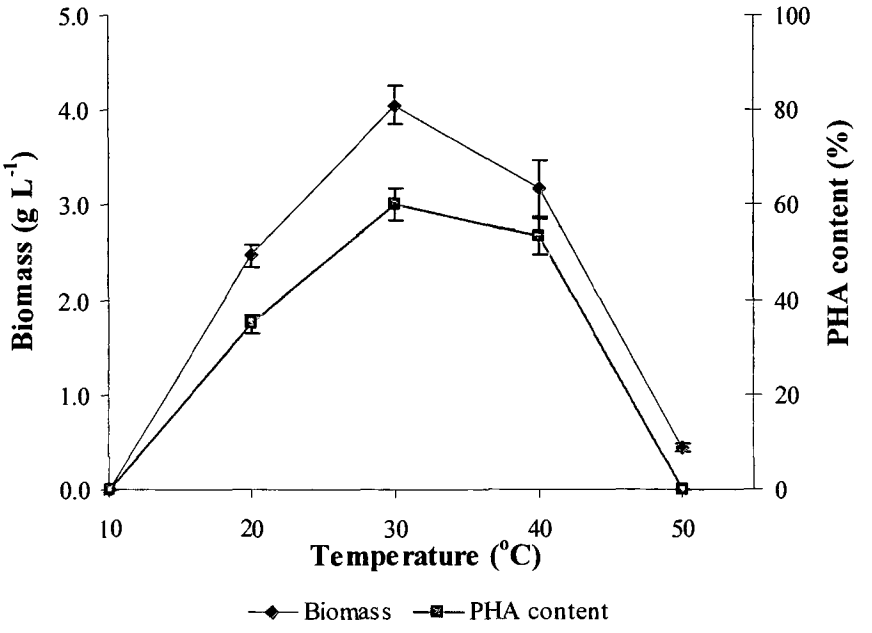
The 16S rRNA sequence analysis of the selected isolates confirmed that these two strains belonged to the genus *Bacillus*. The isolate COL1/A6 displayed maximum homology to *Bacillus megaterium* (97%) whereas the 16S rRNA sequence of the isolate BLQ-2/A7 showed maximum similarity to *Bacillus* sp. (98%). The partial 16S rRNA gene sequences have been deposited in NBCI with the accession numbers EU702754 (COL1/A6) and EU924811 (BLQ-2/A7) assigned to them.

3.I.2.2 Optimization studies

Studies carried out to deduce the effect of temperature on growth and PHA production revealed that both the isolates were able to accumulate PHA between 20°C and 40°C with optimal growth and maximum PHA accumulation at 30°C. The PHA content was found to be higher in isolate COL1/A6 as compared to BLQ-2/A7. Both the isolates were unable to grow at 10°C. No PHA was synthesized at 50°C even though poor growth of the isolates was observed (Fig. 3.1A and 3.1B). Wu *et al.* (2001) has reported growth of *Bacillus* sp. JMa5 at 47°C and emphasized the significance of a thermotolerant strain for industrial applications.

PHA production amounting to 60.25% of the total DCW with isolate COL1/A6 and 55.97% in case of isolate BLQ-2/A7 was observed at the optimum temperature. The

(A)



(B)

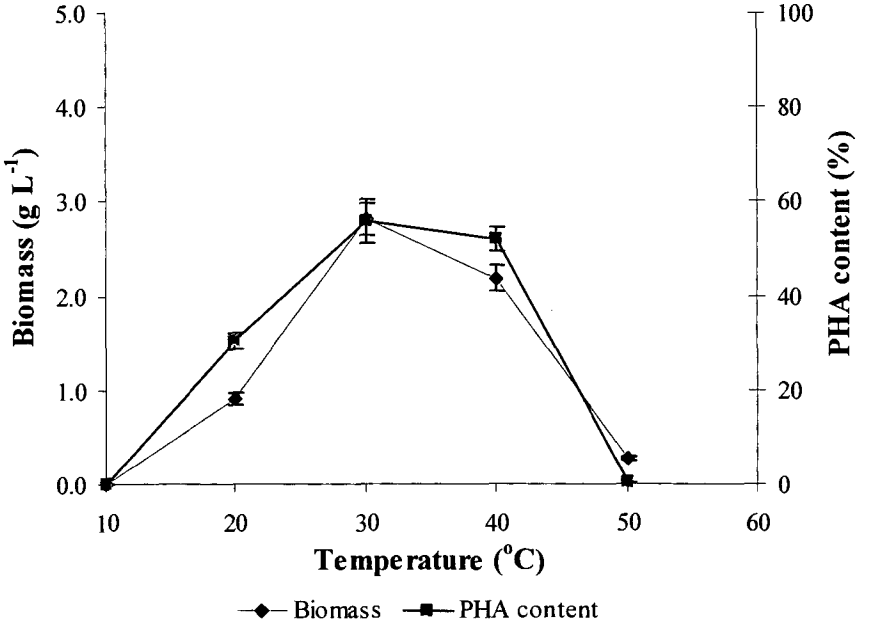


Fig. 3.1 Effect of temperature on the biomass and PHA production **(A)** isolate COL1/A6 and **(B)** isolate BLQ-2/A7

PHA yields obtained on either side of the temperature optima were relatively lower however the polymer production was higher at 40°C than at 20°C in both the isolates. In most of the studies conducted, the optimum temperature reported for PHA fermentations using *Bacillus* species is 30°C (Borah *et al.,* 2002; Omar *et al.*, 2001; Full *et al.*, 2006; Halami, 2008; Philip *et al.*, 2009; Shamala *et al.,* 2003; Aslim *et al.,* 2002; Valappil *et al.,* 2007b; Anil Kumar *et al.,* 2007; Kulpreecha *et al.,* 2009; Prabhu *et al.*, 2009). These fermentations resulted in PHA yields ranging from 11.5 to 69.4% DCW. However, Full *et al.* (2006) have reported extremely high yields of PHA using *Bacillus* sp. strain CL1, a close relative of *B. megateruim,* accounting to about 90% DCW. Other temperatures ranging from 28°C to 45°C have also been reported for growth and PHA accumulation in various *Bacillus* species (Aslim *et al.*, 2002; Rohini *et al.,* 2006; Tajima *et al.*, 2003; Kumar *et al.,* 2009; Wu *et al.*, 2001; Pandian *et al.*, 2010). PHA biosynthesis has been reported at temperatures as high as 45°C for *Bacillus* sp. INT005. However, the PHA yield obtained at this temperature was around 1.7% DCW which was comparatively lower than that obtained at 41°C (28.5% DCW) (Tajima *et al.*, 2003).

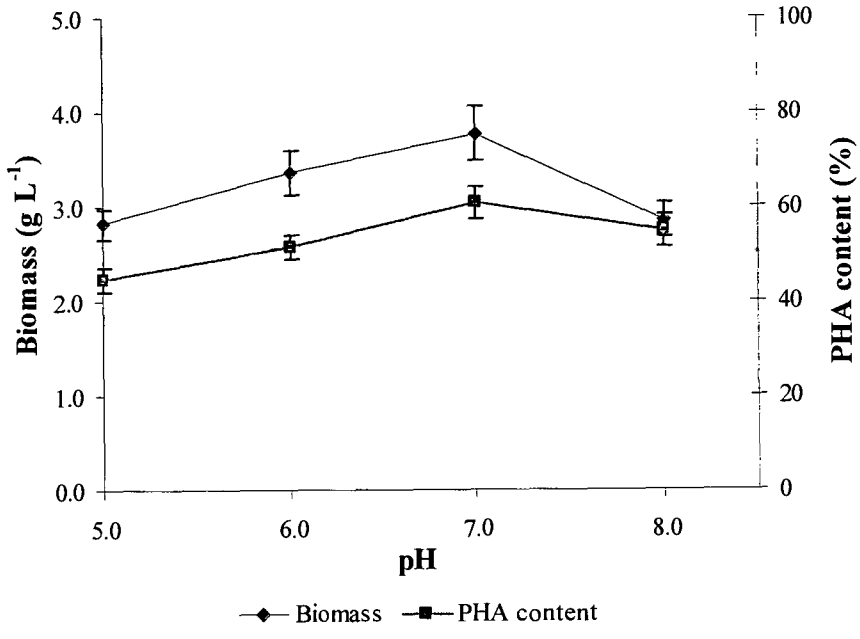
Although the isolates reported in the current study yielded lesser PHA as compared to some of the reported *Bacillus* species (Borah *et al.,* 2002 and Full *et al.,* 2006), their temperature optima ensures that no additional production costs are involved or associated with its growth, thereby reducing the cost of fermentation. In addition, temperature fluctuation of even up to 10°C higher did not adversely affect the polymer producing ability since the PHA yields varied only between 3 to 7% from the optimum.

As the ability of cells to accumulate PHA is greatly influenced by the pH of the culture medium (Kominek and Halvorson, 1965), the effect of initial pH was studied. Results obtained from these studies indicated that both, strain COL1/A6 and BLQ-2/A7 exhibited pH optima of 7.0 for growth as well as PHA production. Highest PHA yields of 60.71% DCW and 56.87% DCW were obtained, respectively (Fig. 3.2A and 3.2B). The optimum pH reported for maximum PHA accumulation using various *Bacillus* species is near neutrality (Borah *et al.*, 2002; Tajima *et al.*, 2003; Anil Kumar *et al.*, 2007; Kumar *et al.*, 2009). Full *et al.* (2006) have reported an optimum pH range of 7.0 to 7.5 for growth of different *Bacillus* species. In contrast, Pandian *et al.* (2010) have reported isolation of a *B. megaetrium* strain from brackish water accumulating maximum PHA at initial pH of 9.0.

Initial pH value of 5.0 was found to affect PHA production. The biomass of the culture obtained at pH 5.0 was less as compared to the other pH values. Similar findings using *Bacillus thuringensis* R1 have been documented by Rohini *et al.* (2006) suggesting medium acidosis responsible for low cell mass formation. However, the isolates COL1/A6 and BLQ-2/A7 were still able to accumulate PHA in such acidic conditions and yields up to 44.65% and 38.03% DCW were obtained, respectively. In contrast, growth of *Alcaligenes eutrophus* is reported to be completely inhibited at pH values below 5.4 with the optimum being near neutrality (Repaske, 1962; Beaulieu *et al.*, 1995).

The isolates used in the present study were able to grow and accumulate significant amounts of PHA in the fermentation medium adjusted to pH values either one unit above or below their pH optima. This is in contrast to the results reported by Ramadas *et al.* (2009) wherein the PHA accumulation by *B. sphaericus* was largely influenced

(A)



(B)

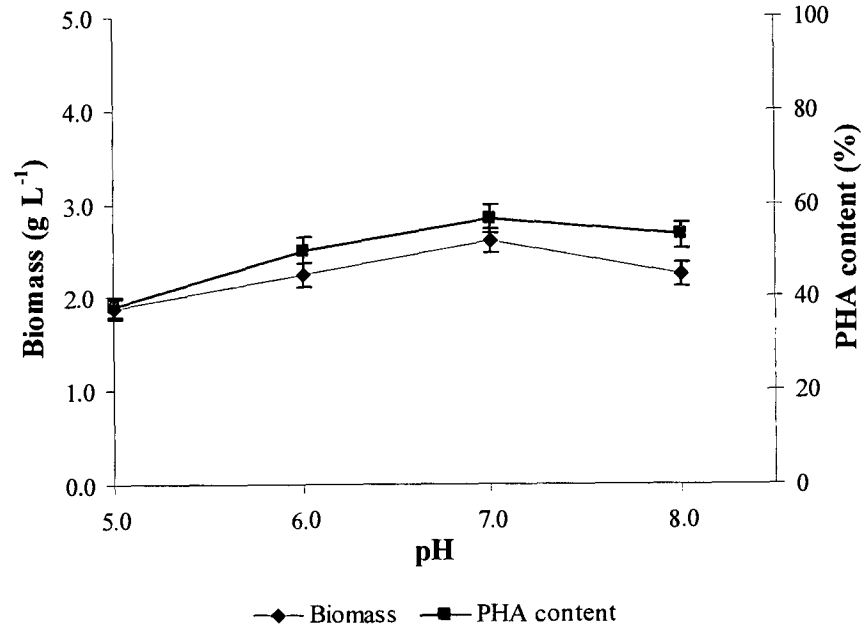


Fig. 3.2 Effect of pH on the biomass and PHA production **(A)** isolate COL1/A6 and **(B)** isolate BLQ-2/A7

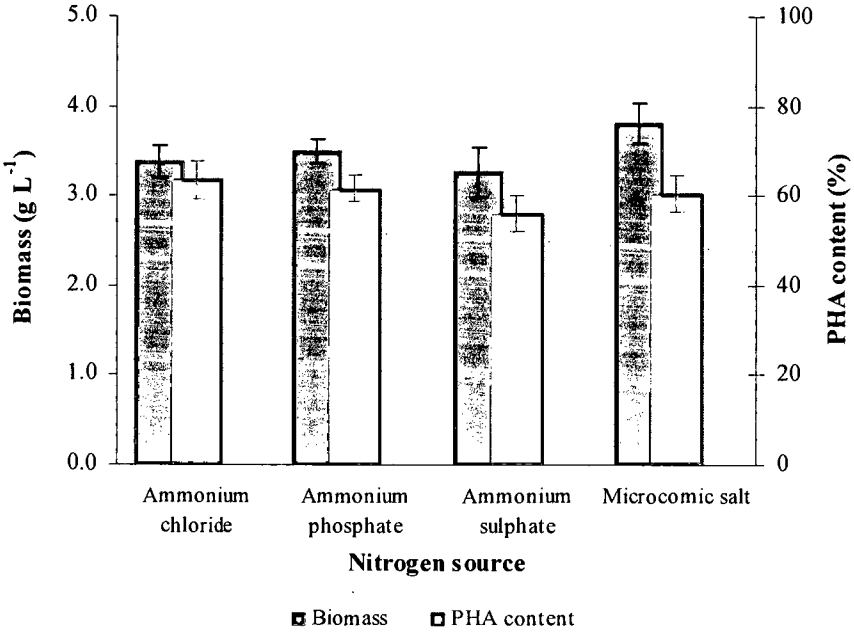
by variation in pH and even a slight deviation of the pH value from the optimum resulted in sudden decline in PHA biosynthesis.

Ammonium salts are widely used as a source of nitrogen for growth and production of PHA in fermentation media supplemented with a variety of carbon sources (Annuar *et al.,* 2008). Hence, the effect of various ammonium salts as a nitrogen source was tested at concentrations equivalent to ammonium provided by microcosmic salt.

Among the various salts tested, highest PHA yields were obtained using ammonium chloride as a nitrogen source. The PHA yield obtained for COL1/A6 was found to be 63.56% DCW and that for BLQ-2/A7 was 59.76% DCW (Fig. 3.3A and 3.3B). Ammonium chloride was also found to be an ideal nitrogen source among the others tested for PHA fermentation using *B. megaterium* by Omar *et al.* (2001). The addition of ammonium chloride almost doubled the polymer accumulation in the cells. Prabhu *et al.* (2009) have also reported maximum PHA production with ammonium chloride as compared with other ammonium salts. In contrast, Borah *et al.* (2002) have reported suppression of PHA synthesis when *B. mycoides* was supplied with ammonium chloride or ammonium nitrate as a nitrogen source. They have reported drastic reduction using the above ammonium salts whereas highest yield of PHA (69.4% DCW) were obtained using ammonium sulphate as a nitrogen source.

Highest biomass production of 3.80 g L^{-1} was obtained in case of COL1/A6 using microcosmic salt and 2.62 g L^{-1} with BLQ-2/A7. Highest accumulation of PHA was also observed with microcosmic salt. However, the overall yield was lower than that obtained using ammonium chloride. The results indicate that the ammonium salt supporting highest growth did not result in highest PHA yields suggesting that the yield of PHA is not always related to increase in growth (Gouda *et al.*, 2001).

(A)



(B)

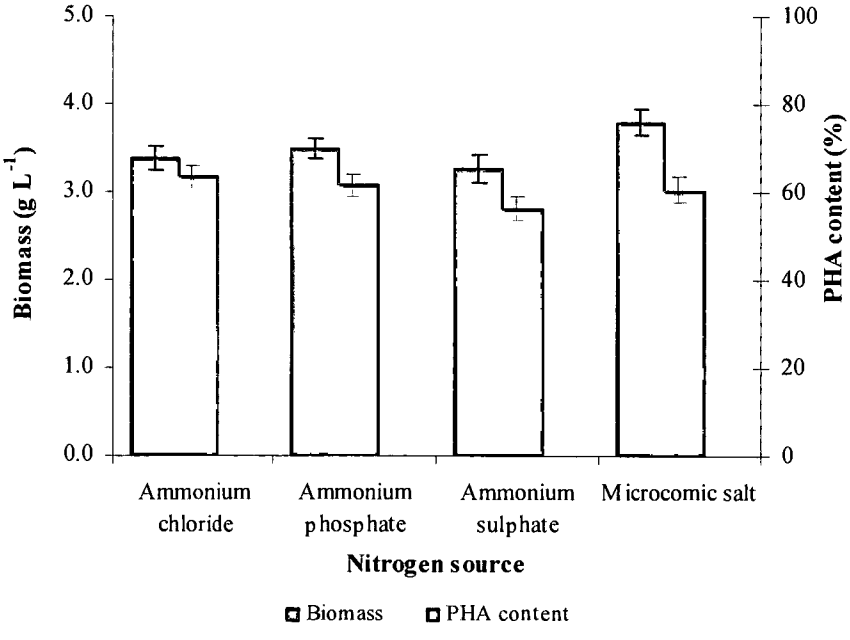


Fig. 3.3 Effect of different ammonium salts as nitrogen source on the biomass and PHA production **(A)** isolate COL1/A6 and **(B)** isolate BLQ-2/A7

Reports suggest enhanced PHA production on supplementation with complex nutrients such as yeast extract, peptone and beef extract (Annuar *et al.,* 2008, Khanna and Srivastava, 2005b). Therefore in this study, effect of one of the most routinely used complex nutrient i.e. yeast extract was studied.

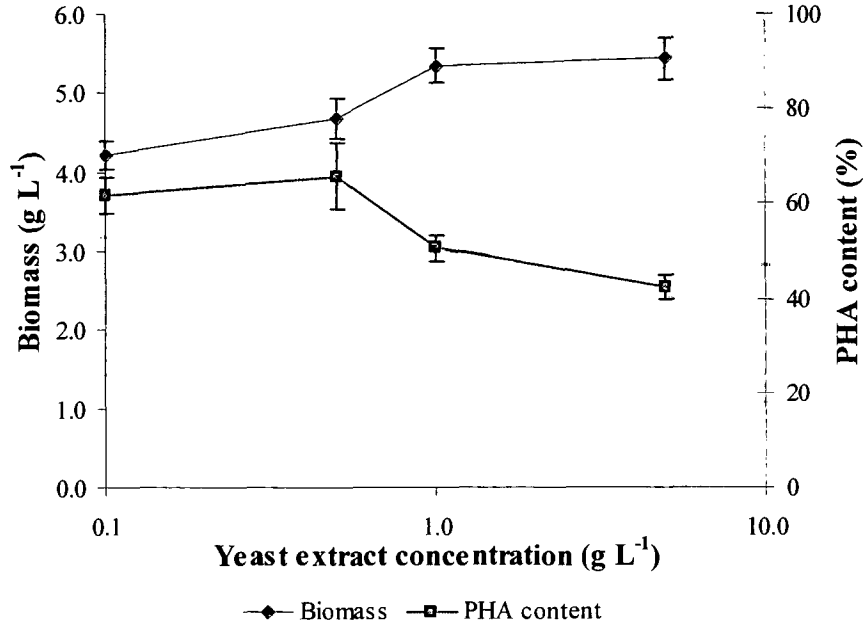
Optimization studies carried out using different yeast extract concentrations indicated that a lower concentration range from 0.1 g L^{-1} to 0.5 g L^{-1} was ideal for PHA production (Fig. 3.4A and 3.4B). Further increase in the concentration up to 5 g L^{-1} resulted in enhanced growth. Highest biomass of 5.4 g L^{-1} was obtained with COL1/A6 whereas 3.8 g L^{-1} was obtained with BLQ-2/A7. However, highest PHA yields of 65.85% DCW and 59.64% DCW were achieved using 0.5 g L^{-1} yeast extract concentration in case of COL1/A6 and BLQ-2/A7, respectively.

The enhanced biomass production with higher yeast extract concentration could be attributed to the synthesis of other cellular material favoring cell growth rather than PHA formation. Berbert-Molina *et al.* (2008) found that yeast extract compounds are preferentially used for biomass production in case of *Bacillus thuringensis* var *israelensis.* There are reports demonstrating that the use of some complex nutrient sources such as yeast extract decreases PHA synthesis in *Bacillus megaterium* upto 70% even though growth is favored (Gouda *et al.,* 2001). Similar results have been cited by Omar *et al.* (2001) wherein the PHA content was reduced to almost half when compared with that in the fermentation medium lacking the complex nutrient.

3.II.2 Batch fermentation using optimized conditions

The batch kinetic results obtained under the optimized conditions for growth and PHA accumulation using **isolate COL1/A6** is displayed in Fig. 3.5.

(A)



(B)

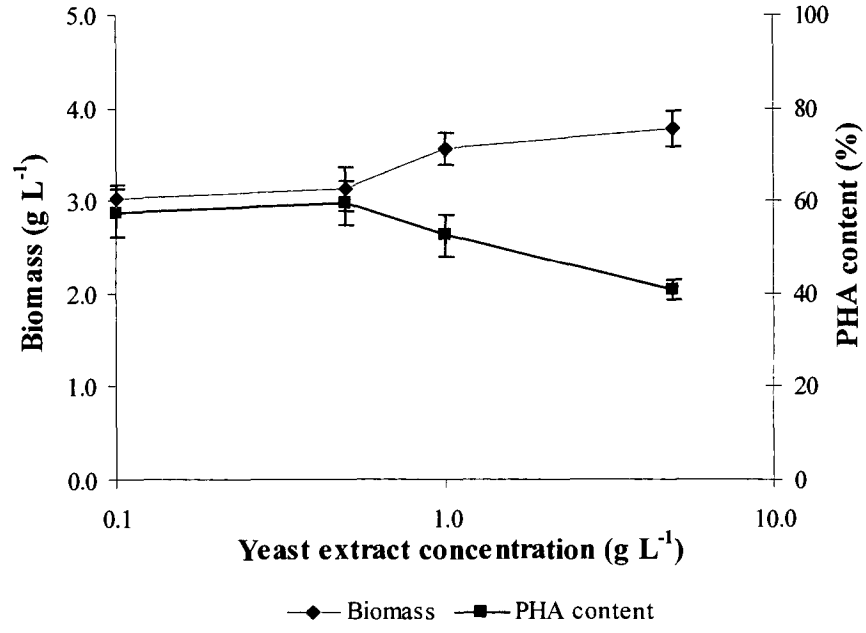


Fig. 3.4 Effect of varying yeast extract concentration on the biomass and PHA production **(A)** isolate COL1/A6 and **(B)** isolate BLQ-2/A7

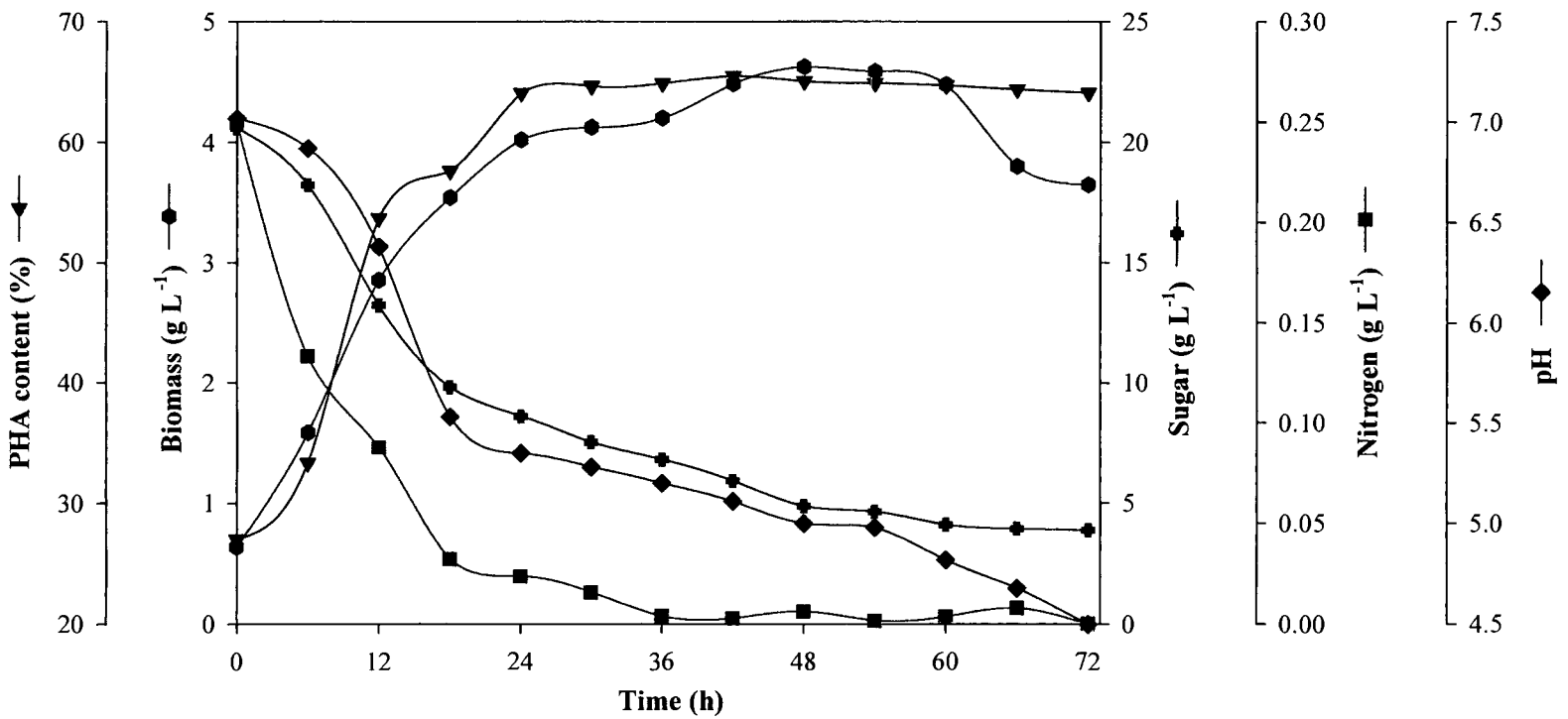


Fig. 3.5 Kinetics of PHA production by *Bacillus* sp. COL1/A6 in shake flask culture under optimized conditions

As seen from the profile, a sharp increase in biomass was observed till 24 h of cultivation indicating active growth. On further incubation, the biomass concentration increased steadily with maximal accumulation of 4.63 g L^{-1} at 48 h. Thereafter a decline in the accumulated biomass was observed.

The PHA content exhibited a similar trend as observed with biomass accumulation. Maximum PHA content of 65.51% DCW was obtained at 42 h of incubation and further cultivation to 72 h did not result in any significant change in the PHA content. Approximately 50% of the sugar was utilized during the first 18 h of growth which coincided with the exponential growth phase of the organism. With incubation till 60 h, a further reduction in the residual sugar was observed and only 20% of the initially supplied sugar was left unutilized in the medium. No further consumption of sugar was observed on incubation beyond 60 h.

The concentration of nitrogen in the medium was also found to decrease as the biomass increased and a rapid decline was observed till 18 h. The pH of the culture broth decreased rapidly during the exponential growth phase. A continuous decrease in the pH was observed as the fermentation time increased. From an initial pH of 7.02, the pH value reduced to a minimum of 4.5 at the end of 72 h.

In case of the isolate **BLQ-2/A7**, the bacterium grew rapidly and entered the stationary phase within 18 h of incubation. The concentration of biomass remained fairly constant till 72 h of cultivation. Maximum biomass concentration of 4.23 g L^{-1} was achieved within 24 h under the optimized conditions (Fig 3.6).

The PHA content accumulated by the cells exhibited a profile similar to growth. The polymer was actively synthesized till 18 h of growth. The PHA content continued to

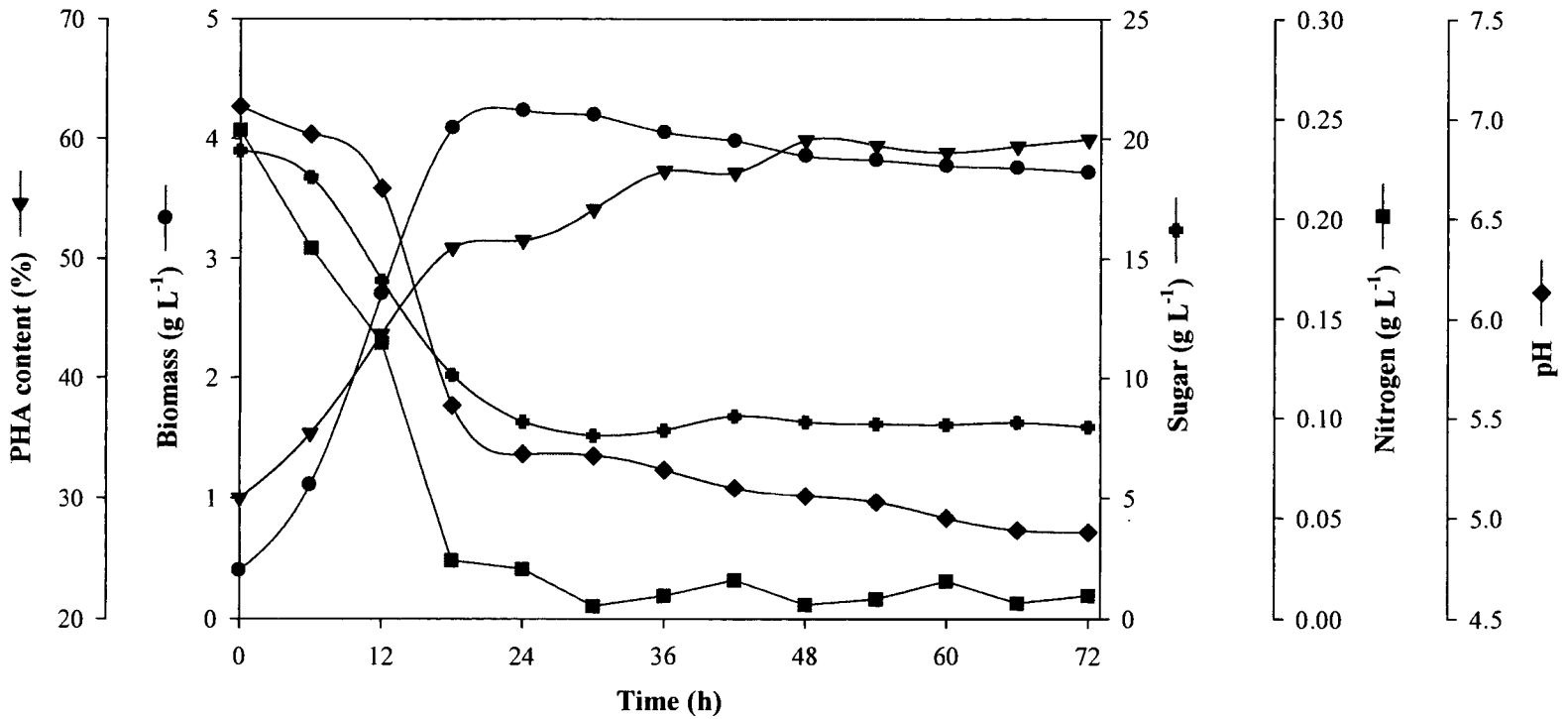


Fig. 3.6 Kinetics of PHA production by *Bacillus* sp. BLQ-2/A7 in shake flask culture under optimized conditions

increase gradually even during the stationary phase. The highest PHA content achieved was 59.87% DCW within 48 h of cultivation.

The isolate showed rapid consumption of sugar within the first 24 h of incubation and approximately 60% of the sugar supplied was consumed. However, no further consumption was observed till the end of the fermentation.

Rapid decrease in the nitrogen concentration was observed during the logarithmic phase of growth. A simultaneous drop in pH of the fermentation broth along with the active growth, PHA accumulation and sugar consumption was observed. The pH dropped from an initial of 7.06 to 4.93 at the end of the fermentation run.

Batch kinetic studies in shake flasks using various *Bacillus* species have been reported by a number of researchers and their results have been complied in Table 3.1. Comparison of the biomass and PHA yield revealed highest biomass production using COL1/A6 followed by BLQ-2/A7, whereas, highest PHA content of 69.4% DCW was exhibited by *B. mycoides* RLJ B-017 (Borah *et al.,* 2002).

The PHA accumulation in isolate COL1/A6 as well as BLQ-2/A7 reached its peak during the stationary phase. The highest polymer accumulation of 3.01 g L^{-1} and 2.32 g L^{-1} was observed in case of COL1/A6 and BLQ-2/A7 at 48 and 36 h respectively. An increase in PHA content during the stationary phase was also observed using *B. cereus* SPV and *B. flexus* (Valappil *et al.,* 2007c; Somashekara *et al.,* 2009).

Sugar and nitrogen utilization pattern studied in different *Bacillus* species by Somashekara *et al.* (2009) and Faccin *et al.* (2009) indicated rapid consumption of carbon and nitrogen sources during the exponential growth phase.

Table 3.1 Batch kinetic studies in shake flasks using various *Bacillus* species

Organism	**Biomass** ($g\ L^{-1}$)	**PHA content** (%)	**Carbon substrate**	**Time** (h)	**Reference**
B. thuringensis	3.10	34.18	Glycerol	36	Rohini *et al.*, 2006
B. cereus	2.00	38.00	Glucose	60	Valappil *et al.*, 2007c
B. sphaericus	1.10	25.00	Fructose	28	Ramadas *et al.*, 2009
B. mycoides	3.60	69.4	Sucrose	24	Borah *et al.*, 2002
B. circulans	0.20	41.00	Sucrose	24	Shamala *et al.*, 2003
B. brevis	0.30	38.00	Sucrose	24	Shamala *et al.*, 2003
B. sphaericus	0.28	40.00	Sucrose	24	Shamala *et al.*, 2003
B. megarterium	0.25	25.00	Sucrose	48	Shamala *et al.*, 2003
B. cereus	1.34	50.00	Glucose	72	Halami, 2008
B. flexus	3.00	50.00	Sucrose	72	Somashekara *et al.*, 2009
COL1/A6	**4.49**	**65.51**	**Glucose**	**42**	**This study**
BLQ-2/A7	**3.85**	**59.87**	**Glucose**	**48**	**This study**

Studies carried out to investigate the effect of initial pH on biomass and PHA production demonstrated a rapid fall in the pH of the fermentation medium. This could be attributed to the production of acidic metabolites during the fermentation of sugar (Slepecky and Law, 1961). A drop in pH from 7.2 to 5.5 has also been reported by Rohini *et al.* (2006) during cultivation of *B. thuringiensis* R1 under uncontrolled pH conditions. The sharp decline in the pH of the culture medium accompanying growth of both the isolates used in this study was found to neither affect growth nor PHA synthesis. This finding is consistent with that of Gouda *et al.* (2001) and Valappil *et al.* (2007c). Hence, these *Bacillus* strains possess a major advantage over the Gram negative organisms such as *Cupriavidus necator* which has been reported to be completely inhibited at pH below 5.4 (Repaske, 1962; Beaulieu *et al.,* 1995).

Previous studies indicate that the accumulated PHA is utilized as a carbon and energy reserve for sporulation process in *Bacillus* species (Wu *et al.,* 2001). In contrast, Valappil *et al.* (2007c) have demonstrated that the acidic conditions generated during the fermentation of sugar under uncontrolled pH conditions led to inhibition of spore formation as well as utilization of the accumulated PHA. These findings are in lieu with that reported by Kominek and Halvorson (1965). Similar phenomenon was observed using isolates COL1/A6 and BLQ-2/A7. Recent studies carried out under controlled pH conditions have revealed that in some *Bacillus* species, the overall PHA content formed is lower than that under uncontrolled pH. Moreover, degradation of the accumulated PHA occurred on further incubation (Philip *et al.,* 2009; Faccin *et al.,* 2009). These results indicate that uncontrolled pH conditions are conducive for PHA accumulation rather than controlled pH systems in *Bacillus* species. This capability of the isolates COL1/A6 and BLQ-2/A7 to accumulate PHA under acidic

conditions is an additional feature favoring the use of these potential PHA producers at an industrial scale.

Cultivation of the isolates under optimized batch conditions revealed that the isolate COL1/A6 entered the stationary phase of growth within 24 h. The isolate BLQ-2/A7 grew rapidly and was able to utilize only half of the sugar supplied for growth and polymer production. Due to the inability of this strain to utilize the supplied carbon source to maximum and also due to its rapidity in growth, isolate BLQ-2/A7 was used for further studies.

Section II

PHA production using fed-batch fermentation

3.II.1 Materials and Methods

Fermentation conditions optimized using the conventional one factor at a time approach described in the earlier section were considered and further experimentations were carried out using initial pH: 7.0, temperature: 30°C, nitrogen source: ammonium chloride, yeast extract concentration: 0.5 g L^{-1}.

3.II.1.1 Determination of interactive effect of carbon and nitrogen source using central composite design

A. Experimental design

A two factor central composite rotary design was employed to find out the interactive effects of two variables, viz. concentrations of glucose (carbon source) and ammonium chloride (nitrogen source) on biomass and PHA production (Appendix 5). Central composite design at the given range of the above variables in terms of coded and uncoded terms is presented in Table 3.2. Five different levels of glucose and ammonium chloride were used in 12 experimental flasks. After the completion of 24 h of incubation on orbitek shaker (170 rpm) at 30°C, the culture broths were analyzed for the biomass and PHA accumulated as described in Chapter II. The concentrations of glucose and ammonium chloride corresponding to the various coded levels including details of the experimental design are given in Table 3.3.

B. Statistical analysis

The experimental data obtained from the design were analyzed by the response regression procedure using the following second-order polynomial equation:

$$Y_i = \beta_0 + \Sigma \beta_i x_i + \Sigma \beta_{ii} x_i^2 + \Sigma \beta_{ij} x_i x_j$$

Where Y_i was the predicted response, $x_i x_j$ were independent variables, β_0 was the offset term, β_i was the ith linear coefficient, β_{ii} was the ith quardratic coefficient and β_{ij} was

Table 3.2 Variables and their levels for Central composite rotatable design

Variables	Symbols	Levels				
		-α	-1	0	+1	+α
Glucose (g L^{-1})	A	1.42	5.00	10.00	15.00	18.58
NH_4Cl (g L^{-1})	B	0.17	0.60	1.200	1.800	2.230

Table 3.3 Central composite design of independent variables for optimization of glucose and ammonium chloride

Run	Coded		Uncoded	
	A	B	A (g L^{-1})	B (g L^{-1})
1	-1	-1	5.00	0.60
2	+1	-1	15.00	0.60
3	-1	+1	5.00	1.80
4	+1	+1	15.00	1.80
5	-α	0	1.42	1.20
6	+α	0	18.58	1.20
7	0	-α	10.00	0.17
8	0	+α	10.00	2.23
9	0	0	10.00	1.20
10	0	0	10.00	1.20
11	0	0	10.00	1.20
12	0	0	10.00	1.20

*ij*th interaction coefficient. In this study, the independent variables were coded as A and B. Thus the second order polynomial equation could be presented as follows:

$$Y_i = \beta_0 + (\beta_1 * A) + (\beta_2 * B) + (\beta_{11} * A * A) + (\beta_{22} * B * B) + (\beta_{12} * A * B)$$

The statistical software package, Sigma-Stat 3.5 was used for regression analysis of the experimental data and the Sigma Plot 11.0 was used to plot the response surface graphs. Analysis of variance (ANOVA) was used to estimate the statistical parameters. The second order polynomial equation was employed to fit the experimental data. The quality of fit of the polynomial model equation was expressed by the coefficient of determination (R^2). The fitted polynomial equation was expressed as three-dimensional surface plots to visualize the relationship between the responses and the experimental levels of each factor used in the design.

3.II.1.2 Growth media and culture conditions for fed batch fermentation

The growth conditions and sample withdrawal pattern was similar to that followed in the batch fermentation. During fed-batch I, concentrated glucose (to provide a final concentration of 10 g L^{-1}) and ammonia (to provide a final concentration of above 0.2 g L^{-1}) solution were administered aseptically into the medium at 18 h.

In fed batch II, only ammonia solution (to provide a final concentration of above 0.2 g L^{-1}) was aseptically added at 12 h after inoculation.

3.II.1.3 Analytical methods

Determination of dry cell weight and PHA was carried out as described in chapter II. Nitrogen and reducing sugar concentrations were estimated using methods described

by Franson (1985) and Miller (1959) (Appendix 3). The pH of the fermented culture broth was analyzed using a pH analyzer (LABINDIA).

All the experiments were carried out in triplicate to examine the reproducibility and the average values are reported.

3.II.2 RESULTS AND DISCUSSION

3.II.2.1 Central composite design

The influence of glucose and ammonium chloride on biomass and PHA production was studied using the optimized conditions (temperature: 30°C, initial pH: 7.0, yeast extract concentration: 0.5 g L^{-1}, nitrogen source: ammonium chloride). In order to study the interaction between glucose and ammonium chloride, central composite design was used. Such statistical data analysis allow the visualization of interaction among several experimental variables, leading to the prediction of data in areas not directly covered by experimentation (Sangkhara and Prasertsan, 2007; Nikel *et al.*, 2005).

The fermentation medium containing glucose and ammonium chloride were taken as two independent variables. The biomass and PHA served as two response variables of the design. The design matrix in actual terms and the experimental results of the two responses by the central composite design (CCD) are presented in Table 3.4. Applying multiple regression analysis, the results fitted in terms of coded factors were obtained as follows:

Biomass (g L^{-1}) = -0.511 + (0.314*A) + (1.643*B) – (0.0118*A^2) – (0.689*B^2) + (0.0423*A*B)

PHA (g L^{-1}) = -0.127 + (0.211*A) + (0.426*B) – (0.00914*A^2) – (0.371*B^2) + (0.0463*A*B)

Table 3.4 The experimental design with predicted and experimental results for biomass and PHA production

Run	Glucose ($g L^{-1}$)	Ammonium chloride ($g L^{-1}$)	Biomass ($g L^{-1}$)		PHA ($g L^{-1}$)	
			Experimental	Predicted	Experimental	Predicted
1	5.00	0.60	2.05	1.63	1.05	0.96
2	15.00	0.60	2.82	2.66	1.59	1.52
3	5.00	1.80	1.83	1.87	0.68	0.68
4	15.00	1.80	3.10	3.41	1.76	1.80
5	1.42	1.20	0.77	0.96	0.18	0.21
6	18.58	1.20	3.29	3.17	1.66	1.65
7	10.00	0.17	1.48	1.78	1.14	1.21
8	10.00	2.23	2.86	2.63	1.25	1.23
9	10.00	1.20	3.06	2.94	1.87	1.60
10	10.00	1.20	2.62	2.94	1.58	1.60
11	10.00	1.20	3.14	2.94	1.53	1.60
12	10.00	1.20	2.62	2.94	1.41	1.60

where, A and B are the coded independent variables representing glucose and ammonium chloride concentrations respectively. The fit of the model which is expressed by the coefficient of determination (R^2) was found to be 0.903 for biomass production, indicating that 90.3% of the variability in the response could be explained by the model (Mokhtari-Hosseini *et al.*, 2009; Hong *et al.*, 2009; Gao *et al.*, 2009).

The regression analysis of the experimental design in case of biomass production demonstrated that the linear model term (glucose) and quadratic model term of glucose as well as ammonium chloride were found to be significant ($p < 0.05$). The linear coefficient of glucose exhibited a positive effect on biomass production. The quadratic effect of both the independent variables displayed a negative effect on biomass production indicating that higher concentrations of these two factors adversely affect biomass production. For biomass synthesis, minimum concentration of ammonium chloride and glucose required was above 0.5 g L^{-1} and 10 g L^{-1}, respectively.

In the model describing the PHA synthesized within the cells, the coefficient of determination (R^2) was 0.948. Therefore 94.8% of the sample variation could be attributed to the independent variables and only 5.2% of the total variation cannot be explained by the model.

It was observed that the linear coefficient of glucose had a positive effect whereas the quadratic coefficient exhibited a negative effect on the response. The quadratic model term for ammonium chloride and the interaction coefficient were not significant ($p > 0.05$). A concentration of glucose below 10 g L^{-1} was not sufficient to support polymer accumulation whereas, in case of nitrogen source i.e. ammonium chloride, a concentration of above 0.5 g L^{-1} is required for polymer production.

The 3D response surface plots are the graphical representation of the regression equation and are shown in Figs 3.7. and 3.8. These plots help to understand the interaction of the variables and to locate the ideal levels of each variable for the maximum response. As seen from the Fig. 3.7, a simultaneous increase in the concentrations of both, glucose and ammonium chloride resulted in decrease in the biomass production. The response surface plot obtained for polymer production also exhibited a similar trend (Fig. 3.8). High concentrations of both the independent variables will result in a decline of polymer produced. From this study it can be clearly indicated that glucose and ammonium chloride concentrations have profound effects on the biomass as well as PHA production.

3.II.2.2 Fed-batch fermentation

Members of the genus *Bacillus* are known to grow rapidly (Full *et al.*, 2006; Faccin *et al.*, 2009). Studies conducted under optimized batch fermentation conditions using BLQ-2/A7 also revealed that this isolate exhibited rapid growth and entered the stationary phase within 18 h. The biomass as well as the rate of PHA synthesis was maximum during the exponential growth phase. Synthesis of PHA continued during the stationary phase but at a reduced rate. It was also seen that within 18 h, there was complete depletion of nitrogen from the medium. Omar *et al.* (2001) has attributed the depletion of nitrogen responsible for lower PHA yields. Similar observations have been opined by Kulpreecha *et al.* (2009). According to these authors, even though the production of PHA is enhanced during nitrogen limiting conditions, the activity of PHA biosynthetic enzymes is considerably restricted under nitrogen deficient conditions.

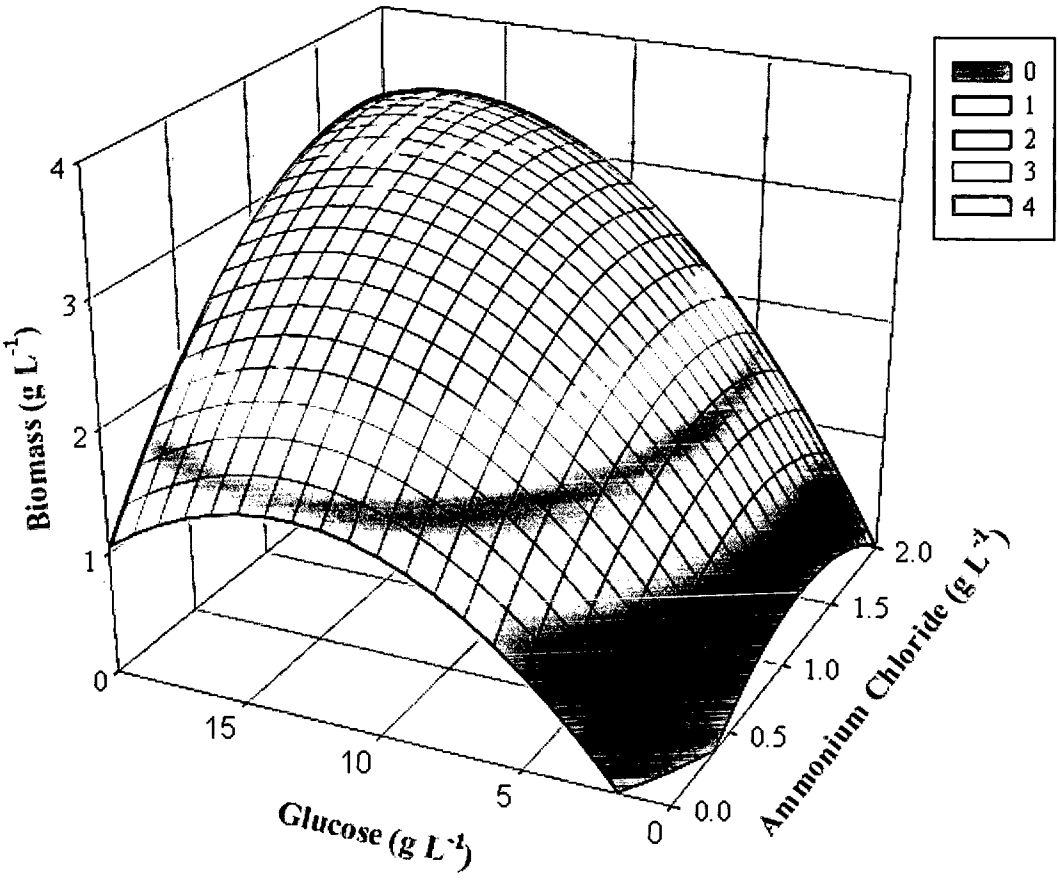


Fig. 3.7 Response surface plot showing the effect of glucose and ammonium chloride on biomass production

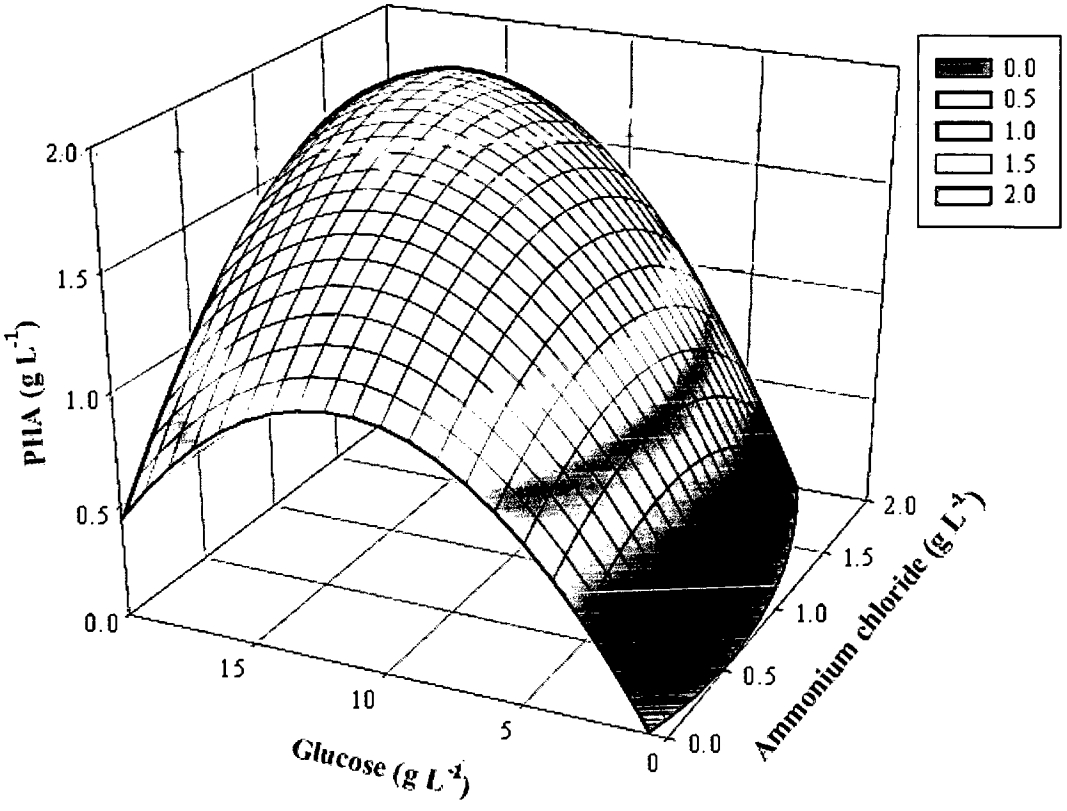


Fig. 3.8 Response surface plot showing the effect of glucose and ammonium chloride on PHA accumulation

In order to maximize the yield of biomass and PHA, it is essential to supply high concentrations of carbon and nitrogen sources to the fermentation medium (Yan *et al.*, 2005). As observed from the RSM studies, higher concentrations of glucose and ammonium chloride were found to adversely affect both the responses (biomass and PHA production). Therefore, the nutrient feed comprising glucose and ammonium chloride was formulated in such a way that the two components were supplied at concentrations to maximize the biomass as well as polymer production without causing any deleterious effect.

Taking the above observations into account, a fed-batch I fermentation system was designed wherein the concentration of glucose and nitrogen was maintained above 10 and 0.2 g L^{-1}, respectively. Since a drastic reduction in the nitrogen concentration was observed within 18 h, this was selected as a timepoint of applying the nutrient feed in order to achieve higher PHA yields.

The profiles of biomass and PHA production as well as the trend of utilization of the supplied carbon and nitrogen sources are displayed in Fig. 3.9. Synthesis of biomass was found to be higher in fed batch I than that in case of batch fermentation. It was seen that the rate of biomass production increased 13 fold as compared to batch fermentation within 6 h after nutrient feed. In contrast, the rate of biomass formation decreased drastically after 18 h in batch fermentation. Further, the rate of PHA formation at 24 h of incubation using fed batch conditions was only 2.5 fold higher than that of batch fermentation reducing the overall PHA content to about 34.78% DCW. Maximum biomass of 5.75 g L^{-1} was synthesized at 24 h whereas a maximum of 2.08 g L^{-1} PHA was obtained at 30 h of cultivation. The biomass as well as the polymer decreased on incubation beyond 30 h. A sharp drop in pH to 4.76 was

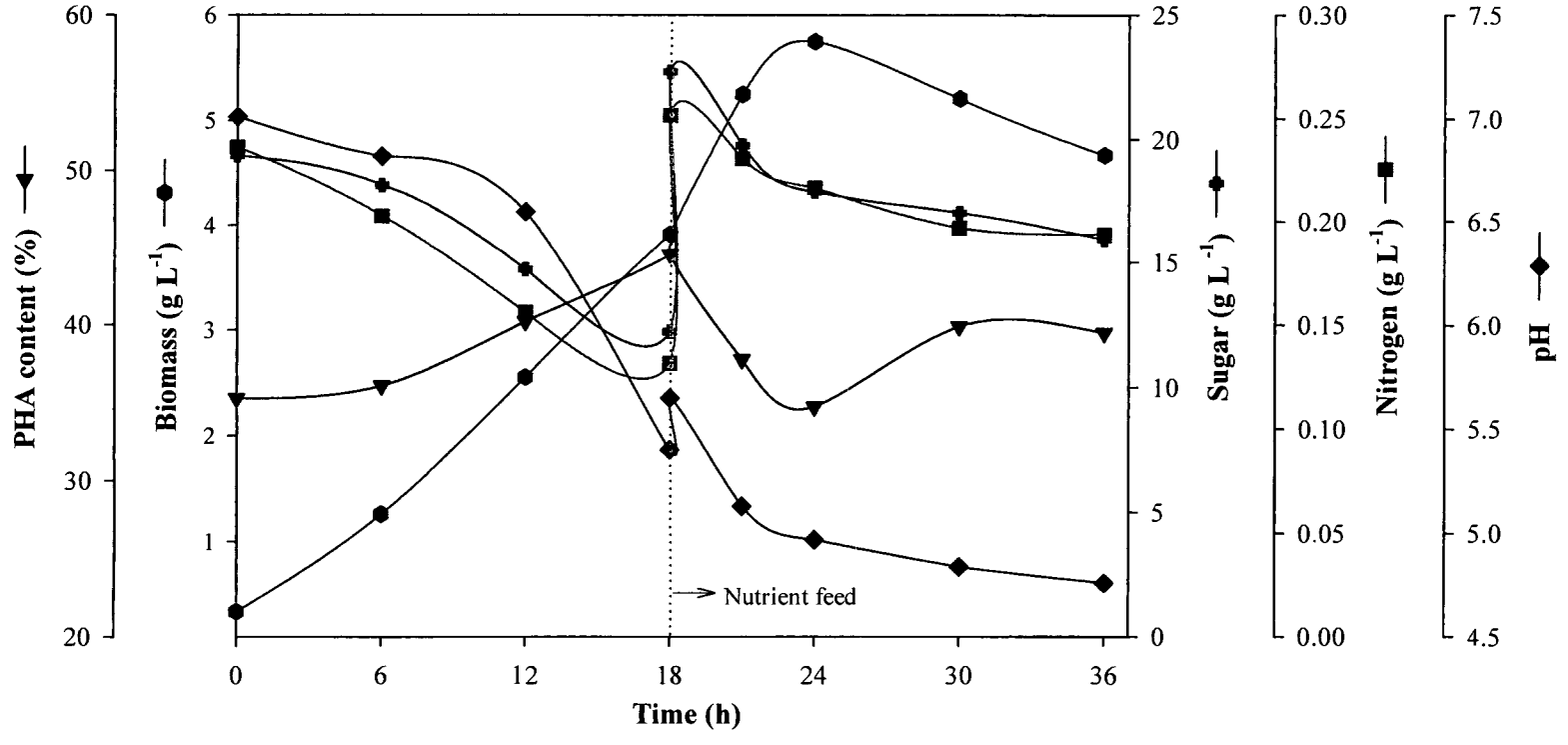


Fig. 3.9 Kinetics of PHA production by *Bacillus* sp. BLQ-2/A7 in shake flask culture under fed-batch I conditions

observed within 36 h of growth. The residual sugar in the medium at the end of 36 h was approximately 16 g L^{-1}.

Since the conditions of fed batch I were found to be ideal for biomass rather than PHA production, a second fed-batch II system was designed with the aim of obtaining high PHA production. In both the earlier fermentations, batch and fed batch-I, 50% of the sugar was left unutilized. Therefore, in this system, only nitrogen was supplied as the nutrient feed and was maintained at a final concentration above 0.2 g L^{-1}.

The results obtained from fed-batch-I indicated that application of the nutrient feed towards the end of the exponential phase enhanced biomass production rather than PHA synthesis (Fig. 3.10). Hence the timepoint of the nutrient feed was preponed by 6 h wherein the bacterium was still in its logarthimic phase of growth. The time point of application of nutrient feed has been demonstrated to have a significant effect on PHA production in various Gram negative bacteria (Lee *et al.*, 2000; Wang and Lee, 1997; Kim *et al.*, 1994). Yan *et al.* (2005) have reported that inadequate nitrogen supplied during the first few hours of fermentation although initiated biosynthesis of PHA restricted the development of the cytoplasmic enzymes, especially those for polymer synthesis.

The data obtained after performing fed-batch II revealed that highest biomass of 4.29 g L^{-1} and PHA up to 2.8 g L^{-1} were accumulated within 24 h of incubation. The highest PHA content of 65.5% DCW was also obtained at 24 h. The concentration of glucose left unutilized in the medium was approximately 5 g L^{-1}.

The trend of biomass and PHA production was similar to that of batch fermentation till 18 h of incubation. At 24 h, the rate of synthesis of biomass was 3-fold higher than that of batch fermentation, whereas, the rate of polymer formation was higher by 7.6

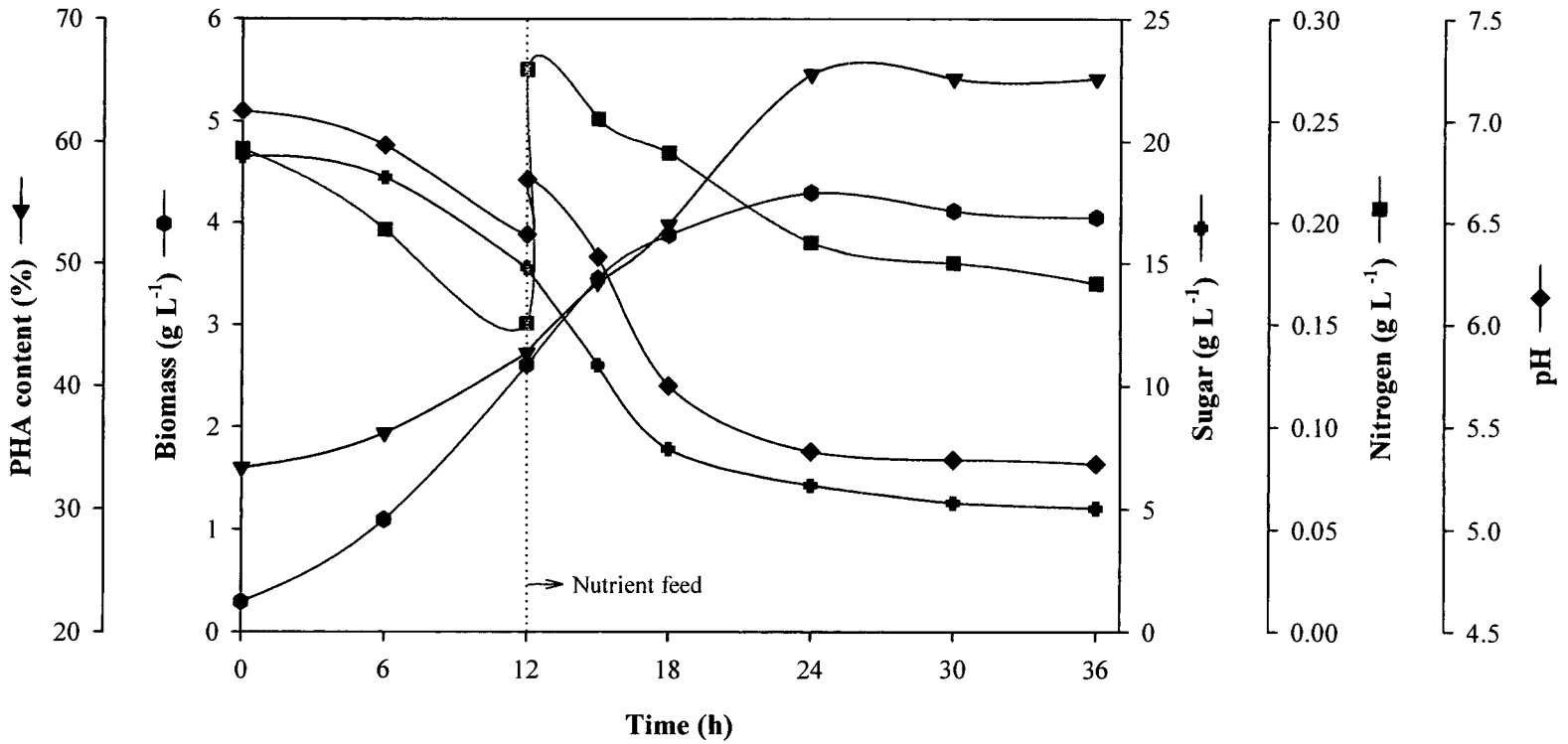


Fig. 3.10 Kinetics of PHA production by *Bacillus* sp. BLQ-2/A7 in shake flask culture under fed-batch II conditions

times. This data when compared with fed-batch-I revealed a 4.35 fold lower rate of biomass production and a 3 fold higher PHA formation rate.

Overall, the percentage yield obtained after performing fed-batch-II was about 5.63% higher than batch fermentation and 30.72% higher than fed-batch-I. Hence, a simple strategy of applying only the nitrogen feed to the culture in its exponential phase was sufficient to improve the PHA yield by 5%. Further, only 25% of the initial sugar was left unutilized as compared to batch fermentation suggesting a higher substrate conversion rate. The biomass, PHA concentration and the PHA yield obtained in the three fermentation strategies is summarized in Table 3.5.

From these studies, it can be concluded that the concentration of glucose and ammonium chloride play a critical role in determining the effect on biomass and PHA production.

The time point of applying the nutrient feed as well as the composition of the nutrient feed is of significance, especially when isolate BLQ-A7 is used for PHA production. By applying a simple nutrient feed strategy, the polymer production in this isolate could increase from 59.87 (under optimized batch fermentation) to 65.5% (using fed-batch II).

Table 3.5 Comparison of the PHA yield obtained in the batch and fed-batch fermentations

Time	Batch			Fed-batch-I			Fed-batch-II		
(h)	**Biomass** ($g\ L^{-1}$)	**PHA** ($g\ L^{-1}$)	**PHA content** (%)	**Biomass** ($g\ L^{-1}$)	**PHA** ($g\ L^{-1}$)	**PHA content** (%)	**Biomass** ($g\ L^{-1}$)	**PHA** ($g\ L^{-1}$)	**PHA content** (%)
0	0.40	0.12	30	0.34	0.12	35.29	0.30	0.10	33.33
6	1.11	0.39	35.37	1.26	0.46	36.11	1.10	0.40	36.16
12	2.70	1.18	43.64	2.56	1.03	40.23	2.60	1.11	42.65
18	4.09	2.08	50.88	3.92	1.75	44.56	3.87	2.06	53.14
24	4.23	2.18	51.51	5.75	2.00	34.78	4.29	2.81	65.51
30	4.20	2.27	54.10	5.21	2.08	39.92	4.11	2.68	65.14
36	4.05	2.32	57.28	4.67	1.85	39.51	4.05	2.64	65.15

Chapter IV

Recovery of PHA from Bacillus sp. COL1/A6

INTRODUCTION

One of the crucial factors influencing the overall PHA cost is the method used for the recovery of the polymer. The PHA isolation procedures and purification methods greatly determine the cost effectiveness and the pharmacological purity of the polymer during commercial production (Yu and Chen, 2006; Jacquel *et al.*, 2008). *Bacillus* is one of the promising genera for the production of PHA suitable for medical applications due to lack of the endotoxin (lipopolysaccharide) that is known to co-purify along with the polymer when extracted from Gram negative bacteria. Since these endotoxins elicit immunogenic reactions, the polymer extracted from Gram positive organisms such as *Bacillus* is now being considered for medical applications (Valappil *et al.*, 2007b).

Different methods for PHA recovery have been described in the past by various research groups but most of them employ Gram negative organisms. Therefore, this study was particularly carried out using the Gram positive isolate, *Bacillus* sp. COL1/A6. Various methods were evaluated for the recovery of PHA synthesized by the isolate grown under optimized batch conditions. These methods involved solubilization of either PHA or non-PHA cellular material. Further, the purity as well as the yield of PHA recovered using various treatments was analyzed.

4.1 MATERIALS AND METHODS

4.1.1Organism used for study

Bacillus sp. COL1/A6 was used for the screening of low-cost downstream processing to obtain high yields of PHA. A number of cheap non-solvent systems and solvent systems were screened for this purpose. Non-solvent systems were screened using cell slurry whereas lyophilized cells were used in case of solvents.

4.1.2 Cultivation of isolate for cell slurry production or lyophilization

The isolate was grown in one liter capacity Erlenmeyer flasks containing 500 ml production medium (Appendix 1). The medium contained glucose (20 g L^{-1}) as sole source of carbon. A total of five liter medium was inoculated with 5% of inoculum (as described in chapter II). The flasks were incubated at 30°C for 48 h on an environmental shaker at 170 rpm.

4.1.3 Preparation and storage of PHA-containing cell slurry

After biomass production, the culture broth was autoclaved at 121°C for 30 min. This was done in order to cause the cell death, denaturation of DNA and inactivation of PHA degrading enzymes. The cell mass in the fermented broth was harvested by centrifugation at 8,000 rpm for 10 min. The cell pellet was washed twice with distilled water and resuspended in the same to produce thick cell slurry. This slurry was stored at 4°C for further use.

4.1.4 PHA recovery by simple digestion of non-PHA cell mass (NPCM)

The various dissolution methods tested included treatment with acid, alkalis, detergent and enzymes. All the dissolution methods were carried out using cell slurry. Cell slurry (10% v/v) in the total digestion volume of 10 ml was treated with various chemicals as summarized in Table 4.1. After the respective treatment, the digestion mixture was centrifuged at 8,000 rpm for 10 min to separate the polymer from the aqueous fraction containing cell debris. The pellets and supernatants obtained were further analyzed using the analytical methods (section 4.1.6).

Table 4.1 Various treatments evaluated for cell mass solubilization

Treatment	Concentration	Time	Temperature
Acid			
Sulphuric acid	2 N	30 min	80 °C
Sulphuric acid	2 N	2 h	80 °C
Sulphuric acid	2 N	4 h	80 °C
Alkali			
NH_3	1 N	15 min	45 °C
KOH	1 N	15 min	70 °C
NaOH	1 N	15 min	70 °C
NaOCl	2%	15 min	30 °C
Detergent			
SDS	1%	1 h	37 °C
SDS	1%	2 h	37 °C
Hydrolytic enzyme			
Protease	0.5 mg ml^{-1}	20 min	30 °C
Lysozyme	0.5 mg ml^{-1}	20 min	30 °C

4.1.5 Treatment of PHA containing cell mass using various solvents

4.1.5.1 Lyophilization of PHA containing cells

The culture broth was autoclaved at 121°C for 30 min. The cell mass in the fermented broth was harvested by centrifugation at 8,000 rpm for 10 min. The cell pellets were washed twice with distilled water and resuspended in minimum amount of the same. The cell suspension was kept overnight in the -20°C freezer. The frozen cell mass was subjected to lyophilization (Freeze dryer 4.5, LABCONCO, USA). The dried cells were collected and stored at -20°C for later use.

4.1.5.2 Treatment of PHA-containing cell mass using various solvents

The solvents used in this study were alcohols (two carbon: ethanol and four carbon: butanol), short chain carboxylic acid (acetic acid), chlorinated hydrocarbon (chloroform), straight chain alkane (hexane) and aromatic hydrocarbon (benzene). Lyophilized cells (1% w/v) were subjected to 10 ml of the above mentioned solvents. The reaction mixture was refluxed in a boiling water bath for 1 h after which it was allowed to cool. The solubilized fraction was separated from the insoluble portion by filtration through a preweighed Whatman filter paper. The filtrate was collected into preweighed tubes and the solvent was allowed to evaporate. The insoluble residual material collected on the filter paper was dried until constant weight was obtained. The PHA content and the extent of solubilization were monitored using analytical methods.

4.1.6 Analytical Methods

A. Dry weight of the recovered fractions

The pellets recovered after separation of the aqueous fraction were gently rinsed with distilled water and centrifuged at 8,000 rpm for 10 min at 4°C. The pellets were collected in preweighed aluminium cups and dried at 60°C till constant dry weight was obtained.

B. PHA estimation in the residual material

Samples of the recovered pellets (5 mg) were analyzed for the quantitative determination of PHA. In case of solvent treatments, the PHA in the recovered fractions as well as the filtrates obtained was determined. Samples were treated with concentrated sulphuric acid (5 ml) and the tubes were placed in boiling water bath (BWB) for 10 min, to hydrolyze and dehydrate PHA to crotonic acid with absorption maxima at 235 nm (Law and Slepecky, 1961; Panda *et al.*, 2006). The spectra were recorded with sulphuric acid as the blank using UV Visible spectrophotometer (SHIMADZU, 1601, JAPAN).

4.1.7 Effect of solubilization

The effect of solubilization on the non-PHA cell mass was observed Fourier-Transform Infrared (FTIR) spectral analysis.

FTIR Spectroscopy

The fractions recovered during various treatments were analyzed using FTIR spectroscopy. The recovered fractions (1 mg) were mixed with FTIR grade potassium bromide (KBr) to form a pellet which was used for analysis. The IR spectra were recorded at 400 to 4000 cm^{-1} on a FTIR spectrometer (SHIMADZU, IR PRESTIGE-21, JAPAN).

In the case of solvent treatments, FTIR spectral analysis was carried out only for the filtrates exhibiting the characteristic crotonic acid peak at 235 nm.

4.1.8 Data analysis

The total cellular concentration (X, g L^{-1}) and the concentration of PHA ([PHA], g L^{-1}) allow the calculation of the residual cellular concentration (X_{NPHA}, L^{-1}), the PHA purity (PHA %), non-PHA biomass solubilization (S_{NPHA}) and the PHA purity increase (Δ purity) in the samples recovered in accordance to Equations 1 to 4 (Kapritchkoff *et al.*, 2006; Suzuki *et al.*, 2008).

The non-PHA biomass concentration (X_{NPHA}) was calculated using the following equation.

$$X_{NPHA} = X - [PHA] \quad (1)$$

PHA purity (PHA %) was expressed as the percentage of PHA mass per total biomass

$$\text{PHA purity} = \frac{[PHA] \times 100}{X} \quad (2)$$

Non-PHA biomass solubilization (S_{NPHA}) was calculated using the following equation

$$(S_{NPHA}) = \frac{(X_{NPHAo} - X_{NPHAt}) \times 100}{X_{NPHAo}} \quad (3)$$

(where X_{NPHAo} and X_{NPHAt} are, the X_{NPHA} at time zero and time t of the experiment respectively).

The PHA purity increase (Δ purity) was calculated using the equation

$$\Delta \text{ purity} = \frac{(PHA\%_t - PHA\%_0) \times 100}{100 - PHA\%_0} \quad (4)$$

(where $PHA\%_t$ and $PHA\%_0$ are the values for PHA % at time t and time zero respectively).

The various treatments involving the non-solvent and the solvent systems were carried out in triplicate to examine the reproducibility, and the average values are reported.

4.2 RESULTS AND DISCUSSION

Among the various treatments reported for the purification of the polymer from cells, the most routinely used methods employing *Bacillus* species include the non selective dissolution of cellular material using sodium hypochlorite solution (Anil Kumar *et al.*, 2007; Halami, 2008; Ramadas *et al.*, 2009) and PHA solubilization using chloroform (Omar *et al.*, 2001; Valappil *et al.*, 2007).

In this study, various methods have been evaluated for polymer purification and the effect of these treatments was analyzed based on the PHA purity obtained, increase in purity achieved and solubilization of the non-PHA cellular material (NPCM). The extent of solubilization of the NPCM was also monitored using the UV spectral profiles of the samples treated with concentrated sulfuric acid (Law and Slepecky, 1961). The concentrated acid hydrolyses and dehydrates the polymer to crotonic acid which gives a characteristic absorption peak at 235 nm.

The residual material recovered after the various digestion treatments was also used for the characterization of functional groups through Fourier Transform Infrared (FTIR) analysis since it is a powerful tool for the rapid and simultaneous

characterization of different functional groups such as lipids, proteins, nucleic acid and polysaccharides in biomolecules and complex structures (Helm and Naumann, 1995; Naumann *et al.*, 1995; Garip *et al.*, 2009). The percentage transmittance (%T) obtained in the spectra represents the transmittance of the sample at each functional group (Kathiraser *et al.*, 2007).

Effect of various non-solvent treatments

The effect of various non-solvent dissolution treatments can be visualized from Fig 4.1. A brown color was obtained for the untreated PHA containing cell mass on drying (Fig. 4.1a). Efficient solubilization of the cells under acidic conditions can be clearly observed in Fig. 4.1b-d. With increase in the reaction time, a progressive improvement in the color of the recovered fraction was observed. Among the different alkaline solutions tested, sodium hypochlorite (NaOCl) was able to dissolve a major portion of the NPCM as can be seen from the whitish color of the residue obtained after treatment (Fig 4.1 h). The positive effect of the other alkaline solutions was also evident from the color of the recovered fractions (Fig. 4.1e-g). The cell mass treated with the anionic detergent sodium dodecyl sulfate (SDS) showed a variation in the color as compared with the untreated cells and further increase in the incubation time improved the appearance of the recovered fraction (Fig. 4.1i-j). Contrasting results were observed with the use of the two hydrolytic enzymes. On one hand, lysozyme displayed a positive effect in the removal of NPCM, while the protease-treated cell mass showed negligible variation in the color as compared to the control (Fig. 4.1k-l).

The use of acidic conditions for PHA purification from Gram positive cells has not been reported earlier. The bacterial cell wall, including the peptidoglycan is susceptible to acidic dissolution thereby releasing proteins and other biological macromolecules into the aqueous medium whereas the PHA is highly resistant to

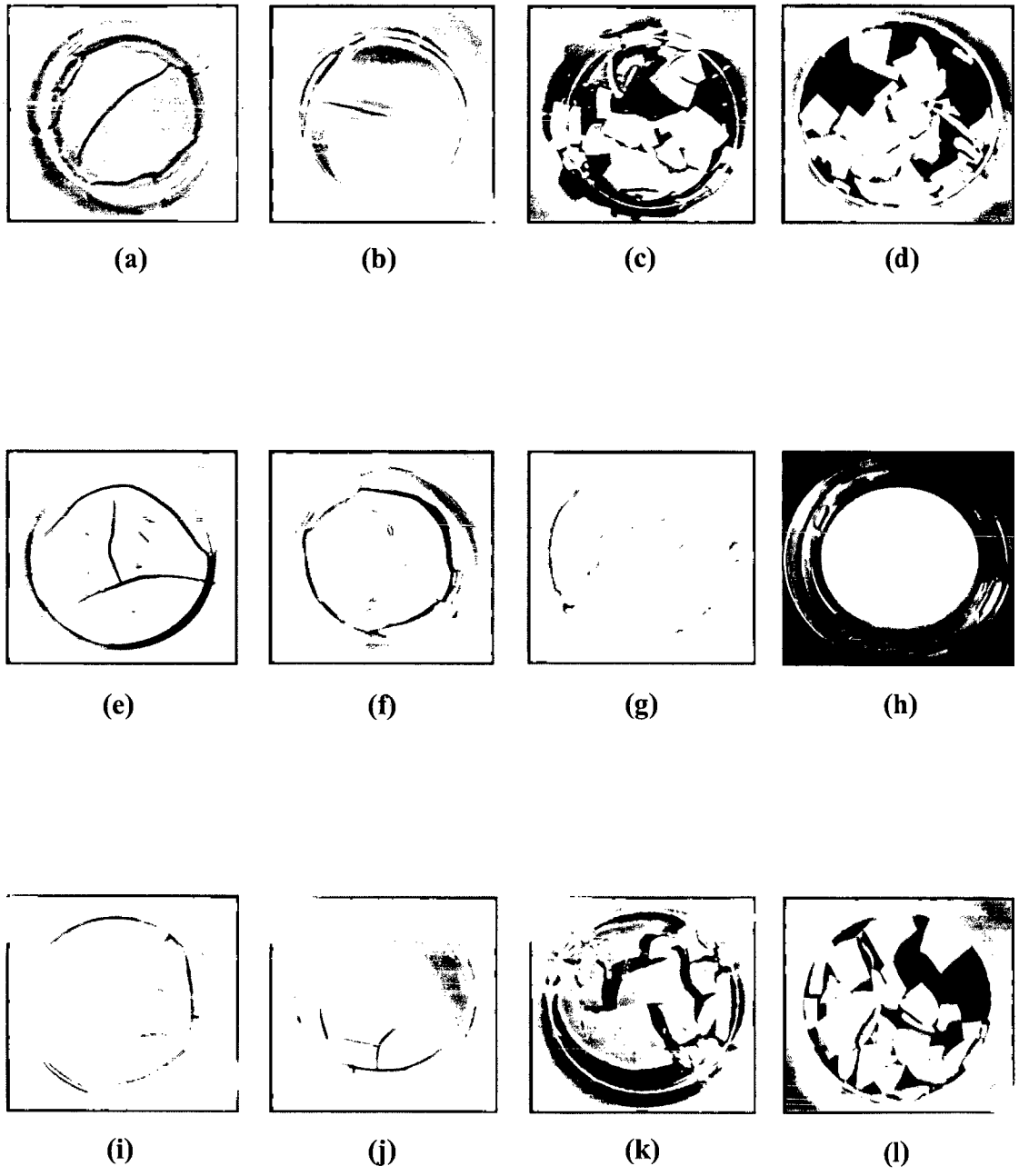


Fig. 4.1 Residual material recovered after treatment with various non-solvent systems

a: untreated cells (control), **b - d**: acid treament (30 min, 2h, 4h respectively)

e - h: alkali treament (NH_3, KOH, NaOH, NaOCl respectively)

i - j: SDS treatment (1h, 2 h respectively),

k - l: hydrolytic enzyme treatment (protease, lysozyme respectively)

acidic hydrolysis and this selective dissolution has been constructively exploited by Yu and Chen (2006) for polymer purification from *Ralstonia eutropha.* In this present study, when cells containing PHA (53.06% DCW) were exposed to acidic conditions, a PHA purity of 71.89% was achieved within 30 min of digestion time (Fig. 4.2). Further incubation up to 2 h resulted in PHA purity of 90.27% whereas incubation of another additional 2 h did not lead to significant increase in PHA purity.

From the current study, it was found that most of the treatments supported increased PHA yield and % purity. A rapid increase in purity of 40.14% was observed when cells were incubated in acid for 30 min. On prolonged exposure of the cells to acidic conditions, the increase in purity was found to be 79.21% and 89.24% at 2 and 4 h, respectively (Table 4.2). Maximum NPCM solubilization of about 93% was observed at 4 h. These results are in contrast to the observations made by Choi and Lee (1999b), who found that acids such as hydrochloric and sulfuric were inefficient for the recovery of PHA from recombinant *E. coli.* Yu and Chen (2006) on the other hand have proposed a new recovery method based on the selective acidic dissolution of cell material. In their study, they demonstrated that acidic conditions had a profound positive effect on NPCM solubilization thereby releasing the protein from *Ralstonia eutropha* with a concomitant increase in the PHA purity to 89.3%.

Among the different alkaline solutions tested, sodium hypochlorite (NaOCl) efficiently solubilized the NPCM yielding a polymer with the highest purity of 97.39% (Fig. 4.3). Treatment with sodium hydroxide (NaOH) and potassium hydroxide (KOH) yielded polymer with slightly lower purity values of 83.53% and 81.60% respectively, whereas lowest purity of 76.67% was observed with ammonia (NH_3). Maximum NPCM solubilization as well as increase in purity was observed with NaOCl treatment (96.94% and 94.41%, respectively; Table 4.2). NaOH and

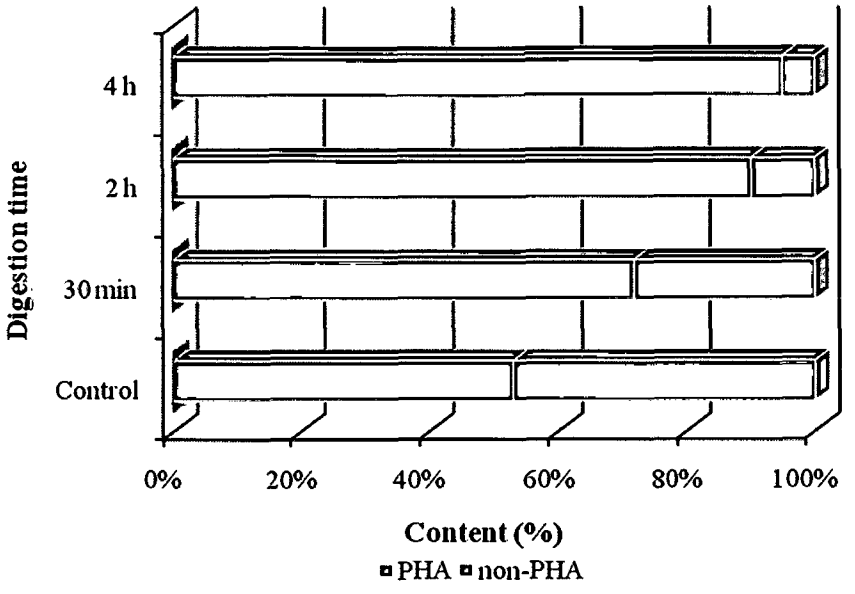


Fig. 4.2 Temporal effect of sulfuric acid on cell mass containing PHA

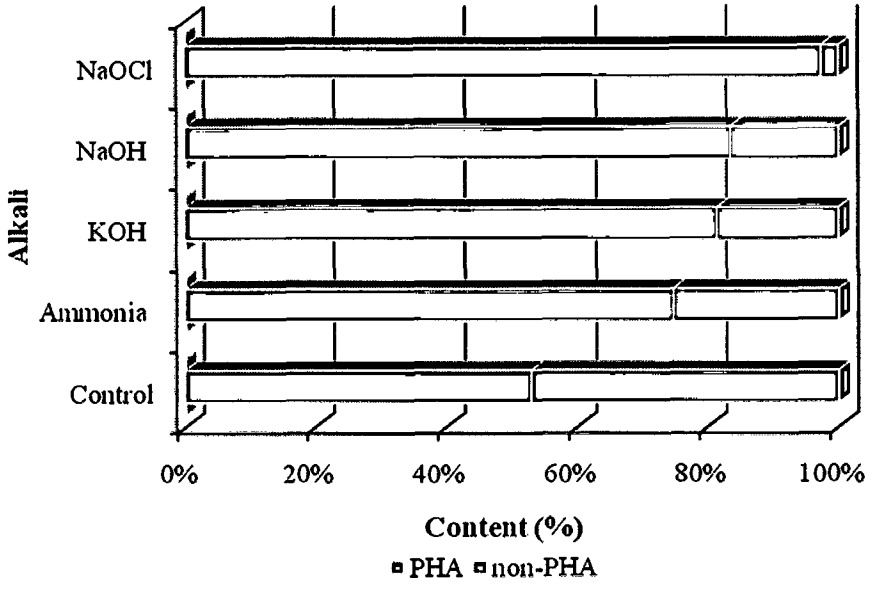


Fig. 4.3 Effect of various alkalis on cell mass containing PHA

Table 4.2 PHA recovery and purification using various treatments

Treatment	Δpurity ± SD	S_{NPHA} ± SD
Acid		
H_2SO_4 (30 min)	40.14 ± 2.52	47.57 ± 3.76
H_2SO_4 (2 h)	79.21 ± 2.96	86.61 ± 2.31
H_2SO_4 (4 h)	89.24 ± 3.01	93.84 ± 1.62
Alkali		
NaOH	64.96 ± 1.90	77.61 ± 1.02
KOH	60.88 ± 3.54	72.65 ± 2.95
NH_3	46.05 ± 1.11	58.48 ± 1.59
NaOCl	94.41 ± 2.60	96.94 ± 1.39
Detergent		
SDS (1 h)	33.26 ± 1.80	48.35 ± 1.25
SDS (2 h)	51.15 ± 1.78	65.50 ± 1.21
Hydrolytic enzyme		
Protease	0	0
Lysozyme	76.10 ± 1.74	85.30 ± 1.53

Δpurity, PHA purity increase; S_{NPHA}, non-PHA solubilization; SD, Standard deviation

Initial PHA%, 53.06 ± 0.58

KOH were capable of solubilizing more than 70% of the non-PHA cell mass (77.61% and 72.65%, respectively) and displayed an increase in purity of 64.96% and 60.88%, respectively however, NH_3 was able to solubilize only 58.48% of non-PHA cell mass and the purity increased only upto 46.05%.

As reported in literature, treatment of bacterial cell walls with alkali dissolves the mucopeptide component in the cell wall as well as solubilizes the teichoic acids (Archibald *et al.*, 1969; Cornett *et al.*, 1979). This effect weakens the cell walls thereby releasing the cell contents into the surrounding environment. Hypochlorite is a known powerful oxidizing agent and is active in killing microorganisms by solubilizing at least 30% of the cell mass (Peng *et al.*, 2002; Koning and Witholt, 1997). The use of alkali for the purification of PHA has been reported earlier by Choi and Lee (1999b) wherein treatment of PHA containing cells of recombinant *E. coli* (with initial PHA content of 77% DCW), resulted in a purity of greater than 91% with NaOH and KOH. The purity of the polymer was lower when treated with ammonium hydroxide (NH_4OH). An increase in the alkali concentration and digestion temperature enhanced the product purity. Use of ammonia for PHA recovery has been described by Page and Cornish (1993). They have reported that concentrations below 1N are ineffective in extracting proteins and other residual material from the cells of *Azotobacter vinelandi.* Further, the use of higher concentrations of ammonia efficiently removed the NPCM and the obtained PHA was found to be 94% pure.

The effectiveness of SDS in purifying the intracellular PHA from the cells is depicted in Fig. 4.4. It was observed that incubating cells for 1 h with SDS enhanced the purity of the polymer recovered from an initial of 53.06% to 68.67%. Further incubation of an additional 1 h increased the purity of the polymer to 77.07% in the recovered fraction. Within 2 h of incubation, the S_{NPHA} and the Δ purity was found to be 51.15%

and 65.50% respectively (Table 4.2). Earlier studies using SDS have demonstrated the solubilization of recombinant *E. coli* cells and PHA purity greater than 95% has been achieved (Choi and Lee, 1999b; Hahn *et al.*, 1998). When cells of *Azotobacter* were used a PHA purity of 90% was obtained with 1% SDS. Further increase in SDS concentration had an adverse effect on the product purity (Zhaolin and Xuenen, 2000). Treating the cells of *Pseudomonas* with SDS resulted in the solubilization of about 20% of the cellular material within 15 min (Koning and Wiltholt, 1997).

Among the various detergents reported for PHA recovery, only anionic detergents were found to be effective. Cationic detergents cause agglomeration of the cell remnants (Koning and Wiltholt, 1997). The surfactants act by incorporating itself into the lipid bilayer of the cellular membrane thereby breaching the membrane when saturated (King, 1991; Ramsay *et al.*, 1990) producing micelles of surfactant and membrane phospholipids. The surfactant also provokes the solubilization of proteins and other non polymeric cellular material (Chen *et al.*, 1999). SDS was preferred over the other detergents evaluated since it efficiently digested the NPCM and produced micelles of smaller diameter (Choi and Lee, 1999b; Koning and Wiltholt, 1997). The other advantages reported in the usage of SDS are: the effective cell lysis without degradation of PHA, high quality of the recovered PHA, less expensive as compared to other detergents and production of fewer pollutants (Suzuki *et al.*, 2008; Kim *et al.*, 2003).

The enzyme lysozyme efficiently improved the purity of PHA-containing cell mass to 88.8%. However, the action of the enzyme protease was negligible as the purity of the fraction after treatment was comparable to that of the control (Fig. 4.5). The quality of the PHA recovered was found to improve using lysozyme treatment as revealed from the Δ purity (76.1%). Lysozyme was found to effectively solubilize upto 85.30% non-

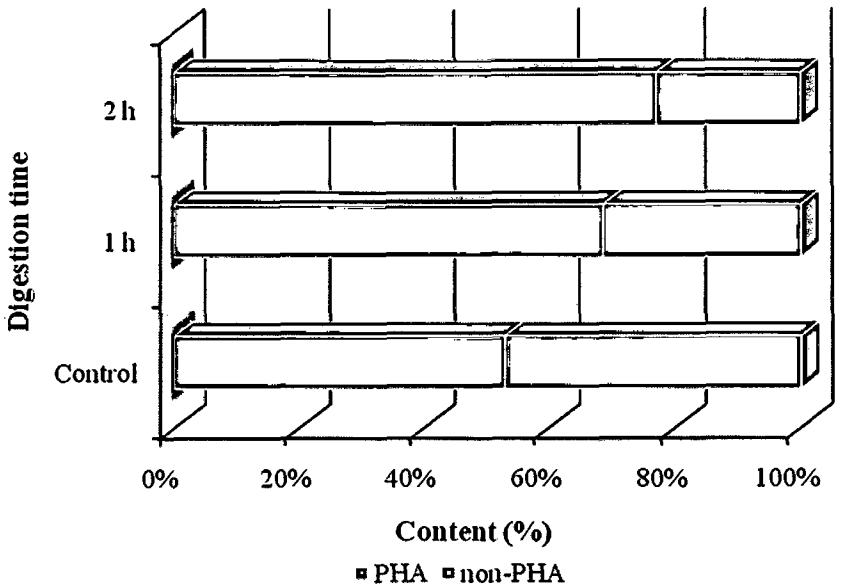


Fig. 4.4 Temporal effect of SDS on cell mass containing PHA

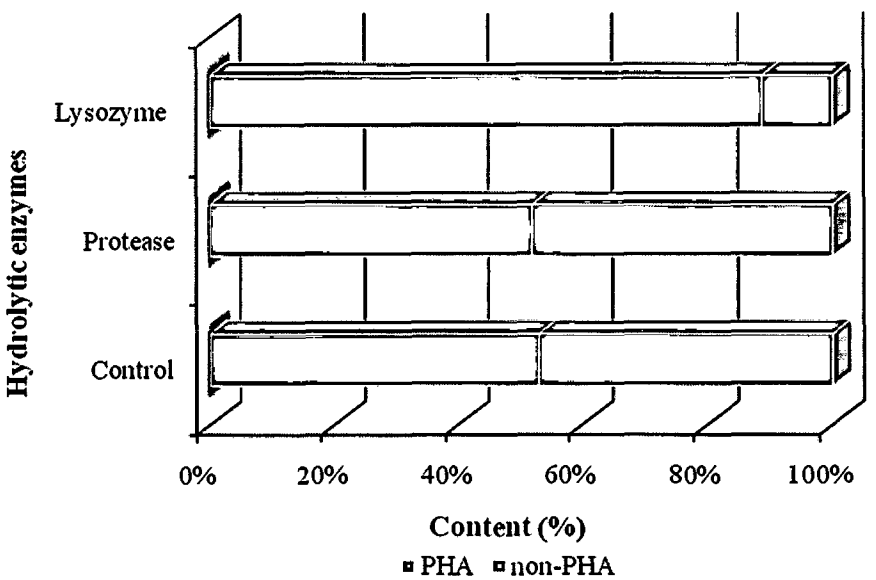


Fig. 4.5 Effect of hydrolytic enzymes on cell mass containing PHA

PHA material (Table 4.2). This positive action of lysozyme for removal of the NPCM has been demonstrated on cells belonging to both Gram character groups. The action of lysozyme on Gram negative cells was found to be enhanced in the presence of EDTA (Koning and Witholt, 1997). McCool *et al.* (1996) reported the use of lysozyme for the isolation of intact PHA granules from *B. megaterium.*

The use of protease in this study did not demonstrate any profound action on the cells. On the other hand, Kapritchkoff *et al.* (2006) have described proteases as the most suitable enzymes for the NPCM solubilization and purification of the polymer from *R. eutropha.* This inability of the protease to degrade the NPCM of *Bacillus* cells could be attributed to the difference in the cell wall structure of Gram negative and Gram positive cells (Prescott *et al.*, 2003).

FTIR spectral profiles of recovered material

The FTIR spectrum of the untreated cells showed presence of bands corresponding to different functional groups (Fig. 4.6a). The major contributors to the various spectral bands reported in bacteria are compiled in Table 4.3. Strong signals at wavenumber values ranging between 3300 and 2800 cm^{-1} correspond to lipids and proteins. Bands near 1650 and 1550 cm^{-1} due the amide I and amide II vibrations of structural proteins are also visible. In addition, a broad band with 3 peaks in the region 1150 to 1050 cm^{-1} due to polysaccharide component is also present (Socrates, 2004).

Cells incubated with acid for 30 min exhibited a prominent band near 1724 cm^{-1} corresponding to the ester (C=O stretch) (Fig. 4.6b). The intensity of the bands in the region from 1700 to 1600 cm^{-1} was found to reduce. Further incubation in acid for 2 h showed the reduction in the bands in 3300 to 2800 cm^{-1} region (Fig. 4.6c). The presence of intense ester group shown by the carbonyl (C=O) stretch at 1750 to 1724

Table 4.3 General band assignment of bacteria available in literature

Wavenumbers (cm^{-1})	**Definition of the spectral assignment**
3307	N-H and O-H stretching vibration: polysaccharides, proteins
3000-2800	CH_3 asymmetric stretch, CH_2 asymmetric stretch, CH_2 symmetric stretch: mainly lipids
1750-1724	Ester C=O stretch: lipids, polyhydroxyalkanoates, triglycerides
1650	Amide I (protein C=O stretch): structural proteins
1550	Amide II (protein N-H bend, C-N stretch)
1452	CH_2 bending: lipids
1391	COO^- symmetric stretch: amino acid side chain, fatty acids
1260-1236	PO_2^- asymmetric stretching: mainly nucleic acids

[a] Adapted from Garip *et al.* (2009); Kathiraser *et al.* (2007); Socrates (2004); Xu *et al.*(2002); Hong *et al.* (1999)

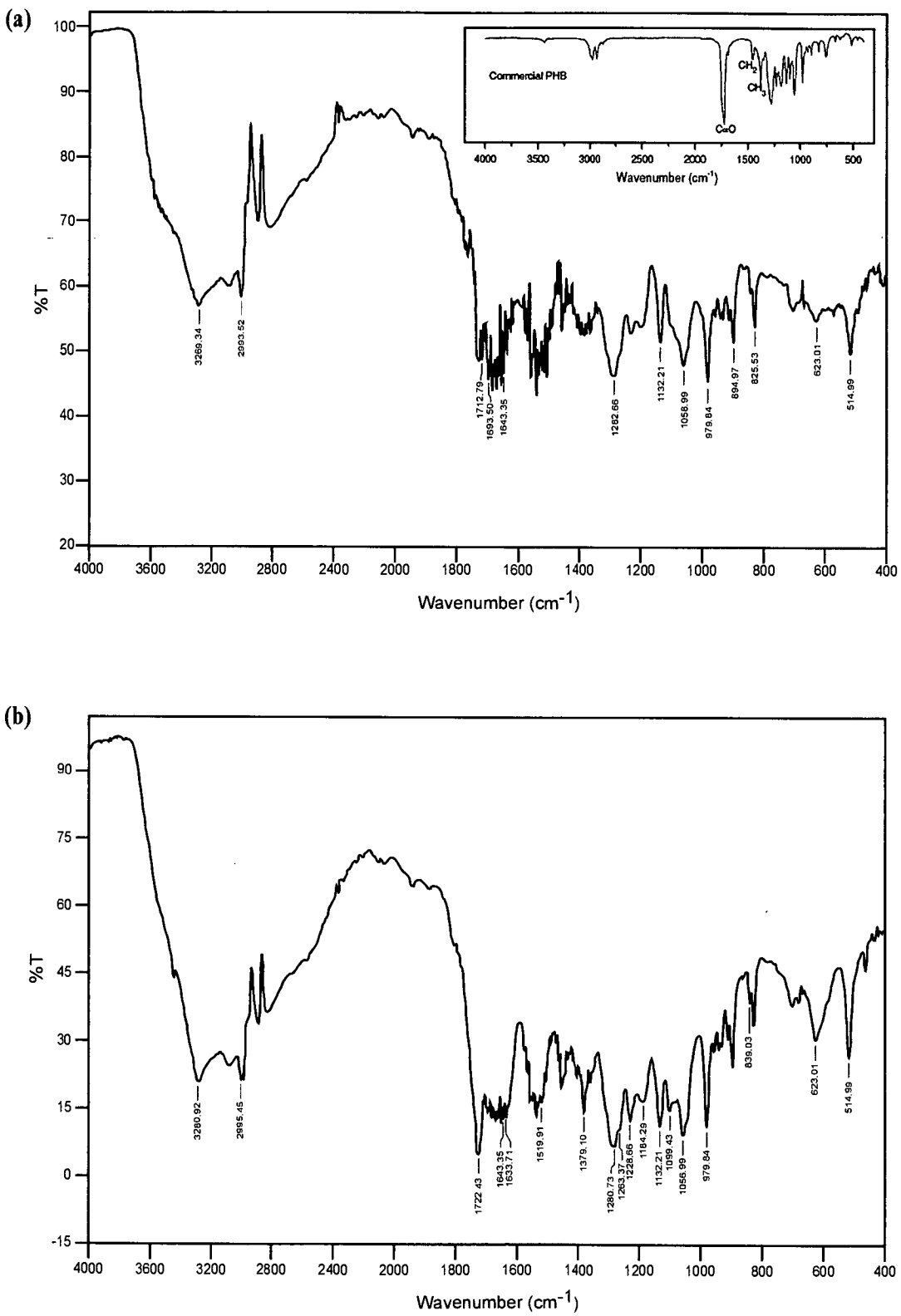


Fig. 4.6 FTIR spectra of the residue obtained after subjecting the cell mass containing polymer to various treatments **(a)** untreated cells (control) and **(b)** acid treatment [30 min] Inset displays FTIR spectrum of commercial PHB (Oliveira *et al.*, 2007)

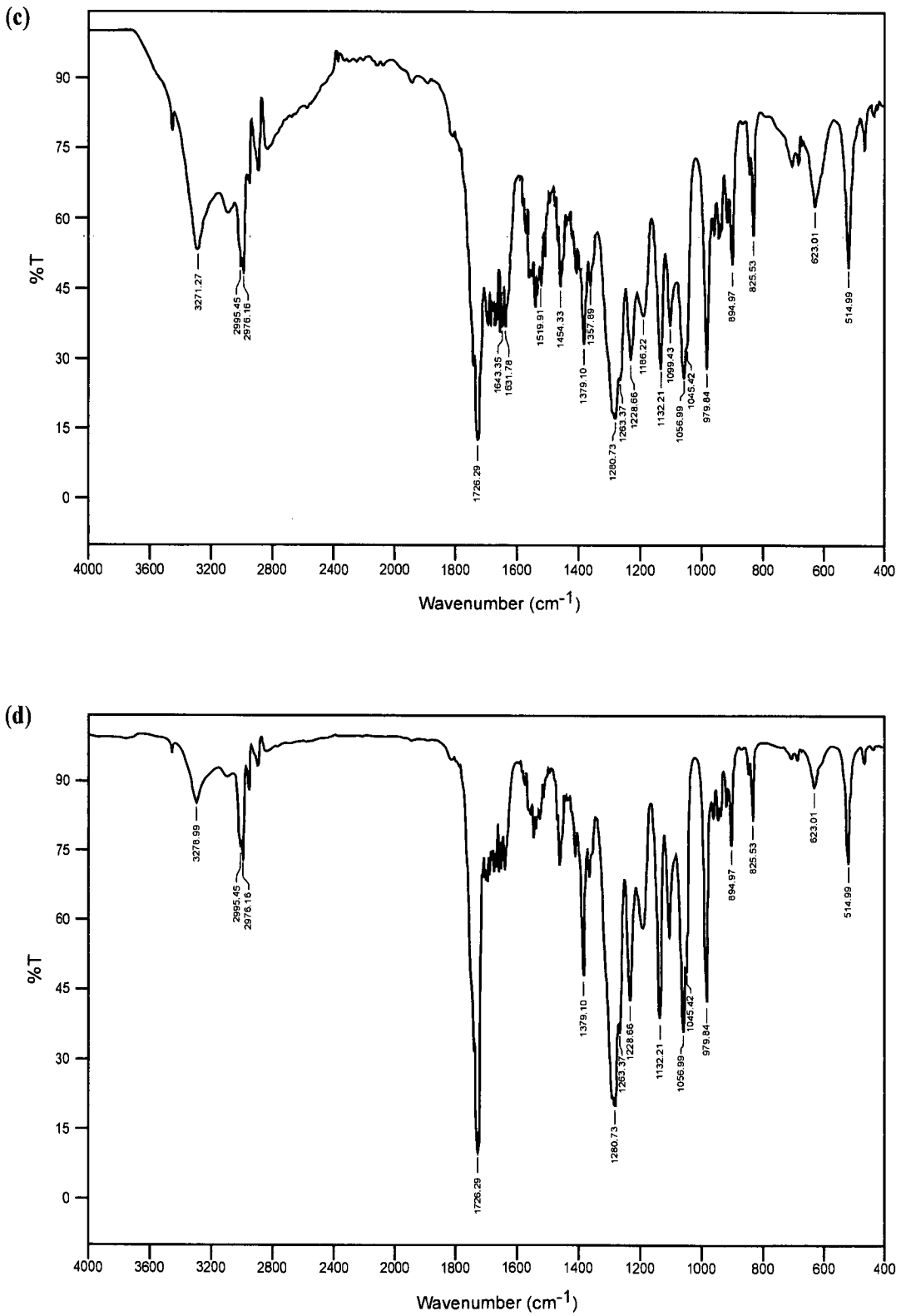
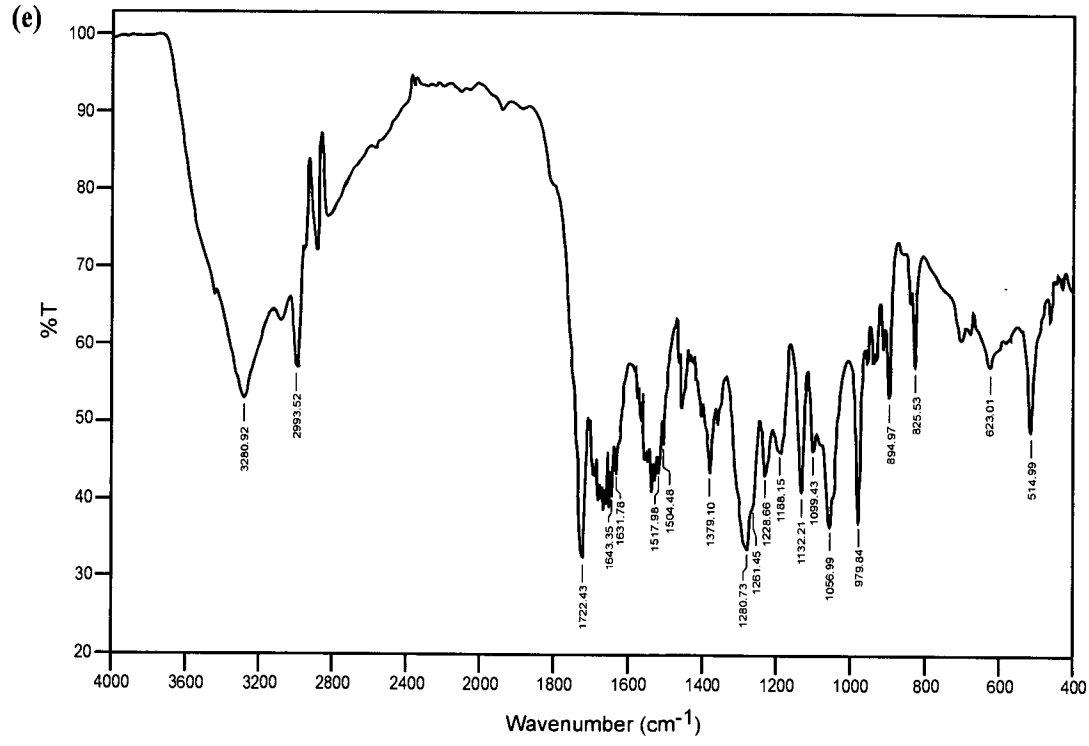


Fig. 4.6 contd. FTIR spectra of the residue obtained after subjecting the cell mass containing polymer to various treatments (**c**) acid treatment [2 h] and (**d**) acid treatment [4 h]



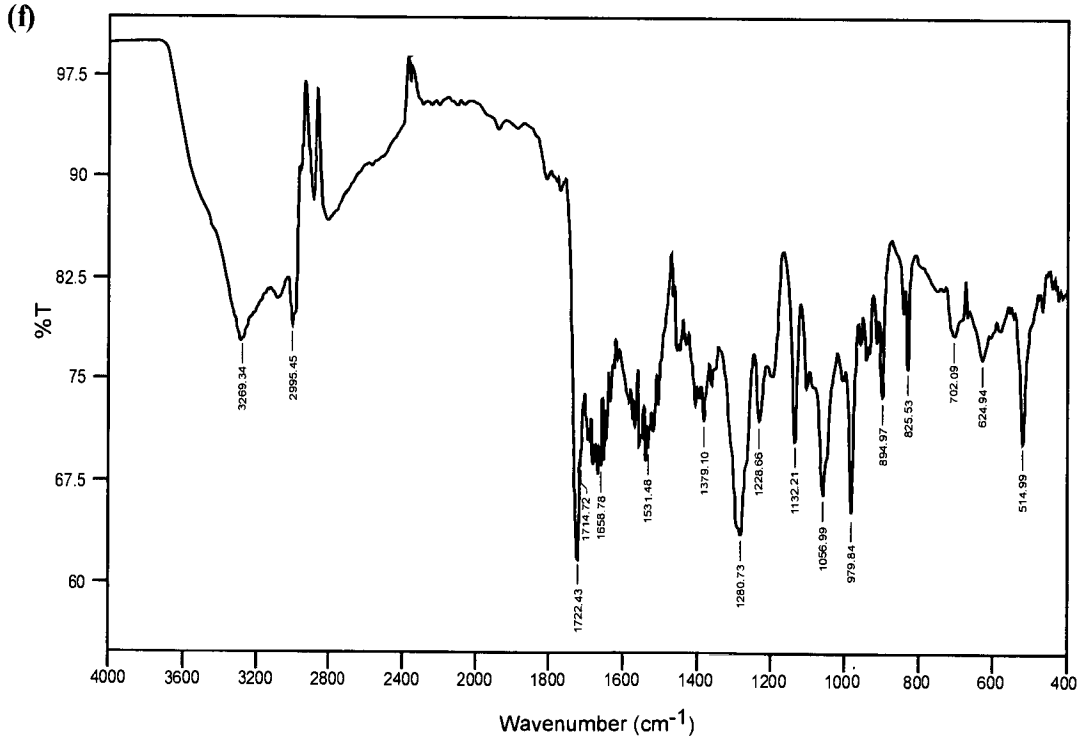


Fig. 4.6 contd. FTIR spectra of the residue obtained after subjecting the cell mass containing polymer to various treatments **(e)** alkali treatment [NH_3] and **(f)** alkali treatment [KOH]

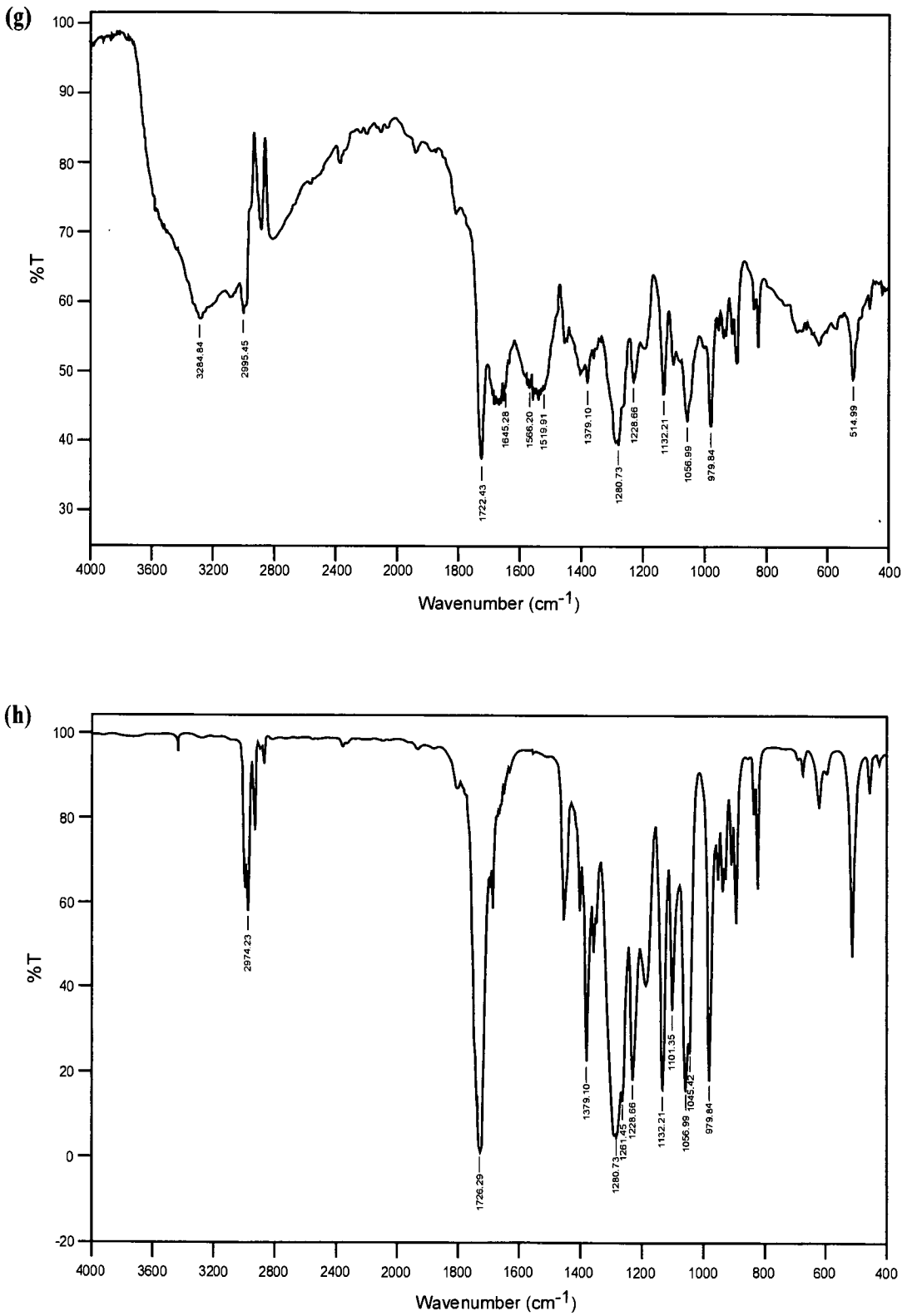


Fig. 4.6 contd. FTIR spectra of the residue obtained after subjecting the cell mass containing polymer to various treatments **(g)** alkali treatment [NaOH] and **(h)** alkali treatment [NaOCl]

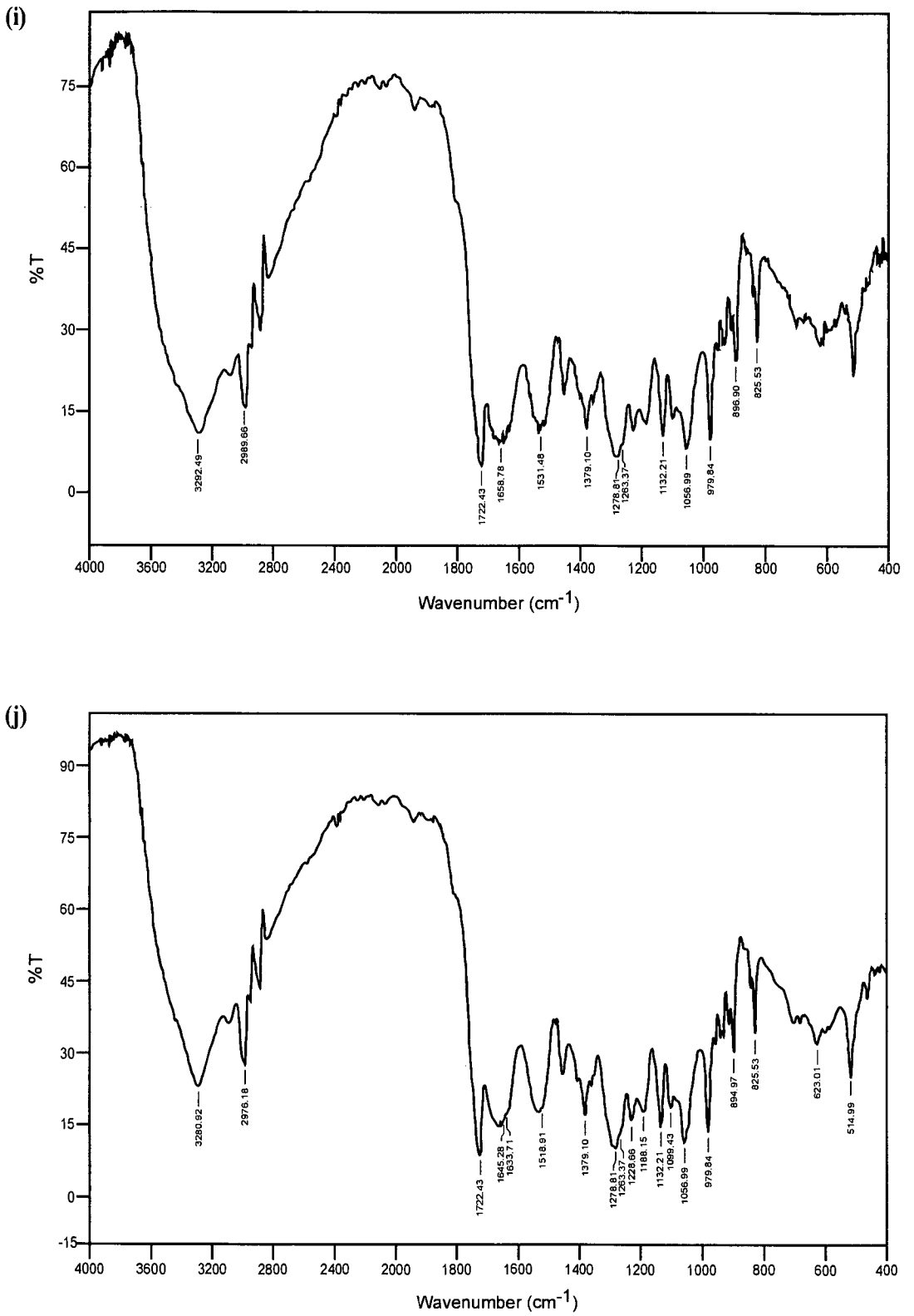


Fig. 4.6 contd. FTIR spectra of the residue obtained after subjecting the cell mass containing polymer to various treatments **(i)** SDS treatment [1 h] and **(j)** SDS treatment [2 h]

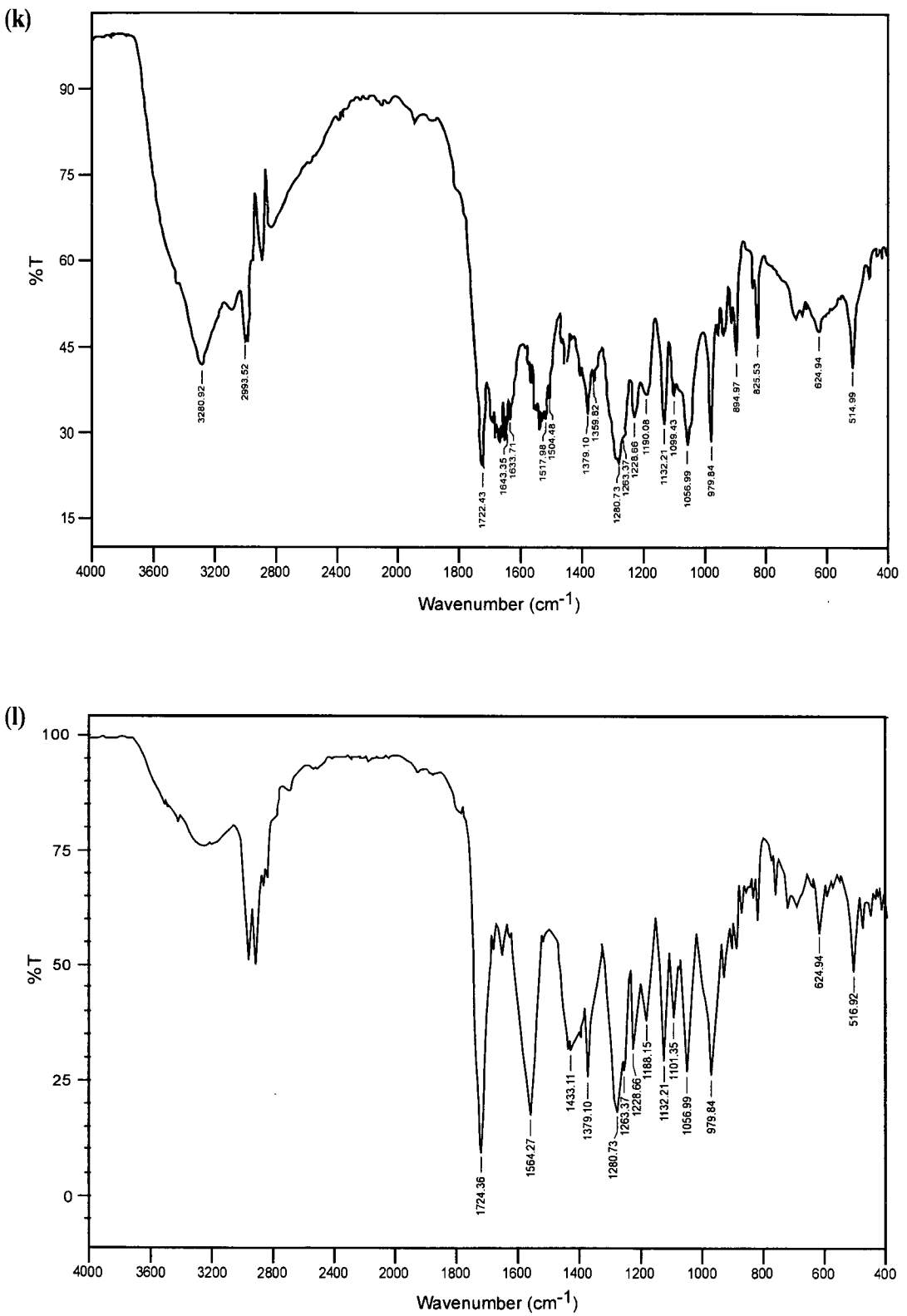


Fig. 4.6 contd. FTIR spectra of the residue obtained after subjecting the cell mass containing polymer to various treatments **(k)** hydrolytic enzyme treatment [protease] and **(l)** hydrolytic enzyme treatment [lysozyme]

cm^{-1}, as well as the characteristic absorption in the fingerprint region at 1300 to 1000 cm^{-1} obtained from the couplings of C-O and C-C stretches was also distinctly visible (Kathiraser *et al.*, 2007). Additional incubation of 2h, further improved the FTIR spectra obtained as a reduction in the number of bands near 3300 to 2800 cm^{-1} as well as 1700 to 1600 cm^{-1} was observed (Fig. 4.6d). Yu and Chen (2006) have also reported the decrease in the intensity of the absorption peaks related to amide I and amide II with increase in reaction time. This removal of protein moiety results in a spectrum almost similar to pure PHA.

The dissolution of NPCM with various alkaline solutions can be seen from Fig. 4.6e to 4.6h. Reduction in the intensities of the bands indicating presence of proteins and lipids was observed. A corresponding increase in the band at 1724 cm^{-1} (ester linkage) was also observed.

On comparing the FTIR profiles obtained on treatment with SDS indicated the positive effect on NPCM solubilization but the extent of dissolution was less as compared to the other treatments (Fig. 4.6i and 4.6j). An improvement in the fingerprint region (1300 to 1000 cm^{-1}) was observed in the detergent treated samples.

Treatment with protease did not show significant difference in the FTIR profile when compared with that of the control cells. However, the lysozyme treated residual material exhibited marked improvement in the FTIR profile obtained (Fig. 4.6k and 4.6l). A significant reduction in the bands corresponding to lipids (3000 to 2800 cm^{-1}) and proteins (amide I and amide II bands; Table 4.3) was observed.

PHA purification using solvents

Among the solvents tested, benzene and hexane did not show any effect on either solubilization of NPCM or the polymer whereas butanol, ethanol, acetic acid and chloroform had a positive effect (Fig. 4.7). The filtrates recovered after treatment with these solvents are displayed in Fig. 4.8. With solvents such as acetic acid and chloroform, 35.33% and 50.67%, respectively of cellular material including polymer was solubilized. The purity of the polymer was found to be 67.53% and 88.42% with acetic acid and chloroform, respectively. Treatment with these two solvents resulted in a white colored filtrate but among the two filtrates, chloroform filtrate was superior. The filtrate obtained on treatment with solvents such as butanol and ethanol was brownish in color (Fig. 4.9).

The filtrates obtained with acetic acid and chloroform displayed a peak at 235 nm indicating the presence of the dehydrated product of PHA ie. crotonic acid (Fig. 4.10). Since the filtrates obtained after treatment with butanol and ethanol did not demonstrate the characteristic peak indicating presence of PHA, they were not considered for further analysis. The mode of action of these solvents was found to be different. Ethanol and butanol were able to solubilize some of the non-PHA cell mass. The insolubility of the polymer in butanol contradicted the results reported by Pal and Paul (2002), who demonstrated PHA to be soluble in butanol. Acetic acid and chloroform were capable of solubilizing the polymer and these results were consistent with the findings of Pal and Paul (2002). On treatment with butanol and ethanol 8.16% and 22.49% of the NPCM was solubilized respectively. Further, the increase in purity was found to be 6.25% and 11.89% respectively. Valappil *et al.*, 2007c described chloroform extraction method in which the freeze-dried cells were stirred in

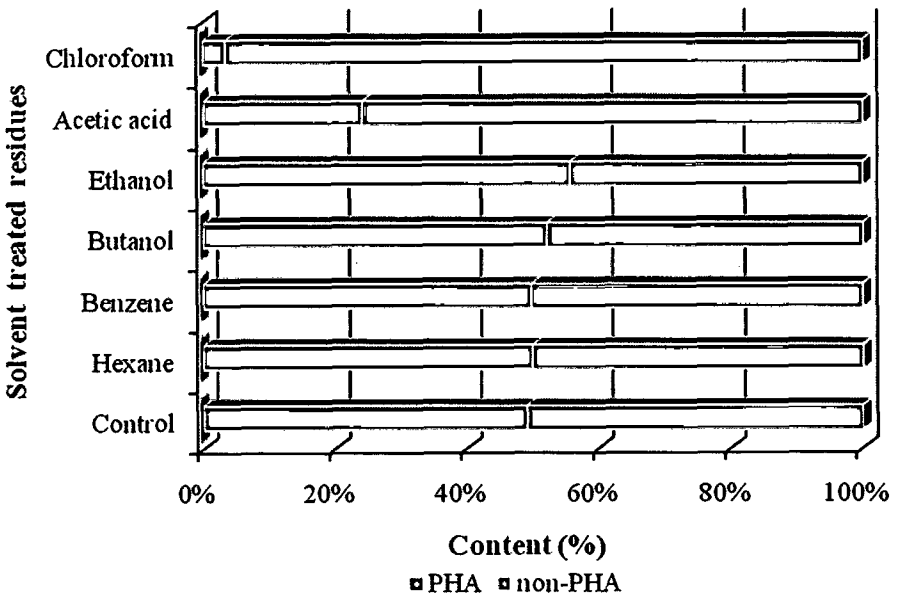


Fig. 4.7 Effect of various solvents on cell mass containing PHA

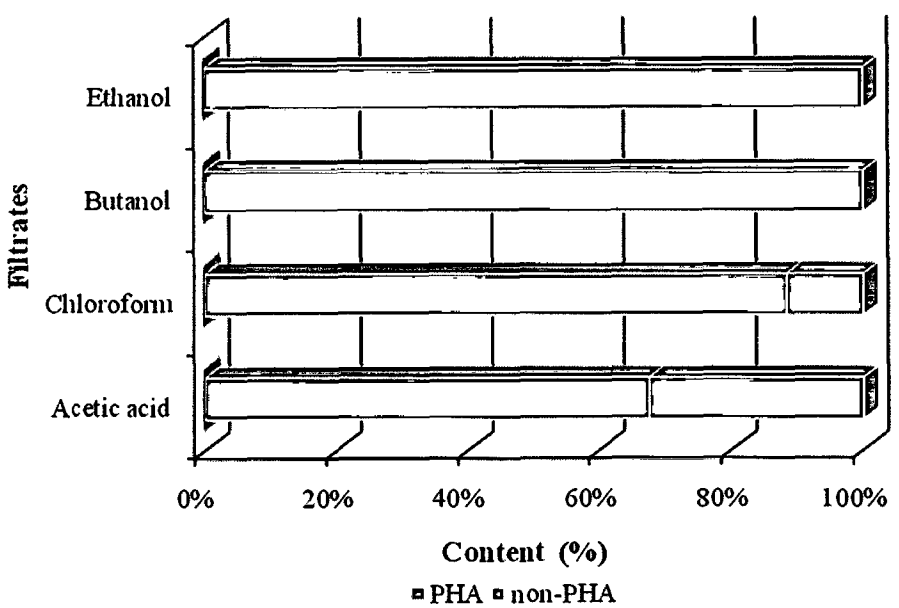


Fig. 4.8 Filtrates obtained after treatment of cell mass containing PHA with different solvents

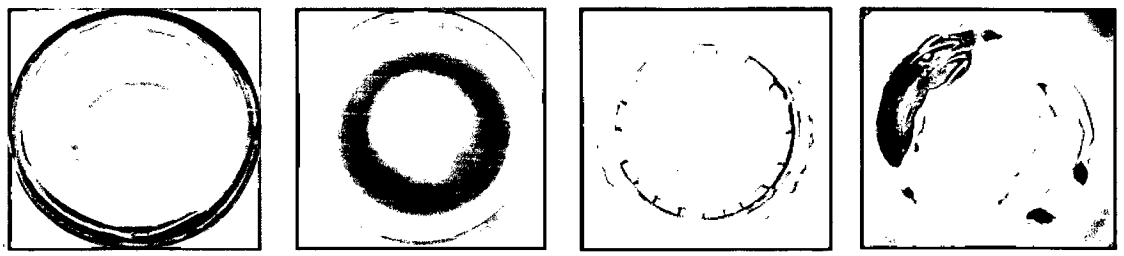


Fig. 4.9 Filtrates recovered after treatment with various solvents **(a)** Butanol, **(b)** Ethanol, **(c)** Acetic acid, **(d)** Chloroform

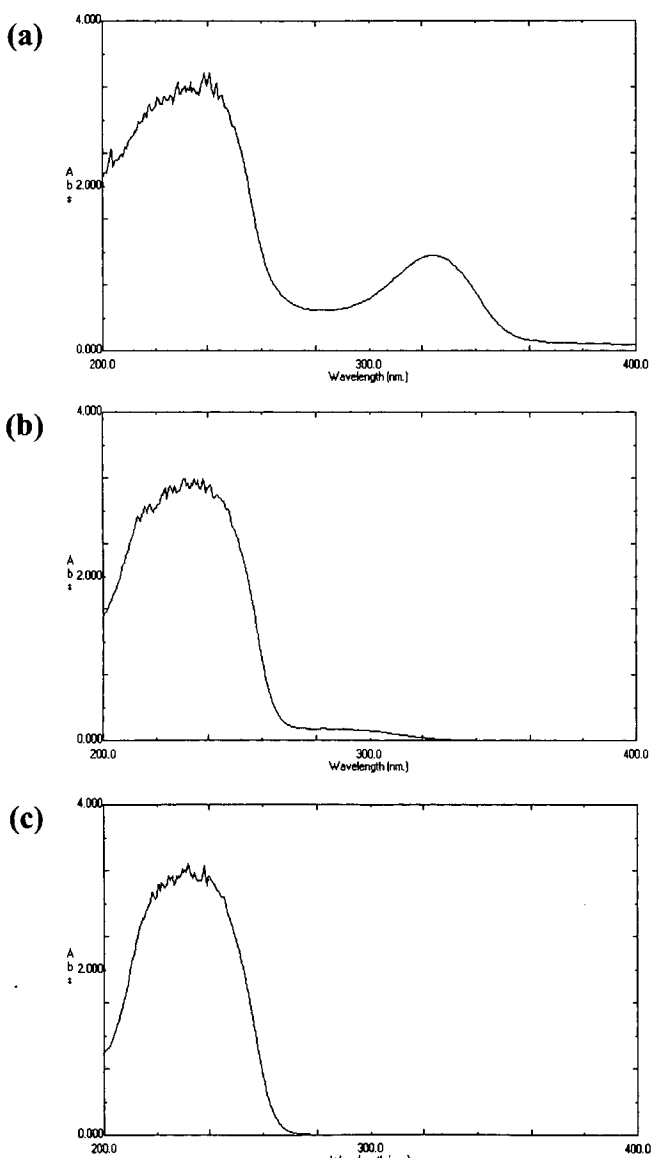


Fig. 4.10 UV absorption spectra of filtrates recovered after treatment with solvents **(a)** Control (untreated cells), **(b)** Acetic acid, **(c)** Chloroform

chloroform for 48 h at 37°C and this method yielded a polymer having a purity of 92%.

The FTIR profiles of the filtrates displayed the characteristic peak near 1724 cm^{-1} corresponding to the ester (C=O) linkage confirming the presence of PHA in the solubilized fraction (Fig. 4.11). FTIR profile of acetic acid solubilized filtrate exhibited the presence of bands near 3300 cm^{-1} indicating the presence of lipids (Table 4.3) along with the polymer.

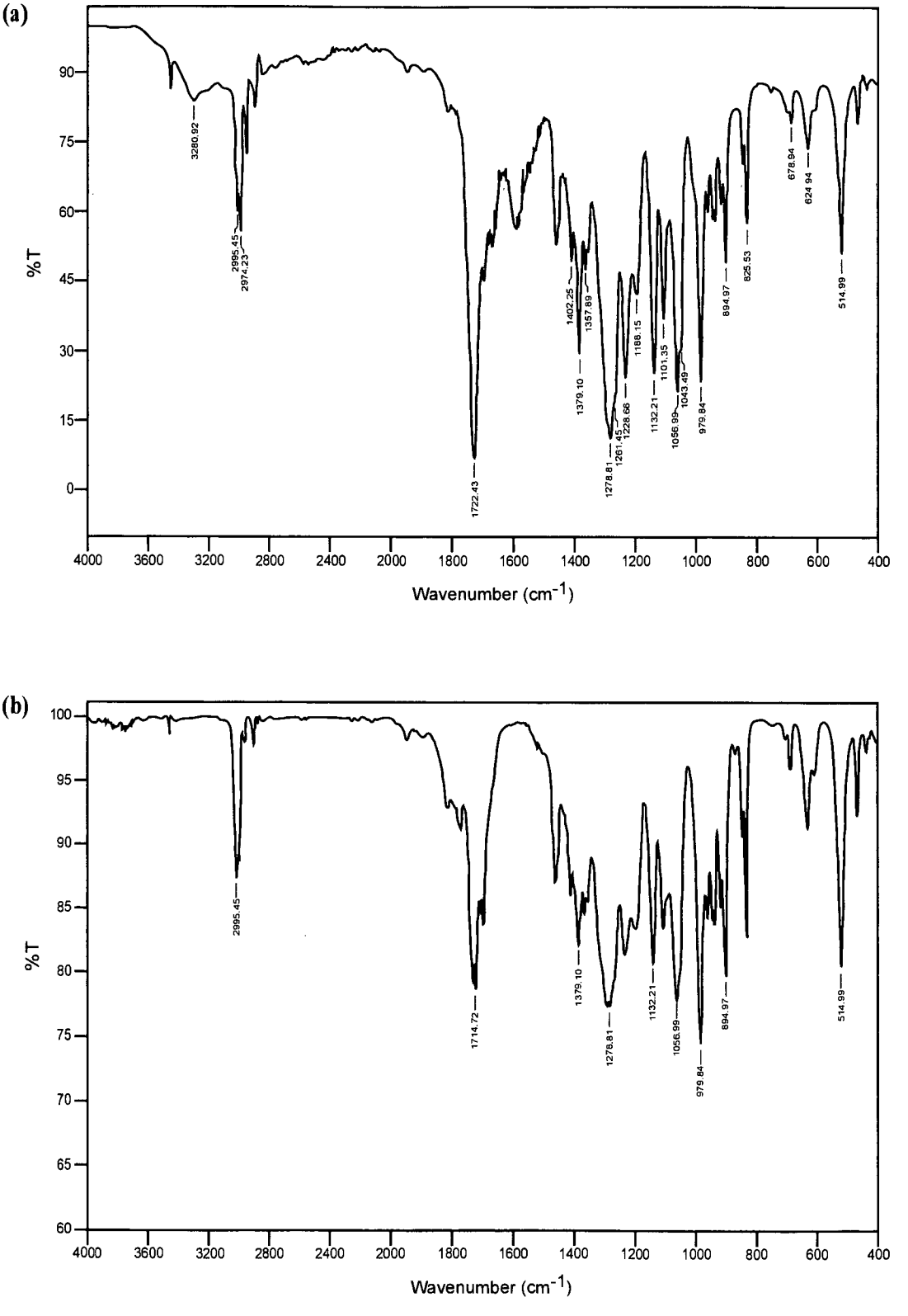


Fig. 4.11 FTIR spectra of filtrate obtained after treatment of cell mass containing PHA with various solvents **(a)** acetic acid and **(b)** chloroform

Summary and Future prospects

SUMMARY

- *Bacillus* spp. isolated from the marine and coastal environments were used for the study.

- These isolates exhibited temporal variation in their PHA accumulation pattern. Based on the PHA content accumulated within the cells, the isolates could be categorized into four groups.

- Isolates (BLQ-2/A7, COL1/A6, L2/A1 and L4/A4) belonging to one of these groups were capable of consistently maintaining the accumulated PHA without significant intracellular degradation during the entire experimentation time. This property is crucial in large scale PHA production as a variation in the time of harvest will not adversely affect the product yield.

- The *Bacillus* spp. used in this study were found capable of utilizing various carbon substrates for polymer production and exploitation of this ability resulted in the selection of some agro-industrial by-products (such as molasses, starch based residues, citrus pulp waste, coconut oil cake, bagasse and rice chaff) as potential carbon feedstock for PHA production.

- Majority of the isolates were capable of producing PHA using the agroindustrial substrates tested.

- Dilute acid hydrolysis of the agroindustrial by-products not only improved the ability of the isolates to assimilate the released fermentable sugars as PHA but also avoided the interference caused by insolubles present in the wastes during downstream processing when cultivated under submerged fermentation conditions.

- Isolates exhibiting maximum PHA fluorescence intensity on respective substrates when stained with Nile blue A were selected for further studies on PHA production under submerged cultivation conditions.

- Using molasses as the carbon source, all the isolates tested were able to accumulate PHA ranging between 51.23% and 68.56% DCW. Starch based residue was also efficiently utilized by the isolates as more than 55% DCW PHA was produced within the cells. Citrus pulp waste was able to support a PHA content ranging between 38.87% and 48.86% DCW in the *Bacillus* spp. tested. Coconut oil cake also served as a potential carbon source for PHA production with a highest PHA content of 42.4% DCW (COL1/A11).

- Isolates COL1/A6 and BLQ-2/A7 were selected for further based on their maximum and consistent production of PHA and ability to utilize diverse agro-industrial by-products.

- Optimization studies revealed that both the isolates were able to accumulate PHA between 20 to 40°C with the optimal temperature for growth and maximum PHA accumulation at 30°C.

- Strain COL1/A6 and BLQ-2/A7 exhibited pH optima of 7.0 for growth as well as PHA production. These isolates were able to grow and accumulate significant amounts of PHA in fermentation medium adjusted to pH values either one unit above or below their pH optima.

- Among the various ammonium salts tested, both the isolates produced highest PHA yields using ammonium chloride as a nitrogen source.

- Optimization studies carried out using different yeast extract concentrations indicated that a lower concentration range between 0.1 g L^{-1} to 0.5 g L^{-1} was ideal for PHA production. Further increase in the concentration to 5 g L^{-1} resulted in enhancement of growth only.

- Growth of the isolate BLQ-2/A7 under optimized conditions revealed that the isolate grows rapidly with maximum PHA of 59.42% DCW obtained at 48 h. However, nearly half of the sugar supplied was found unutilized in the medium at the end of the fermentation run.

- In the case of isolate COL1/A6, time course studies using batch cultivation showed that the isolate grows slowly but accumulates more amount of PHA (65.51% DCW) than isolate BLQ-2/A7 with only 25% of the supplied sugar left in the medium after the fermentation run.

- Studies on the improvement of the PHA yield were investigated with isolate BLQ-2/A7 since this isolate grows rapidly and is unable to efficiently utilize the sugar supplied.

- The model obtained employing response surface methodology indicated a positive interaction between glucose and ammonium chloride in case of biomass as well as PHA production. The studies also indicated that the concentration of glucose and ammonium chloride is critical for growth and PHA production.

- The conditions described for fed-batch I fermentation revealed that the addition of glucose and ammonia at 18 h of incubation created conditions conducive for biomass production rather than PHA formation.

- The fed-batch II strategy of fermentation (addition of ammonium at 12 h) resulted in an increase in the PHA content. Maximum content of 65.51% DCW within 24 h was achieved and the PHA content was found to be relatively stable for another additional 24 h.

- Fed-batch fermentation studies highlighted the importance of a simple nitrogen feeding strategy for improved substrate utilization and PHA production. The PHA yield using the isolate BLQ-2/A7 increased from an initial of 56.2% DCW at 48 h under non-optimized fermentation conditions to 65.5% DCW at 24 h by optimization of batch fermentation conditions coupled with a simple nitrogen feeding strategy.

- Screening of various non-solvent and solvent systems for the solubilization of cell material containing PHA revealed that treatment with sodium hypochlorite and sulphuric acid (4 h) resulted in the recovery of the polymer (purity 97.39% and 94.93% respectively). Among the solvents, chloroform proved to be a better solvent for solubilization of the polymer. Based on FT-IR profiles of the polymer obtained using various treatments, the best method was found to be treating the cells with sodium hypochlorite followed by acid treatment (4 h) and chloroform.

FUTURE PROSPECTS

The present study indicates the potential of *Bacillus* spp. as PHA producers. In this study, *Bacillus* spp. were able to unequivocally produce PHA ranging from 29.45% DCW (COL2/A2 on coconut oil cake hydrolysate) to 68.56% DCW (ICP-1/A3 on molasses) from low-cost agroindustrial residues. Further characterization of the produced polymer is necessary as this genus is known to produce PHAs with different

monomer composition from a wide variety of substrates (Valappil *et al.*, 2007a; Tajima *et al.*, 2003; Labuzek and Radecka, 2001). Based on these investigations further studies are envisaged for

- PHA production and high-cell density fermentation with intensive studies on fed-batch mode of operation involving pH, dissolved oxygen (DO) regulated system and glucose supplementation need to be investigated.

  The high-cell density is vital for the economic viability of the production process (Madison and Huisman, 1999). The advantages of this cultivation system include higher product concentration, reduced investment in equipment, decreased production costs and increased volumetric productivity.

- Use of solid state fermentation (SSF) for production of PHA.

  This is another attractive and emerging fermentation strategy that can be employed. SSF allows the use of inexpensive feedstock such as agro-industrial residues.

  The advantage of employing such fermentation strategy provides solutions to the disposal of these residues with the simultaneous production of value-added products. The added advantage is that these residues can be directly incorporated in the fermentation media without any pretreatment necessary unlike submerged fermentation (SMF). The fermented solids containing PHA products can be used directly without downstream processing to prepare composite materials of increased biodegradability (Castilho *et al.*, 2009).

Appendices

APPENDIX 1:- MEDIA AND STAINS

Nutrient Agar (NA)

Ingredients	**g L^{-1}**
Peptone	10.0 g
NaCl	5.0 g
Beef Extract	3.0 g
Agar	20.0 g
Distilled water	1.0 L
pH	7.4

Nutrient Broth was prepared with all the ingredients except agar.

Use: Used for isolation, maintenance and cultivation of microorganisms.

E2 mineral medium (Lageveen *et al.*, 1988)

Ingredients	**g L^{-1}**
Basal medium	
Microcosmic salt ($NaNHPO_4$)	3.5 g
Dipotassium hydrogen phosphate	7.5 g
Potassium dihydrogen phosphate	3.7 g
$MgSO_4.7H_2O$ (100 mM)	10 ml
MT stock	1.0 ml
Yeast extract	0.004 g
Glucose	20 g
Distilled water	989 ml
pH	7.2

MT stock	
$FeSO_4.7H_2O$	2.78 g
$MnCl_2.4H_2O$	1.98 g
$CoSO_4.7H_2O$	2.81 g
$CaCl_2.2H_2O$	1.47 g
$CuCl_2.2H_2O$	0.17 g
$ZnSO_4.7H_2O$	0.29 g
Distilled water	1.0 L

The basal medium, glucose, magnesium sulfate solution, yeast extract solution and MT stock were sterilized separately and added to medium prior to use.

E2 mineral medium agar was prepared by adding 20 g agar to basal medium before sterilization.

Production medium

Ingredients	**g L^{-1}**
Ammonium chloride	0.895 g
Potassium phosphate buffer	0.07 M, pH 7
Yeast extract	0.05 g
Magnesium sulphate (100 mM)	10 ml
Mineral trace element solution	1 ml
Glucose	20 g

Ammonium chloride solution, Potassium phosphate buffer, Yeast extract solution, Magnesium sulphate solution, mineral trace element solution and glucose solution were sterilized separately and added to medium prior to use.

Glucose or the hydrolyzed carbon wastes were autoclaved separately and added to the medium prior to inoculation.

Saline

NaCl	0.85 g
Distilled water	100 ml

Sodium hypochlorite solution

Sodium hypochlorite (4% active chlorine)	50 ml
Distilled water	50 ml

Stain

Nile Blue A stain for plate assay

Nile Blue A	0.05 g
Ethanol	100 ml

APPENDIX 2:- DILUTE ACID HYDROLYSIS OF SELECTED BY-PRODUCTS

Hydrolysis using dilute acid of selected by-products was carried out as shown in Table A1.

The hydrolysis was carried out with 0.75% v/v sulphuric acid (Buchner and Agblevor 2001) in different conditions as described below:

Treatment I: 100°C for 60 min

Treatment II: 100°C for 120 min

Treatment III: 121°C with 15 lbs pressure for 30 min

Treatment IV: 121°C with 15 lbs pressure for 60 min

The amount of waste used in all the above treatments was 7 % (w/v). After hydrolysis the reaction mixture was centrifuged. The undigested components which pelleted out were collected in pre-weighed aluminium cups and dried at 60°C until constant weight was obtained. The percentage of hydrolysis was calculated based on the weight of the pellet recovered after the various hydrolysis treatments. The hydrolysate separated on centrifugation was neutralized with sodium hydroxide to pH 7.0. The neutralized hydrolysates were analyzed for their reducing sugar content and total sugar content (Miller, 1959; Dubois *et al.*,1956) (Table A1).

Table A1. Dilute acid hydrolysis of selected by-products

By-product	Treatment	Total sugar ($g\ g^{-1}$ ± SE)	Reducing sugar ($g\ g^{-1}$ ± SE)	% Hydrolyzed (± SE)
Citrus pulp	I	0.560± 0.019	0.471± 0.017	62.99± 1.6
	II	0.35 ± 0.02	0.342 ± 0.013	66.86± 1.5
	III	0.376± 0.024	0.405± 0.025	63.03± 1.5
	IV	0.350± 0.01	0.358± 0.011	65.05± 1.8
Wafer	I	1.073± 0.031	0.761± 0.021	93.99± 0.29
	II	ND	ND	ND
	III	0.607± 0.011	0.584± 0.011	95.63± 0.31
	IV	ND	ND	ND
Bagasse	I	0.087± 0.008	0.045± 0.004	7.38± 1.25
	II	0.257± 0.017	0.085± 0.008	13.64± 3.59
	III	0.188± 0.007	0.157± 0.007	9.27± 1.49
	IV	0.214± 0.014	0.133± 0.008	7.73± 1.97
Rice chaff	I	0.125± 0.006	0.09±0.003	22.37± 3.56
	II	0.257± 0.022	0.138± 0.05	32.51± 2.16
	III	0.230± 0.006	0.215± 0.008	34.35± 4.80
	IV	0.257±0.017	0.175± 0.008	35.95± 2.70
Coconut oil cake	I	0.192± 0.005	0.194± 0.006	34.33± 3.58
	II	0.453± 0.024	0.219± 0.009	48.31± 2.05
	III	0.198± 0.008	0.186± 0.007	40.16± 1.70
	IV	0.270± 0.01	0.209± 0.009	47.73± 1.03
Pectin	I	0.735± 0.02	0.385± 0.01	66.30± 0.41
	II	ND	ND	ND
	III	0.717± 0.022	0.430± 0.012	63.85± 2.22
	IV	ND	ND	ND
Starch	I	0.788± 0.024	0.962± 0.03	98.99± 0.28
	II	ND	ND	ND
	III	1.233± 0.034	1.06± 0.029	99.25± 0.2
	IV	ND	ND	ND

Key: SE- Standard error

ND- Not done

APPENDIX 3:- ESTIMATION METHODS

Estimation of Inorganic Phosphate (Fiske and Subbarao, 1925; Buchanan, 1975)

Reagents:

Solution A (Sulfuric acid solution)

9 N solution of H_2SO_4 prepared by dissolving 24.8 ml of 98% concentrated H_2SO_4 to 100 ml double distilled water.

Solution B (Ammonium molybdate solution)

Dissolved 6 g of ammonium molybdate in distilled water to a final volume of 100 ml

Solution C (Reducing solution)

8 g of $FeSO_4.7H_2O$ dissolved in minimal volume of distilled water in 100 ml volumetric flask. To this solution, 1 ml of solution A added, mixed thoroughly and volume made up to 100 ml.

Protocol:

3.5 ml of diluted sample

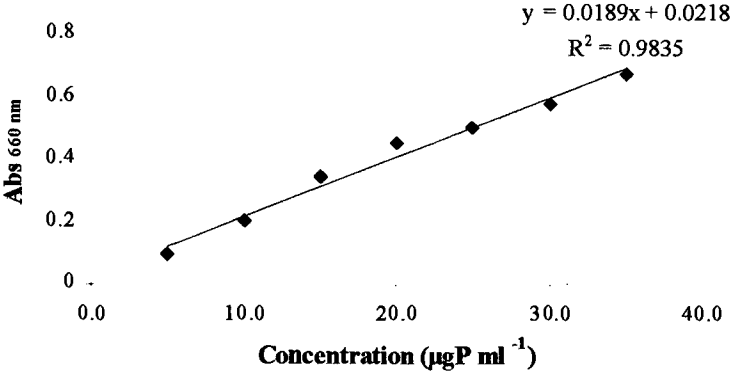
↓

0.5 ml of solution A, B and C added sequentially, mixed and incubated in dark

↓

Absorbance recorded at 660 nm after 20 min

The calibration curve was prepared by using KH_2PO_4 as the standard.



Standard curve for inorganic phosphate estimation

Estimation of reducing sugars (Miller, 1959)

Reagents: (Modified method)

Dinitro salicylic acid solution (DNSA)

2 g of NaOH dissolved in 50 ml double distilled water. Add 0.2 g phenol and 0.2 g of DNSA, add with constant stirring. Make up the volume to 100 ml with distilled water. Before using, add 0.05 g of Sodium sulfite to 100 ml of DNSA solution and mix thoroughly.

Tartarate solution

Dissolve 33.0 g of Potassium sodium tartarate to a final volume of 100 ml in double distilled water.

Protocol:

2.0 ml of diluted sample + 1.0 ml of DNSA solution

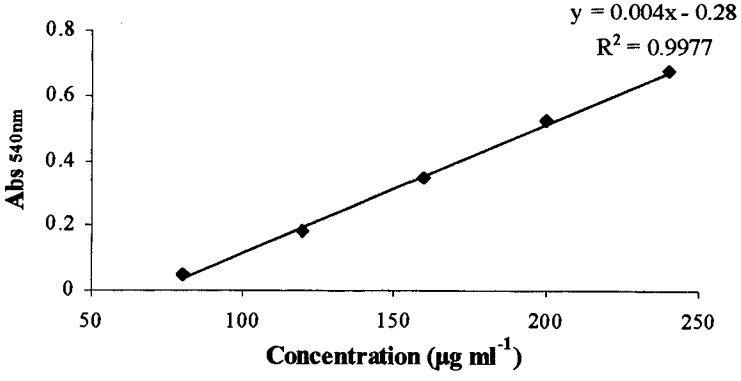
↓ Boiling water bath for 10 min

After cooling, add 0.4 ml of tartarate solution and mix thoroughly

↓

Absorbance recorded at 540 nm

The calibration curve was prepared by using glucose as the standard.



Standard curve for reducing sugar estimation

Estimation of total sugars (Dubios *et al.*, 1956)

Reagents:

Phenol solution (5%)

Dissolve 5 g of phenol in 100 ml of double distilled water.

Protocol:

1.0 ml of diluted sample

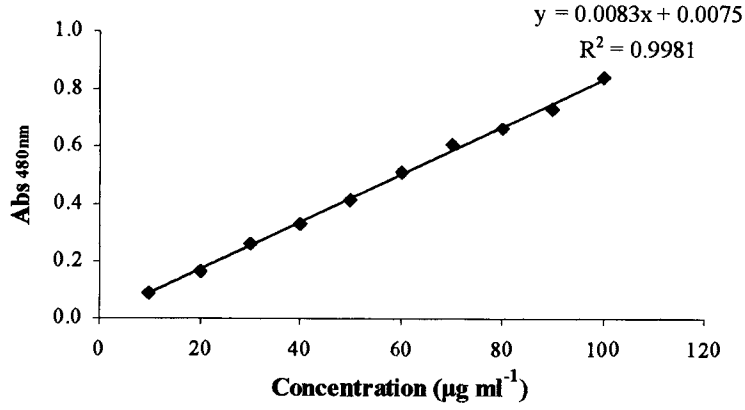
↓

1.0 ml 5% phenol and 5.0 ml of concentrated H_2SO_4

↓ Ice bath for 10 min

Absorbance recorded at 480 nm

The calibration curve was prepared by using glucose as the standard.



Standard curve for total sugar estimation

Estimation for Ammonia-nitrogen (Phenate method; Franson, 1985)

Reagents:

Ammonia-free water

Boil distilled water for 2 h on burner or 30 min on gas. Use to make all reagents.

Hypochlorous acid reagent

12.5 ml of sodium hypochlorite (4%), adjust the pH to 7.0 and bring up the volume to 50 ml with ammonia-free water.

Manganous sulphate solution (0.006N)

Dissolve 50 mg $MnSO_4.H_2O$ in 100 ml ammonia-free water.

Phenate solution

Dissolve 2.5 g NaOH and 10 g phenol in 100 ml ammonia-free water.

Protocol:

10.0 ml of diluted sample

↓

0.05 ml of $MnSO_4$ solution

↓

Place on magnetic stirrer

↓

0.5 ml Hypochlorous acid reagent

↓

Immediately add, a drop at a time 0.6 ml Phenate reagent

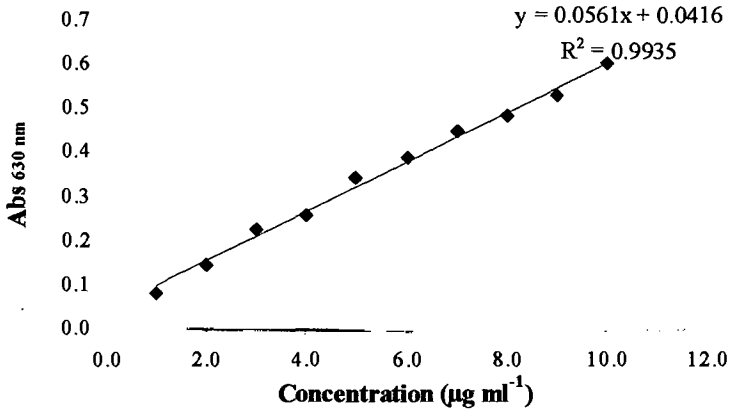
↓

Stir vigorously during addition of reagents

↓

Absorbance recorded at 630 nm after approximately 18 h

The calibration curve was prepared by using anhydrous ammonium chloride as the standard.



Standard curve for ammonia-nitrogen estimation

Estimation for PHA (Law and Slepecky, 1961)

Reagents:

Sulfuric acid: Concentrated

Protocol:

Sample containing polymer in chloroform transferred to clean test tube

↓

Chloroform evaporated

↓

5.0 ml of concentrated H_2SO_4

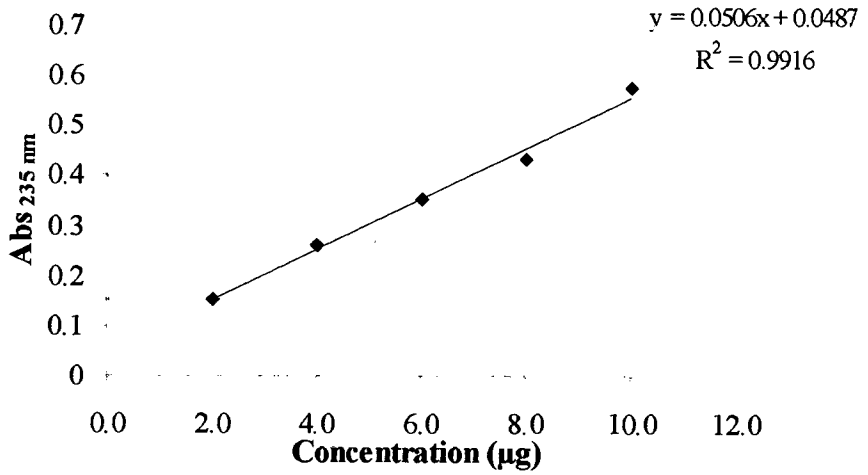
↓ Boiling water bath for 10 min

Solution cooled and mixed thoroughly

↓

Absorbance recorded at 235 nm

The polymer used as the standard was extracted and purified using methods described by Law and Slepecky (1961). The calibration curve was prepared by using the polymer prepared by above method as the standard.



Standard curve for PHA estimation

APPENDIX 4:- GENOMIC DNA EXTRACTION AND PCR AMPLIFICATION

Saline-EDTA buffer

Sodium chloride	0.15 M
Sodium-EDTA (pH 8.0)	0.1 M

TE Buffer

Tris (pH 7.3)	0.01 M
Sodium-EDTA	0.001 M

Phenol-chloroform-isoamyl alcohol (PCI)

Phenol (pH 8.0)	25.0 ml
Chloroform	24.0 ml
Isoamyl alcohol	1.0 ml

PCR amplification

The 16S rRNA gene was amplified using isolated chromosomal DNA as the template. The primers used for the amplification were S-D-Bact-0011-a-S-17 5'-GTTTGATCCTGGCTCAG-3' as forward and S-*-Univ-1392-b-A-15 5'-ACGGGCGGTGTGTTC-3' as reverse primer (Alm *et al.*, 1996). All PCR reactions were performed in a reaction mixture of 100 µl total volume using 1 µg template DNA. The reaction mixture was heated at 94°C for 3 min followed by 40 cycles of 94°C for 1 min, 52°C for 1 min and 72°C for 1 min. The final elongation step was carried out at 72°C for 5 min. The extracted chromosomal DNA was sent for sequencing at Macrogen Inc, Korea.

APPENDIX 5:- RESPONSE SURFACE METHODOLOGY (RSM) (Montgomery, 1991; Myers *et al.*, 1989)

Knowledge about the relationship between the treatments and their effects is critical if one wants to find the treatment combination for optimal (or maximum or minimum) effect. Methods that are directed towards this kind of investigation, using tools from experimental design and regression analysis, are usually referred to as Response surface methodology (RSM).

RSM is an effective statistical technique used for investigation of complex processes whose mechanisms are not completely known. The objective of this methodology is to optimize a *response* (output variable) which is influenced by several *independent variables* (input variables). RSM consists of a series of tests, called *runs*, wherein changes are made in the input variables in order to identify the reasons for changes in the output response.

The response can be represented graphically, either in the three-dimensional space or as *contour plots* that help visualize the shape of the response surface. These surfaces actually explain individual, cumulative, and interactive effects of test variables on response.

An important aspect of RSM is the *design of experiments* (Box and Draper, 1987), usually abbreviated as DoE. The objective of DoE is the selection of the points where the response should be evaluated. A particular combination of runs defines an *experimental design*. The possible settings of each independent variable in the N dimensional space are called *levels*. The choice of the design of experiments can have a large influence on the accuracy of the approximation and the cost of constructing the response surface.

Among the various methodologies for the DoE, **Central composite design (CCD)** is frequently used for optimization studies in biotechnology. A second-order model can be

constructed efficiently with this design (Montgomery, 1991). CCD are first-order ($2N$) designs augmented by additional centre and axial points to allow estimation of the regression coefficients of a second-order model. In Figure A1, central composite design involving $2N$ factorial points, $2N$ axial points and one central point is shown.

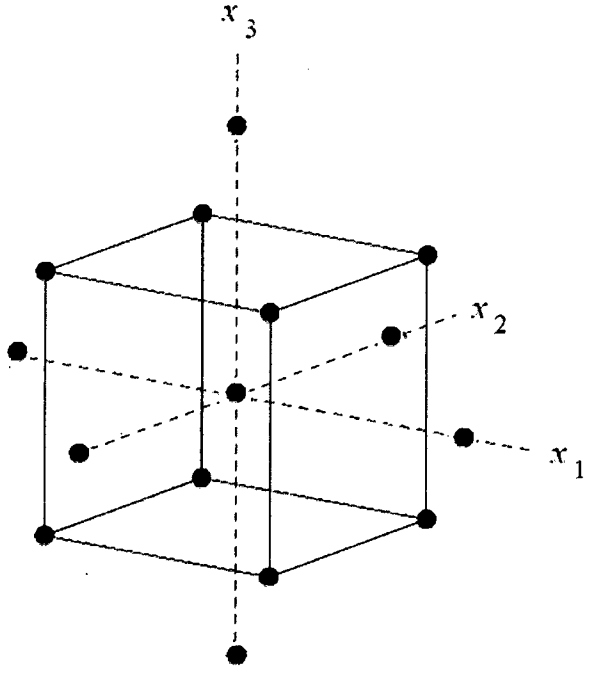


Fig. A1 Central composite design for three design variables

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List of Publications

LIST OF PUBLICATIONS

1. Prabhu, N.N., **Santimano, M.C.**, Bhosle, S.N., Mavinkurve, S. and Garg, S. (2010) Native granule associated SCL-PHA synthase from a marine derived *Bacillus* sp. NQ-11/A2. *Antonie van Leeuwenhoek* 97, 41-50.

2. **Santimano, M.C.**, Prabhu, N.N. and Garg, S. (2009) PHA production using low-cost agronomic wastes by *Bacillus* sp. strain COL1/A6. *Res J Microbiol* 4 (3), 89-96.

3. **Santimano, M.C.**, Prabhu, N.N. and Garg, S. (2009) A simple method for PHA production by *Bacillus* sp. COL1/A6 utilizing waste coconut oil cake. *J Curr Sci* 14 (1): 274-282.

4. Prabhu, N.N., **Santimano, M.C.** and Garg, S. (2009) Studies on polyhydroxyalkanote production by a marine *Bacillus* sp. NQ-11/A2 isolated from continental shelf sediment. *J Curr Sci* 14 (1): 265-273.

PAPERS PRESENTED AT CONFERENCES

1. **Santimano, M.C.**, Prabhu, N.N., Chari, L.J., Bhosle, S.N. and Garg, S. (2006) Waste rich organic contents as low cost substrate for the production of polyhydroxyalknaote. AMI conference on "Microbiology: The challenges ahead". 6th to 8th December, Barkatullah University, Bhopal, India.

2. **Santimano, M.C.,** Prabhu, N.N., Kowshik, M., Nair, D., Bhosle, S.N., Mavinkurve, S. and Garg, S. (2005) Screening of Coastal and Marine environments for polyhydroxyalkanoate accumulating microorganisms.

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3. Prabhu, N.N., **Santimano, M.C.**, Kowshik, M., Bhosle, S.N., Mavinkurve, S. and Garg, S. (2005) Diversity of PHA accumulating bacterial isolates belonging to *Bacillus* spp. obtained from marine and coastal environments" International conference on "Microbial Diversity: Current perspectives and potential applications" 16th to 18th April, University of Delhi, South Campus, New Delhi, India.

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ORIGINAL PAPER

Native granule associated short chain length polyhydroxyalkanoate synthase from a marine derived *Bacillus* sp. NQ-11/A2

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Abstract A rapidly growing marine derived *Bacillus* sp. strain NQ-11/A2, identified as *Bacillus megaterium*, accumulated 61% polyhydroxyalkanoate by weight. Diverse carbon sources served as substrates for the accumulation of short chain length polyhydroxyalkanoate. Three to nine granules either single or attached as buds could be isolated intact from each cell. Maximum activity of polyhydroxyalkanoate synthase was associated with the granules. Granule-bound polyhydroxyalkanoate synthase had a K_m of 7.1×10^{-5} M for DL-β-hydroxybutyryl-CoA. Temperature and pH optima for maximum activity were 30°C and 7.0, respectively. Sodium ions were required for granule-bound polyhydroxyalkanoate synthase activity and inhibited by potassium. Granule-bound polyhydroxyalkanoate synthase was apparently covalently bound to the polyhydroxyalkanoate-core of the granules and affected by the chaotropic reagent urea. Detergents inhibited the granule-bound polyhydroxyalkanoate synthase drastically whilst glycerol and bovine serum albumin stabilized the synthase.

Keywords Polyhydroxyalkanoate (PHA) · Granule bound PHA synthase (GBPS) · Marine derived *Bacillus* · Short chain length—PHA (SCL-PHA)

Introduction

Bioplastics have received tremendous attention in the recent years due to their high molecular weight, thermoplastic/elastomeric properties, biodegradability, biocompatibility, non-toxicity and its production from renewable carbon sources. Polyhydroxyalkanoates (PHAs) are simple macromolecules synthesized by a wide variety of Gram-positive and Gram-negative bacteria, and members of family Halobacteriaceae of the Archaea (Philip et al. 2007; Hezayen et al. 2002). The synthesis of the polymer is initiated when acetyl-CoA is restricted from entering the tricarboxylic acid cycle due to the nutrient limitation, shunting the acetyl units from the TCA cycle into the production of polyhydroxybutyrate (PHB), which is an ideal carbon and energy storage polymer. Currently more than 150 different hydroxyalkanoic acids have been identified as substrates by PHA synthases, thereby determining the type of PHA produced by the organism.

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PHA Production Using Low-Cost Agro-Industrial Wastes by *Bacillus* sp. Strain COL1/A6

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Abstract: Recycling of wastes generated from agro based industries for polyhydroxyalkanoate production is not only crucial for waste management but also in economizing and commercializing the polymer. In this study, the heterotrophic bacterium *Bacillus* sp. strain COL1/A6 isolated from humus was biologically characterized and explored for its potential to synthesize PHA using agroindustrial wastes. Qualitative analysis using Nile blue A staining revealed that starch, wafer residue, citrus pulp and cane molasses proved to be excellent carbon substrates for PHA accumulation. Growth and PHA producing ability of the isolate on cane bagasse and rice chaff improved after dilute acid hydrolysis. Highest cellular PHA content was obtained using wastes such as hydrolyzed wafer residue (62.41±1.04% of dry cell wt.) followed by cane molasses (54.68±1.36% of dry cell wt.) and hydrolyzed citrus pulp (47.5±1.01% of dry cell wt.). This is the first report wherein a *Bacillus* sp. has been reported to grow and utilize wastes such as wafer residue and citrus pulp as carbon feedstock for PHA production.

Key words: *Bacillus* sp., humus, low-cost, polyhydroxy alkanoate, agroindustrial wastes

INTRODUCTION

The extensive usage of petrochemical plastics due to their versatile properties especially durability is causing severe problem in waste management affecting the aesthetic quality of cities, water bodies and natural areas (Full *et al*., 2006). As a result, lot of research is now focused on the production of biodegradable plastics. Polyhydroxyalkanoates (PHAs) are the only naturally occurring polymers that are 100% biodegradable (Khanna and Srivastava, 2005). The wide spread use of this polymer is restricted only to areas where conventional plastics find limited applications such as the medical field due to its high production cost (Verlinden *et al*., 2007; Valappil *et al*., 2007a).

Process economics reveal that the use of inexpensive and renewable carbon substrates viz. agro industrial wastes and byproducts as PHA feedstock can contribute to as much as 40-50% reduction in the overall production cost (Choi and Lee, 1999; Kim, 2000). Other parameters which also influence the total production cost are bacterial strains, fermentation strategies and recovery processes. Currently, Gram negative microorganisms such as *Cupriavidus necator*, *Alcaligenes latus* and recombinant *Escherichia coli* are used for commercial polymer production (Valappil *et al*., 2007a). They contain Lipopolysaccharide (LPS) endotoxins which co-purify with PHA. This limits the application of the polymer in medical field as LPS can elicit severe immunological reactions (Valappil *et al*., 2007a; Chen and Wu, 2005). Synthesis of LPS free polymer requires additional purification step increasing the production cost. Gram positive bacteria such as *Bacillus* sp. are ideal candidates for industrial scale PHA production due to the lack of LPS layer. Members of this genus are known to grow rapidly, possess various hydrolytic enzymes and produce copolymers from structurally unrelated carbon sources (Valappil *et al*., 2007b; Halami, 2007). These very characteristics of *Bacillus* sp. can be exploited for the production of PHA with desirable material properties from various low-cost agricultural feedstocks.

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A simple method for PHA production by *Bacillus* sp. COL1/A6 utilizing waste coconut oil cake

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ABSTRACT

The bacterial strain, COL1/A6 used in this study was phenotypically characterized and identified as *Bacillus* sp. Ability of this isolate to utilize waste coconut oil cake as carbon feedstock for polyhydroxyalkanoate (PHA) production was exploited. The innovative method developed for polymer synthesis yielded 41.92 % PHA of total cell dry weight. The polymer purity was confirmed by detecting the presence of a single crotonic acid peak at 235 nm. This is the first report on the use of waste coconut oil cake as sole source of carbon for PHA production.

Keywords: *Bacillus, humus, polyhydroxyalkanoate, biodegradable, carbon feedstock, coconut oil cake*

INTRODUCTION

The global production of oil seeds for the year 2007-08 is estimated to be about 392.6 million tonnes with India being one of the world's leading oil seed producers (FAO 2008). During the oil extraction process, the major by-product generated is the oil cake which contributes to almost 50 % of the discarded waste. These cakes can be used as fertilizers, fuel or animal feed due to their rich protein content, but are mostly disposed off as waste in the environment (Ramachandran *et al.* 2007).

Utilization of oil cakes as potential raw materials in bioprocesses is advantageous since they can serve as excellent substrates for the growth of microorganisms. With increasing emphasis on cost reduction of industrial processes, attempts are now being made to exploit such agro-industrial wastes for production of value added commodities such as enzymes, amino acids, organic acids and mushrooms (Joo and Chang 2005; Pandey 2003; Sircar *et al.* 1998; Shashirekha *et al.* 2002). In the recent past, attempts have been made for producing polyhydroxyalkanoates (PHAs) using these wastes (Singh and Mallick 2008; Oliveira *et al.* 2004).

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Studies on polyhydroxyalkanoate production by a marine *Bacillus* sp. NQ-11/A2 isolated from continental shelf sediment

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ABSTRACT

The marine bacterial strain *Bacillus* sp. NQ-11/A2 isolated from continental shelf sediment exhibited bright orange fluorescence indicating PHA production. Optimum temperature for growth and PHA accumulation was found to be 30 °C. Out of the five diverse carbon rich wastes tested as substrates for PHA production, maximum growth and PHA accumulation was obtained using sugarcane molasses. Among the different inorganic nitrogen sources supplied, ammonium chloride supported excellent PHA production. The polymer accumulated by the isolate using glucose as sole carbon source was characterized. Fourier transform-infrared (FT-IR) spectroscopy confirmed the aliphatic nature of the polymer with a low Crystallinity Index and a thermal melting temperature (T_m) of 167 °C.

Keywords: *Polyhydroxyalkanoate, Bacillus, continental shelf sediment, glucose, carbon rich wastes, feedstock.*

INTRODUCTION

Biodegradable plastics are gaining tremendous attention globally not only due to the rising oil prices but also because of the problems associated with the disposal of conventional plastics. Polyhydroxyalkanoates (PHAs) are polyesters synthesized by a wide range of bacteria when a carbon source is present in excess and one of the essential growth nutrients is limiting (Rehm 2007). The basic unit and the most common member of PHA is poly-3-hydroxybutyric acid [P(3HB)], a homopolymer made up of repeating units of (R)-3HB. It is hard and brittle unlike P(3HB-co-3HV), a copolyester containing randomly arranged units of (R)-3-hydroxybutyrate and (R)-3-hydroxyvalerate, which is an elastomer. The copolymer possesses superior material properties and finds application in many fields including manufacture of consumer products such as plastics, films and fibres (Rehm 2003). This copolymer was produced at an industrial scale for the first time in the year 1982 by Imperial Chemical Industries Ltd. (ICI), England using the Gram-negative bacterium, *Alcaligenes eutrophus* and the commercialized product was trade named "Biopol" (Lenz and Marchessault 2005).