UTILIZATION OF RENEWABLE AGRICULTURAL RESOURCES FOR ECTOINE, XYLANASE AND CELLULASE PRODUCTION FROM NEWLY ISOLATED HALOPHILIC BACTERIA

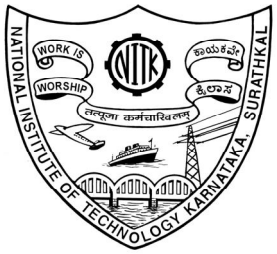
Thesis

Submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

by

POOJA S.



DEPARTMENT OF CHEMICAL ENGINEERING,

NATIONAL INSTITUTE OF TECHNOLOGY KARNATAKA,

SURATHKAL, MANGALORE – 575 025

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DECLARATION

I hereby *declare* that the Research Thesis entitled **"Utilization of renewable agricultural resources for ectoine, xylanase and cellulase production from newly isolated halophilic bacteria"** which is being submitted to the **National Institute of Technology Karnataka, Surathkal** in partial fulfilment of the requirements for the award of the Degree of **Doctor of Philosophy** in Chemical Engineering is a *bonafide report of the research work carried out by me*. The material contained in this Research Thesis has not been submitted to any University or Institution for the award of any degree.

(Signature of the Research Scholar)

Name: **POOJA S.**

Register Number: **CH09F02**

Department of Chemical Engineering

Place: NITK-Surathkal

Date:

CERTIFICATE

This is to *certify* that the Research Thesis entitled **"Utilization of renewable agricultural resources for ectoine, xylanase and cellulase production from newly isolated halophilic bacteria"** submitted by **Pooja S.** (Register Number: 090705CH09F02) as the record of the research work carried out by her, is *accepted as the Research Thesis submission* in partial fulfilment of the requirements for the award of degree of **Doctor of Philosophy**.

Research Guide

Dr. Gopal Mugeraya

Chairman- DRPC

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ABSTRACT

Industrial production of high value biomolecules is frequently limited by substrate costs. The present investigation deals with an economically alternative strategy of utilizing renewable agricultural resources as substrates for halophilic bacteria. Several biopolymer degrading halophilic bacteria were isolated from saline regions of coastal Karnataka, India. *Halomonas* sp. PS6 (GenBank ID: KC295600) produced ectoine (mg/gdw) - on glucose (71.3), xylan (45.2), CMC (22.6), inulin (11.8), chitin (8.1) and carob (22.7). This shows the ability of the halophilic bacterium to utilize *bio-polymers* in the *synthetic medium* for growth and ectoine production. Strain PS6 produced substantial amounts of ectoine on agro-residues (mg/gdw) - rice bran (60.2), wheat bran (36.2), sugarcane bagasse (34.7), corn cobs (25.5), groundnut shells (18.3) and coir pith (14.1). 85.4 mg/gdw ectoine was produced at 12% w/v NaCl and 70.5 mg/gdw ectoine at 37 ºC. Using response surface methodology, two-fold increase in ectoine was seen (112.3 mg/gdw) at rice bran 50 g/l, NaCl 110 g/l and temperature 37 ºC with validity of 98%.

Brachybacterium sp. PS3 (GenBank ID: JQ425852) produced xylanase (1.37 U/ml) in MM63 medium. Higher activity (4.2 U/ml) was seen with xylan, yeast extract and peptone. Strain PS3 produced xylanase (U/ml) - on wheat bran (0.9), corn cobs (0.75), rice bran (0.7 U/ml), sugarcane bagasse (0.44), groundnut shells (0.48) and coir pith (0.4). Using Plackett-Burman design and response surface methodology, a maximum xylanase activity of 8.23 U/ml at pH 9.0, wheat bran 40 g/l, NaCl 90 g/l and corn cobs 30 g/l was obtained with validity of 95.2% and over four-fold increase. PS3 xylanase exhibited highest activity at pH 9.0 and 55 °C and stability up to 4 M NaCl.

Halomonas sp. PS47 (GenBank ID: JQ425853) produced cellulase (0.0076 U/ml) on basal MM63 medium. Higher activity (0.14 U/ml) was seen with CMC and combination of yeast extract and peptone. Strain PS47 produced higher cellulase activities (U/ml) - on wheat bran (0.079), corn cobs (0.06), rice bran (0.059 U/ml), sugarcane bagasse (0.04), and groundnut shells (0.049). By statistical optimisation, cellulase activity of 0.35 U/ml at wheat bran 50 g/l, yeast extract 3 g/l and $MgSO_4.7H_2O$ 0.4 g/l was observed with validity of 95.8% and 3 three-fold increase. PS47 cellulase exhibited highest activity at pH 7.5 and 50 °C and stability up to 4 M NaCl.

The present investigation assumes significance in the ability of halophilic bacteria to survive in a wide range of salinity and yield optimum levels of high value biomolecules like compatible solutes and saline hydrolases using cheap agricultural (lignocellulosic) resources.

Keywords: Halophilic bacteria; Agricultural residues; Ectoine; Xylanase; Cellulase

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NOMENCLATURE

%	percent
(k,c,m,µ,n) m	(kilo, centi, milli, micro, nano) meter
(m) M	(milli) molar
(m,µ) l	(milli, micro) liter
(m,n) g	(milli, nano) gram
ANOVA	analysis of variance
BLAST	basic local alignment search tool
bp	base pair
CFU	colony forming unit
CMC	carboxy methyl cellulose
CMCase	carboxy methyl cellulase
D_2O	deuterium oxide (heavy water)
cdw	cell dry weight
DNA	deoxyribonucleic acid
dNTPs	deoxynucleoside triphosphate (dATP, dCTP, dGTP, dTTP)
DOE	design of experiments
EDTA	ethylene diamine tetraacetic acid
et al.	et alia (and others)
h	hour
HPLC	high performance liquid chromatography
kDa	kilo Dalton
MM63	minimal medium

Na-TMSP	3-(trimethylsilyl)propionic acid – sodium salt
NCBI	national center for biotechnology information
NMR	nuclear magnetic resonance
ºC	degree Celsius
OD	optical density
PBD	Plackett-Burman design
PCR	polymerase chain reaction
pH	potential of hydrogen
pp	pages
ppm	part per million
psi	pound per square inch
rDNA	ribosomal deoxyribonucleic acid
RNA	ribonucleic acid
rpm	revolutions per minute
RSM	response surface methodology
RT	room temperature
SmF	submerged fermentation
SSF	solid state fermentation
t	Time
Taq	*Thermus aquaticus*
Tris	Tris(hydroxymethyl)aminoethane
U/ml	Unit of enzyme activity (µmol/ml/min)
UV	ultraviolet

v/v	volume/volume
w/v	weight/volume
w/w	weight/weight
ΔH	change in enthalpy (J)
ΔS	change in entropy (J K^{-1})
μ_w	chemical potential of water (J)

Chapter 1

Introduction

1.1 RENEWABLE AGRICULTURAL RESOURCES

Lignocellulosic biomass represents the most abundant renewable resources produced by photosynthesis on earth. These residues are mainly composed of (on dry weight basis) - cellulose (40-60%), hemicellulose (xylan - 20-40%) and lignin (10-25%) (Saha 2003; Yuan et al. 2013). Substrate resources used in industrial production processes are, in most cases, non-renewable in nature. Availability of raw material resources is one of the major needs for development of process biotechnology. This has resulted in search for alternate resources. The use of renewable agricultural residues represents a long term solution to the problem of dwindling petroleum reserves. Thus, shifting the raw material base from fossil to renewable resources would ensure sustainable means of production.

The heterologous structure of lignin is recalcitrant to bacterial degradation. Hence pretreatments are required to facilitate its removal. Alkali treatment is reported to be an effective method in pretreating biomass. However, the resulting substrates are alkaline and neutralization results in high amount of salt (Begemann et al. 2012; Palmqvist and Hahn-Hägerdal 2000). Degradation of naturally abundant lignocellulosic polymers is essential for maintaining carbon cycle and food web (Fernández-Luqueño et al. 2008; Betty Anita et al. 2013). Halophilic bacteria capable of utilizing cheap renewable agricultural residues could be exploited as an economic alternative to the existing production processes.

1.2 HALOPHILIC BACTERIA

Halophiles are microorganisms that adapt to moderate and high salt concentrations. They are found in all three domains of life: Archaea, Bacteria and Eukarya (Baxter et al. 2005). Halophilic *bacteria* grow over an extended range of salt concentrations (3-15% NaCl, w/v and above) unlike the truly halophilic *archaea*

whose growth is restricted to high saline environments (Ventosa et al. 1998). Addition of salts like NaCl to water interferes with its ordered structure, increasing the randomness of solvent molecules. The chemical potential of water μ_w (in J) can be expressed as:

$$\Delta\mu_w = \Delta H_w - T\Delta S_w, \quad \text{.....(1.1)}$$

Where, ΔH (in J) is the change in enthalpy, T (in K) is temperature and ΔS (in J K^{-1}) is the change in entropy (Wood 1999; Sweeney and Beuchat 1993). Positive entropy results in reduction of chemical potential. Hence water flows from high chemical potential, inside the cytoplasm of a cell which is exposed to saline environment, to low potential outside. This results in cell shrinkage and subsequent cessation of growth (Kunte 2006).

In order to maintain an osmotic equilibrium, the cell has to reduce the chemical potential of the cytoplasmic water. Halophiles cope with this osmotic stress either by uptake of salts from the medium or by synthesizing organic osmolytes which are compatible to the cell machinery. Halophilic bacteria majorly employ the compatible solute strategy, which makes them versatile in adaptation to salinity (Roberts 2005).

1.3 PRODUCTS FOR BIOTECHNOLOGY

Microorganisms are potential sources of high value products. Based on his lifetime research experience, Louis Pasteur had rightly framed an everlasting doctrine: 'the role of the infinitely small in nature is infinitely great'. Halophilic bacteria usually accumulate osmoregulatory compatible solutes like polyols such as glycerol, sugars and their derivatives, amino acids and their derivatives, and quaternary amines such as glycine betaine and ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid) (Galinski 1995).

In addition to their stabilizing effects, these solutes are used as salt antagonists, stress protective agents, cosmetic actives and therapeutics, thus having a

high commercial value (Oren 2010). Ectoines have gained much attention in biotechnology because of the potential commercial applications.

Halophilic bacteria when grown on agro-residues preferentially synthesize *halostable* cellulases and hemicellulases (xylanases) for substrate utilization (Deka et al. 2011). Cellulases and xylanases are active de-polymerising enzyme systems that work through concerted action and synergism to convert biomass polysaccharides into fermentable monosachharides (Rashamuse 2013).

These hydrolases obtained from a halophilic source are expected to retain activity and also sometimes function optimally in the presence of salt. Moreover, halophilic enzymes are usually polyextremophilic in nature, exhibiting stability at higher ranges of temperature and pH (Zhang et al. 2012, Gomez and Steiner 2004). Cellulases and xylanases have increasing industrial applications: food and beverages, pulp and paper, textiles and animal feed (Beg et al. 2001).

Large scale production of solutes and enzymes is hampered by high substrate costs (Huang et al. 2005). In spite of the considerable success achieved in the recent past in elucidating the mechanism of biopolymer-degrading enzymes like cellulases and xylanases, the challenge of screening new improved strains remains active. There has been an increase in search for alternate sources of energy in recent times. However, studies on using biopolymer media for the growth of halophiles have been less investigated till date.

India being largely an agricultural economy, huge amounts of agricultural wastes is generated every year. Moreover, this peninsular country has vast stretches of saline reserves. In salt marsh ecosystems, complex polymers derived from plants are degraded by microorganisms.Survival with a ‘pinch of salt’ offers high-value products for biotechnology. Hence production of high value compounds like compatible solutes and biopolymer-degrading saline hydrolases from halophilic bacteria utilising cheap, renewable agricultural resources has been the aim of the present doctoral work.

1.4 SCOPE OF THE PRESENT INVESTIGATION

- Studies on the growth of halophilic bacteria on synthetic biopolymer medium.
- Investigation on agricultural residues necessitates the study of de-branching enzymes, cellulases and xylanases.
- Studying the production of compatible solute (ectoine) and essential enzymes (xylanases and cellulases) from halophilic bacteria.
- Designing an alternative economic strategy of production by using renewable agricultural residues in the medium which in turn would add value to the waste.

1.5 OBJECTIVES

1. Isolation of halophilic bacteria from sea coasts and solar salterns on biopolymeric substrates.
2. Screening for compatible solute (ectoine) producers.
3. Screening for producers of extracellular hydrolases (xylanases and cellulases).
4. Identification of potential isolates.
5. Pre-treatment of agricultural residues to be used as medium component(s).
6. Growth and production studies of potential strains on different renewable agricultural residues.
7. Growth and production studies of potential strains at different salt concentrations, pH and temperature.
8. Optimization of agro-residue medium by response surface methodology for enhanced production.

1.6 ORGANISATION OF THE THESIS

The study of three biomolecules (ectoine, xylanase and cellulase) requires different conditions of microbial growth and production. Hence, these molecules are produced from three different halophilic bacterial strains. Accordingly, the thesis is divided into three parts.

Part one – deals with *ectoine* production and includes chapters 4 and 5

Part two – deals with *xylanase* production and comprises chapters 6 and 7

Part three – deals with *cellulase* production and contains chapters 8 and 9

Chapters 1, 2, 3 and 10 are common to all the parts. The break-up of chapters is as follows:

Chapter 1: Introduction

In the current chapter, an introduction to the present investigation is given. The widespread applications of high value biomolecules like ectoine, halostable xylanase and cellulase is hampered by production costs. The importance of using renewable agricultural resources for solute and enzyme production from halophilic bacteria is highlighted. The scope and objectives of research have been stated.

Chapter 2: Review of literature

This section comprehensively covers the information on halophilic bacteria and biomolecules they produce. A detailed account is given on halophiles, osmoregulation, compatible solutes, physiological aspects of ectoine synthesis from halophiles and their properties, saline hydrolases including xylanases and cellulases, biopolymers, agricultural residues, different methods of pretreatment and utilization of agro-residues as medium components, multivariate process optimization like Plackett-Burman design, response surface methodology and analytical aspects. The current challenge in utilization of renewable resources for growth of halophilic bacteria is highlighted. Relevant tables and figures have been incorporated.

Chapter 3: Isolation of halophilic bacteria and pretreatment of agricultural residues

This chapter describes the details of experimental methods used in sample collection, isolation of biopolymer degrading halophilic bacteria from saline regions, screening and maintenance of pure cultures. An account on pretreatment methods for agricultural residues to be used as medium components, like alkaline and enzymatic hydrolysis, has also been described. The detailed procedures of culture and experimental techniques are given.

Chapter 4: Screening and production of ectoine using biopolymeric substrates

In this chapter, an account on the screening of potential strains for ectoine production is given. Detailed extraction procedures of ectoine and analytical techniques like HPLC, NMR have been discussed. Ectoine production is shown on different biopolymeric substrates. The strain capable of utilizing different biopolymers has been shortlisted for further investigations. Morphological and genetic identification of the potential bacterium have been reported.

Chapter 5: Production of ectoine from *Halomonas* sp. PS6 using agricultural resources

This section describes the production and optimization of ectoine from the selected strain *Halomonas* sp. PS6 using cheap, renewable raw materials like wheat bran, rice bran, corn cobs etc. The process has been optimized to get higher amount of ectoine using response surface methodology. The effect of rice bran, NaCl and temperature on the accumulation of the solute has been studied and optimized for maximum production and subsequently validated in shake flasks.

Chapter 6: Screening and production of halostable xylanase

In this chapter, an account on the screening of strains grown on xylan medium for higher xylanase activity is given. Qualitative analysis using Congo Red dye and quantitative DNS method for estimation of enzyme activity has been discussed. The potential strain has been identified by gene sequence analysis. Growth study of the

bacterium in MM63 medium is reported. Accounts of xylanase production on different agro-residues and varying conditions of salinity, pH and temperature have been investigated.

Chapter 7: Optimisation of halostable xylanase production from *Brachybacterium* sp. PS3

This section explains the selection of significant variables influencing xylanase production from the selected strain *Brachybacterium* sp. PS3 by Plackett-Burman design. Optimisation of the significant parameters like pH, wheat bran, NaCl and corn cobs has been studied by central composited design. Analysis of variance and response surface graphs have been plotted and discussed. The optimum conditions for xylanase production are validated.

Chapter 8: Screening and production of halostable cellulase

In this chapter, an account on the screening of strains grown on CMC medium for higher cellulase activity is described. Qualitative analysis using Congo Red dye and quantitative DNS methods has been studied for cellulase activity. The potential strain has been identified using gene sequence analysis. Bacterial growth studies and different conditions influencing cellulase activity have been discussed.

Chapter 9: Optimisation of halostable cellulase production from *Halomonas* sp. PS47

In this chapter, an attempt to optimise the cellulase production from the potential strain, *Halomonas* sp. PS47 is discussed. This section explains the selection of significant variables influencing cellulase production by Plackett-Burman design. Optimisation of significant parameters like wheat bran, yeast extract and $MgSO_4.7H_2O$ has been done by central composited design. The optimum conditions influencing cellulase production are validated.

Chapter 10: Summary and conclusion

This section offers a brief summary of the presented research. The findings of the research work on the production of ectoine, xylanase and cellulase from newly isolated halophilic bacteria are concluded with some recommendations for the future work. This will pave the way for development of media using agricultural residues and bioconversion of these renewable, cheap resources into value-added products.

Chapter 2

Review of literature

2.1 HALOPHILES

Halophiles are a group of microorganisms that live in saline environments and indeed in many cases require salinity to survive. Extreme environments and their inhabitants open up a fascinating area of research. First isolation of marine bacteria is reported to be done by Bernard Fischer in the 1880s (Dunlap and Kita-tsukamoto 2006). In the late 1940s Claude E. Zobell devised aseptic sampling and isolation of halophilic bacteria from marine environments and thereby opened up the marine facet of microbiology (Zobell 1941). Microorganisms able to grow in the absence as well as in the presence of salt are designated halotolerant and those that are able to grow above approximately 15% (w/v) NaCl (2.5 M) are considered extremely halotolerant (Kushner and Kamekura 1988).

According to Kushner (1978), many marine organisms are slight halophiles [seawater contains about 3% (w/v) NaCl]. Moderate halophiles optimally grow at 3-15% (w/v) NaCl; extreme halophiles at 25% (w/v) NaCl (*halobacteria* and *halococci*) and borderline extreme halophiles require at least 12% (w/v) salt (Margesin and Schinner 2001). Halophiles include a great diversity of organisms including moderately halophilic aerobic bacteria, cyanobacteria, sulphur-oxidizing bacteria, anaerobic bacteria and archaea, halobacteria, other phototrophic bacteria, protozoa, fungi, algae and multicellular eukaryotes (DasSarma 2001).

2.1.1 Moderately halophilic bacteria

This group of bacteria has been reviewed extensively by Ventosa et al. (1998) in their article, ‘Biology of moderately halophilic aerobic bacteria’. The occurrence of non-pigmented halotolerant bacteria was probably first mentioned in 1919 by LeFevre and Round in their study of the microbiology of cucumber fermentation brines. Kushner (1978) clearly states: “Though they are less exciting at first glance than the extreme halophiles the moderately halophilic bacteria, and solute-tolerant

microorganisms in general, pose quite sufficiently interesting questions, especially those implied by their ability to grow over wide ranges of solute concentrations. Further work on these relatively little-studied microorganisms may be expected to bring dividends in the form of insight on the relation of internal and external solute concentrations, and on the state of cell-associated ions within the cytoplasm. If the last decade has been that of the extreme halophiles, we can hope that the next one will see their more modest, moderate cousins (in the spiritual sense only) take their proper place in the scientific canon."

These halophilic species are found in salt marshes, marine ecosystems, salted meats, hypersaline seas, salt evaporation pools and salt mines. Most of the important groups of bacteria are able to live in concentrations up to about 15% salt and many groups are physiologically active even at much higher salt concentrations (Hof 1935). Halophilic bacteria form a versatile group and are adapted to life at the lower range of salinities, with the possibility of rapid adjustment to changes in the external salt concentration. In contrast, the halophilic archaea are strictly dependent on the constant presence of high salt concentrations (3 to 4 M) for survival.

2.1.2 Osmoregulation

In their review, 'Life in extreme environments', Rothschild and Mancinelli (2001) say that organisms live within a range of salinities, from essentially distilled water to saturated salt solutions. Osmophily refers to the osmotic aspects of life at high salt concentrations, especially turgor pressure, cellular dehydration and desiccation. Halophily refers to the ionic requirements for life at high salt concentrations. Many microorganisms respond to increase in osmolarity by accumulating osmotica in their cytosol, which protects them from cytoplasmic dehydration and desiccation (Yancey et al. 1982).

There are two strategies adapted for survival in saline conditions: 'compatible solute' strategy and 'salt-in' strategy (Ventosa et al. 1998). Both strategies work by increasing the internal osmolarity of the cell. In the "compatible solute" strategy, employed by the majority of moderately halophilic bacteria, some archaea, yeasts,

algae and fungi; cells maintain low concentrations of salt in their cytoplasm by balancing osmotic potential by the synthesis or uptake of organic, compatible solutes. This a more flexible strategy found in bacteria that grow over a wide range of salt concentrations (Roessler and Müller 2001). Compatible solutes include polyols such as glycerol, sugars and their derivatives, amino acids and their derivatives, and quaternary amines such as glycine betaine. In fact; the compatible solutes often act as more general stress protectants as well as just osmoprotectants.

The second, more radical, "salt-in" adaptation involves the selective influx of K^+ ions into the cytoplasm. This adaptation is restricted to the moderately halophilic bacterial Order *Halanerobiales*, the extremely halophilic archaeal Family *Halobacteriaceae* and the extremely halophilic bacterium *Salinibacter ruber*. The presence of this adaptation in three distinct evolutionary lineages suggests convergent evolution of this strategy, it being unlikely to be an ancient characteristic retained in only scattered groups or through massive lateral gene transfer. To use this strategy all enzymes and structural cell components must be adapted to high salt concentrations to ensure proper cell function.

2.1.3 Halotolerance

Halotolerance is the adaptation of living organisms to conditions of high salinity. Proteins of halophilic bacteria are either resistant to high salt concentrations or require salts for activity. These organisms contain an excess ratio of acidic to basic amino acids, a feature required for activity at high salinity. Surface negative charges are thought to be important for salvation of halophilic proteins and to prevent the denaturation, aggregation and precipitation that usually results when non-halophilic proteins are exposed to high salt concentrations (DasSarma, 2001).

Adaptation to hypersaline conditions is interesting from an evolutionary standpoint. The likelihood of concentration of brines during prebiotic evolution, suggests that adaptation to salts may have been among the earliest evolutionary times (Dundas 1998).

An understanding of halotolerance can be applicable to areas such as arid-zone agriculture, xeriscaping, aquaculture (of fish or algae), bioproduction of desirable compounds (such as enzymes, phycobiliproteins or carotenoids etc.) using seawater to support growth, or remediation of salt-affected soils. In addition, many environmental stresses involve or induce osmotic changes, so knowledge gained about halotolerance can also be relevant to understanding tolerance to extremes in moisture or temperature (Kastritis et al. 2007).

2.1.4 Potential of halophilic bacteria for biotechnology

Halophilic bacteria offer potential applications in various fields of biotechnology (Margesin and Schinner, 2001). Although moderately halophilic bacteria have many industrial applications, only a few studies have been carried out concerning their industrial applications. These organisms can be used as a source of metabolites, compatible solutes and other compounds of industrial value.

Conventional agricultural species could be made more halotolerant by gene transfer from naturally halotolerant species (by conventional breeding or genetic engineering) or by applying treatments developed from an understanding of the mechanisms of halotolerance. In addition, naturally halotolerant plants or microorganisms could be developed into useful agricultural crops or fermentation organisms (Ventosa et al. 1998).

The green alga *Dunaliella salina*, growing at moderately high salinities is widely used for the commercial production of β-carotenes, which it produces in response to solar radiation; and glycerol, to counterbalance external osmotic pressure. Direct uses include marketing of dried *probiotics* as a nutritional supplement, primarily as an antioxidant. Antifreeze proteins show potential as cryoprotectants of frozen organs (Rothschild and Mancinelli 2001).

Although current commercial uses of halophiles are quite significant (fermentation of soy and fish sauces, β-carotene production, and aquaculture); the many novel and unique properties of many of these organisms suggest that they have enormous potential for biotechnology (Thongthai and Sontinanalert 1991). Novel

halophilic biomolecules may also be used for specialized applications, e.g. bacteriorhodopsin for biocomputing, gas vesicles for bioengineering floating particles, pigments for food colouring and compatible solutes as stress protectants (DasSarma 2001).

The industrial and environmental applications of halophilic microorganisms have been reviewed by Oren (2010). Accordingly, halophiles are involved in centuries-old processes of manufacturing solar salt from seawater and production of traditional fermented foods. β-carotene production by *Dunaliella* and ectoine synthesis using *Halomonas* and other moderately halophilic bacteria; have been two highly successful processes involving halophiles. The potential use of bacteriorhodopsin, the retinal protein proton pump of *Halobacterium* is being explored in optoelectronic devices and photochemical processes.

Other possible uses of halophilic microorganisms such as treatment of saline and hypersaline wastewaters, and the production of exopolysaccharides, poly-β-hydroxyalkanoate bioplastics and biofuel are being investigated, but no large-scale applications have yet been reported. Biodegradation of organic pollutants by halophilic bacteria and archaea has been recently reviewed (Borgne et al. 2008). These microorganisms are good candidates for the bioremediation of hypersaline environments and treatment of saline effluents. Understanding the degradation process would also shed light on the enzymes involved and the metabolism regulation.

Halophilic bacteria are a potential source of extracellular hydrolases like proteases, xylanases and cellulases with a wide array of industrial applications. These enzymes exhibit stability over a range of saline conditions. Moreover, they are often also exhibit at varying conditions of temperature and pH. Hence they find applications in industries and treatment plants where harsh process conditions generally prevail (Shivanand and Jayaraman 2009).

2.2 COMPATIBLE SOLUTES

Compatible solutes are low molecular weight osmoregulatory compounds which are highly water-soluble sugars, alcohols, amino acids, betaines, ectoines or their derivatives (Ventosa et al. 1998; Sand et al. 2013). They are compatible with cellular functions even at molar concentrations by maintaining cell volume, turgor and electrolyte concentrations. These solutes often act as more general stress protectants as well as just osmoprotectants.

Compatible solutes are useful as stabilizers of biomolecules and whole cells, salt antagonists, or stress-protective agents. Due to their stabilizing effects they can be for various research and industrial processes. Moderate halophiles are expected to produce polar uncharged and zwitterionic solutes and halo-thermophilic microbes produce anionic compatible solutes.

Compatible solutes have protein-stabilizing properties that help in proper folding of polypeptide chains (Arakawa and Timasheff 1985). Due to their stabilizing effect on protein molecules they are also sometimes referred to as chemical chaperones (Chattopadhyay et al. 2004).

Conformational shift of protein towards folded, native-like states induced by preferential exclusion of the solute is responsible for the chaperone-like effects (Kolp et al. 2006). These solutes exert their effect through changes in solvent structure and/or subtle changes in the dynamic properties of the protein rather than by changing the structure of the protein itself (Lamosa et al. 2003).

Compatible solutes also interact with nucleic acids and can influence protein-DNA interactions (Pul et al. 2007; Kurz 2008). Compatible solutes can be detected and analysed by various methods like HPLC, NMR etc. (Roberts 2006). It has been recently shown that Raman spectroscopy is a suitable tool to assess the mode of osmotic adaptation used by halophilic microorganisms (Jehlička et al. 2012).

Biochemical and molecular characterization of genes encoding compatible solutes is being done by several researchers (Suam et al. 2006; Burkhardt et al. 2009).

The distribution of some major compatible solutes in prokaryotes is given in Table 1.1 (Shivanand and Mugeraya 2011).

Table 1.1 Distribution of some major compatible solutes in prokaryotes

Compatible Solutes:	Occurrence:
Ectoine	*Halomonas elongata, Ectothiorhodospira halochloris, Halomonas boliviensis, Brevibacterium epidermis, Chromohalobacter israelensis, Chromohalobacter salexigens*
Hydroxyectoine	*Halomonas elongata, Nocardiopsis halophila, aerobic heterotrophic bacteria*
Betaine	*Actinoployspora* sp., *Halorhodospira halochloris, Thioalkalivibrio versutus*
Proline	*Streptomyces,* halophilic/halotolerant *Bacillus* strains
α-Glutamate	*Marine bacteria, some methanogenic archaea*
Mannosylglycerate	*Methanothermus fervidus, Pyrococcus furiosus, Rhodothermus marinus, Thermus thermophilus, Pyrococcus furiosus, Thermococcus*
Diglycerol phosphate	*Archaeoglobus fulgidus*
Mannosylglyceramide	*Rhodothermus marinus*
Glucosylglycerate	*Agmenellum quadruplicatum, Erwinia chrysanthemi, Stenotrophomonas maltophilia*
Trehalose	*Pyrobaculum aerophilum, Thermoplasma acidophilum, Actinoployspora halophila, Rubrobacter xylanophilus*
Sucrose	*Anabaena, Nitrosomonas europaea* and proteobacteria
Mannitol	*Pseudomonas putida*

2.2.1 Ectoines

(a) (b)

Figure 2.1 Compatible solutes (a) Ectoine and (b) Hydroxyectoine

Ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid) is one of the most common osmotic solutes in the domain Bacteria. It was discovered in the extremely halophilic phototrophic bacterium *Halorhodospira halochloris* (formerly *Ectothiorhodospira halochloris*) and characterized by ^{13}C-NMR spectroscopy, mass spectrometry and infrared spectroscopy (Galinski et al., 1985). Later a great variety of halophilic and halotolerant bacteria were found to produce this compound, often together with its 5-hydroxy derivative (Pastor et al. 2010).

Ectoines have gained much attention in biotechnology as protective agents for enzymes, DNA and whole cells against stresses such as freezing, drying and heating. Ectoines are common in aerobic heterotrophic eubacteria (Roberts 2006). In recent years additional properties of interest were found for ectoine. It is claimed that it counteracts effects of ultraviolet UV-A-induced and accelerated skin ageing, and therefore it is being used as a dermatological cosmetic additive in moisturizers for the care of aged, dry or irritated skin (Motitschke et al. 2000).

They are used as potential therapeutic agents for certain diseases like lung inflammation (Sydlik et al. (2009). Ectoine also inhibits aggregation and neurotoxicity of Alzheimer's β -amyloid, and recently a clinical trial was initiated to test its efficacy in inhalations against bronchial asthma (Oren 2010).

Ectoines are used for increasing the stability and freshness of foods by stabilizing food components. Ectoines have been patented as moisturisers in cosmetics (Motitschke et al. 2000). Ectoines also find applications in the treatment of

the mucous membranes of the eye against unfavourable factors. Ophthalmologic preparations containing these molecules are useful for eye treatment to decrease the dryness syndrome and for more rapid healing after surgery. Introduction of ectoine and its derivatives into preparations for oral care has also been suggested (Detkova and Boltyanskaya 2007).

The entry molecule into ectoine biosynthesis is aspartate semialdehyde, which is an intermediate in amino acid metabolism. As shown in Fig. 2.2, the aldehyde is converted to L- 2,4-diaminobutyric acid, which is then acetylated to from Nγ-acetyldiaminobutyric acid (NADA). The final step is the cyclization of this solute to form ectoine. Ectoine synthesis is carried out by the products of three genes: *ectABC*. The *ectA* gene codes for diaminobutyric acid acetyltransferase, *ectB* codes for diaminobutyric acid aminotransferase and *ectC* codes for ectoine synthase.

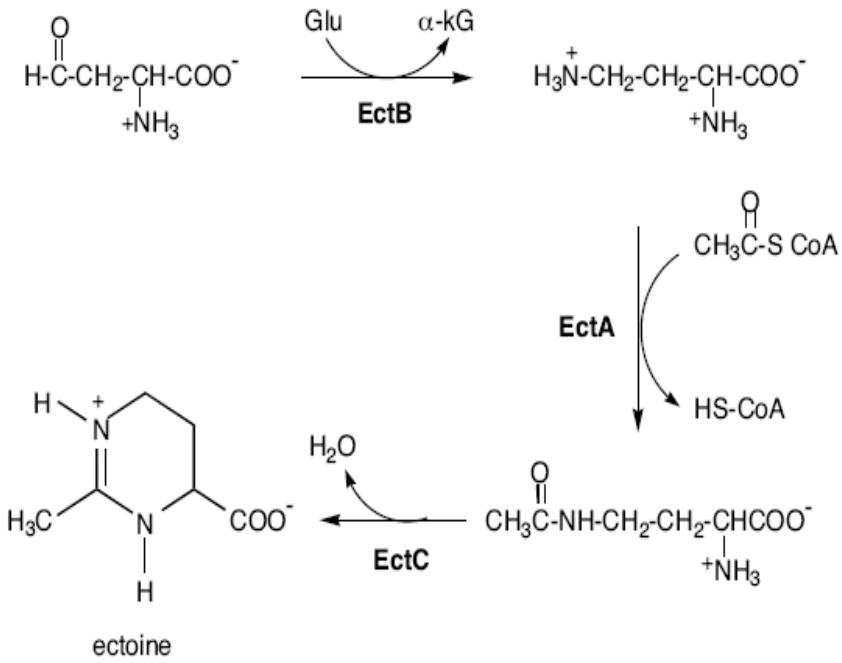

Figure 2.2 Biosynthetic pathway for ectoine

2.2.2 Betaines

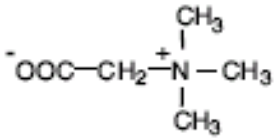

Figure 2.3 Structure of Betaine

Betaines are the compatible solutes occurring in halophilic phototrophic bacteria, chemotrophic bacteria and archaebacteria. They have therapeutic potential for the treatment and prophylaxis of the adipose infiltration of the liver which are the initial stages of cirrhosis (Detkova and Boltyanskaya 2007). Betaines decrease side effects of anti-inflammatory preparations. Their anticoagulant properties prevent thrombus formation and decrease the probability of heart attacks, infarctions and strokes (Messadek 2005). They are useful in PCR amplification of GC-rich DNA templates to increase product yield and specificity (Roberts 2006). Betaine was shown to be a more effective cryo-protectant than serum albumin or trehalose/dextran, particularly under conditions stimulating long-term storage (Cleland et al. 2004).

2.2.3 Mannosyl-glycerate

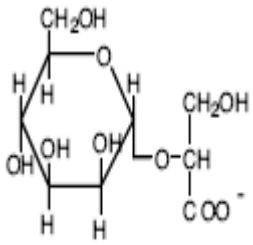

Figure 2.4 Structure of Mannosyl-glycerate

Mannosyl-glyerate (MG) is a novel compatible solute widely found in the halotolerant *Methanothermus fervidus, Pyrococcus furiosus and Rhodothermus marinus*. This compound has also been detected in many hyperthermophilic archaea, where it accumulates concomitantly with increasing salinity of the medium (Santosh and da Costa 2002). Although present in many red algae, the apparent restriction of MG to thermophilic bacteria and hyperthermophilic archaea led to the hypothesis that

MG plays a major role in thermal adaptation (Empadinhas and da Costa 2008). In R. marinus, there is a direct condensation of GDP-mannose and D-glycerate to form MG catalyzed by mannosylglycerate synthase (Roberts 2005). Possible applications are the utilisation as protectants for enzymes against physical or chemical stress, as additive in PCR and as excipient in pharmaceuticals.

2.2.4 Diglycerol phosphate

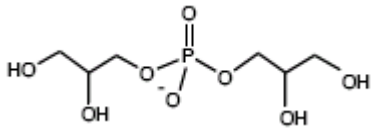

Figure 2.5 Structure of Diglycerol phosphate

Diglycerol phosphate accumulates under salt stress in the hyperthermophile *Archaeoglobus fulgidus*. This new compatible solute is a potentially useful protein stabilizer, as it exerted a considerable stabilizing effect against heat inactivation of various dehydrogenases and a strong protective effect on rubredoxins (with a fourfold increase in the half-lives) from *Desulfovibrio gigas* and *Clostridium pasteurianum* (Litchfield 2002).

2.2.5 Trehalose

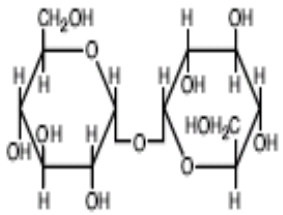

Figure 2.6 Structure of Trehalose

The non-reducing glucose disaccharide trehalose is used by organisms to counteract drying, but it also serves as an osmolyte (Roberts 2006). It occurs in a wide variety of organism, from Bacteria and Archaea to fungi, plants and invertebrates.

Trehalose could not only be useful as a cryoprotectant for the freeze-drying of biomolecules, but also for long-term conservation of microorganisms, as the membrane structure is preserved in the presence of this disaccharide (Empadinhas and da Costa 2008).

2.2.6 Production studies

The increasing commercial demand for compatible solutes like ectoine has led to multiplication of attempts to improve production processes. For practical applications reasonable quantities of compatible solutes have to be generated either in vitro or in vivo. A novel bioprocess for production of ectione from *Halomonas elongata* called 'Bacterial milking' (Sauer and Galinski 1998) has been the basis of German biotechnology company Bitop which develops products from osmolytes.

In the process of "bacterial milking", cells are concentrated several folds using cross-flow filtration after a high-cell-density fermentation as shown in the flow diagram Fig. 2.7. Bacteria in high concentrations of NaCl are subjected to osmotic shock by transferring the cell biomass to low osmolarity medium where they rapidly excrete the now excess solutes in the medium to maintain the osmotic equilibrium. After each dilution step the medium (containing the solutes) has to be removed prior to re-exposure to high salt. Subsequent reincubation in a medium of higher salt concentration results in resynthesis of these compatible solutes.

The process could be repeated several times after a defined generation time. Similar to *H. elongata*, an osmotic downshock based method made by centrifuging the biomass and resuspending the cell pellets on distilled water has been described using the *Brevibacterium* sp. JCM 6894 strain (Nagata et al. 2008). Ectoine production in *Brevibacterium epidermis* did not use periodic downshocks, but a final extraction process using distilled water and ethanol and salt concentration as low as 1 M (Onraedt et al. 2005).

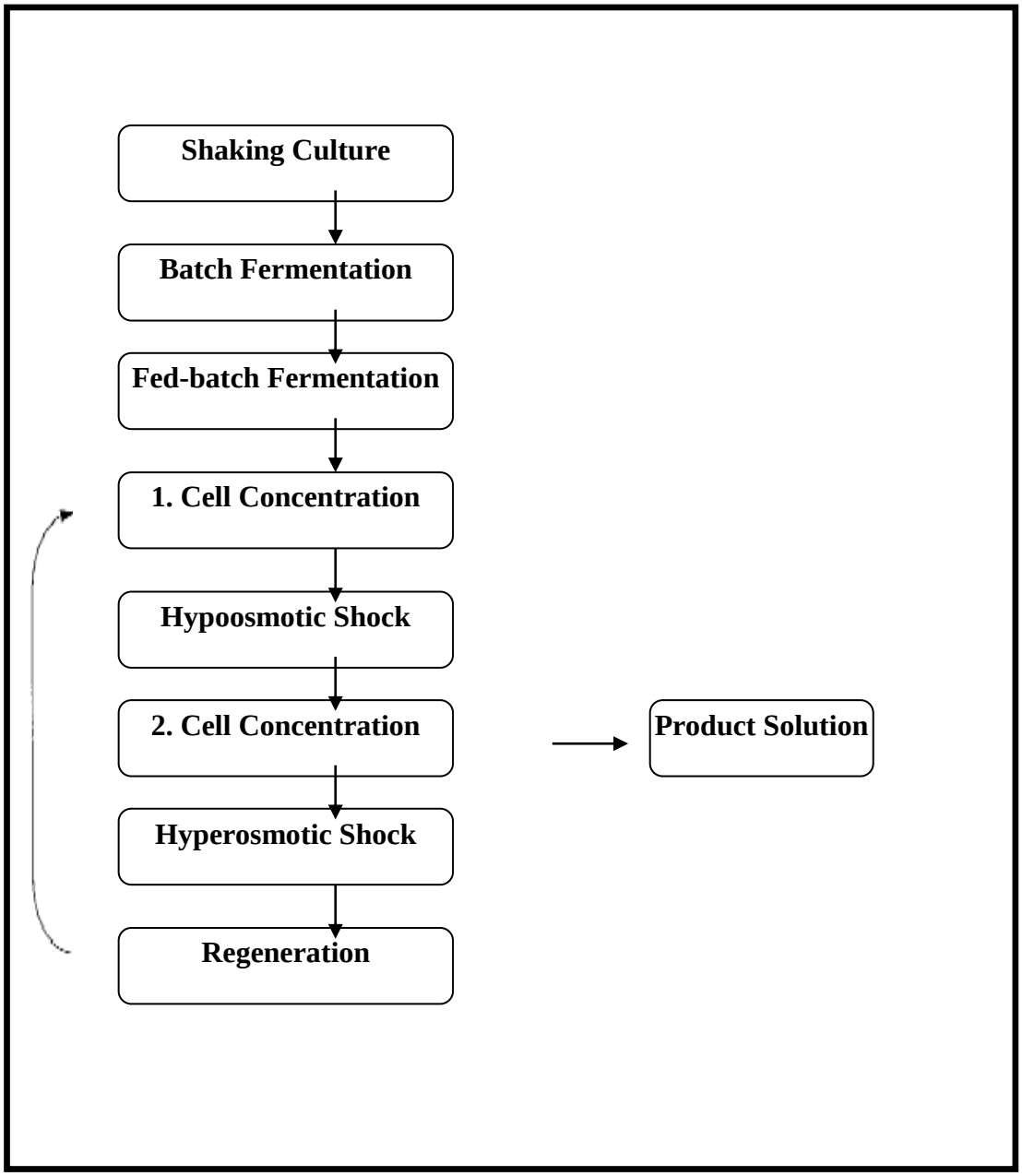

Figure 2.7 Flow chart of Bacterial Milking process for ectoine production. Reference: (Sauer and Galinski 1998)

After a high-cell-density fermentation cells are fivefold concentrated using cross flow filtration. Bacteria in high concentrations of NaCl are subjected to osmotic shock by transferring the cell biomass to low osmolarity medium where they rapidly excrete the now excess solutes in the medium to maintain the osmotic equilibrium. After each dilution step the medium (containing the solutes) has to be removed prior to re-exposure to high salt. Subsequent reincubation in a medium of higher salt concentration results in resynthesis of these compatible solutes. The process could be repeated several times after a defined generation time (Sauer and Galinski 1998).

After nine repetitions, 155 mg ectoine/g cell dw per cycle was produced. This process may also be useful for the production of other low molecular weight compounds. The required microorganism must withstand osmotic shocks and must have a broad salt tolerance. Production parameters can be optimized for enhanced yield.

A process comprising two fed-batch cultures for the production of compatible solute ectoine and biopolyester poly (3-hydroxybutyrate) by a moderate halophile, *Halomonas boliviensis* is reported (Guzman et al. 2009). The co-production process as described has been proposed for lowered production costs of the respective molecules. Application of statistical design involving response surface methodology allowed a quick optimization of medium components for ectoine production by *H.* boliviensis (Van-Thuoc et al. 2010). An overall ectoine volumetric productivity of 6.3 g/l/day was obtained. The optimized medium also showed improvement in ectoine productivity when used in fed-batch fermentation.

An alternative, economically viable production method was proposed by demonstrating that a metabolic bottleneck for ectoine production in the non-halophilic recombinant *E-coli* DH5α can be relieved by co-expression of deregulated aspartate kinase from *C. glutamicum* (Bestvater et al. 2008).

2.2.7 Purification and structure elucidation

Moderate halophiles generally produce polar uncharged and zwitterionic solutes and halo-thermophilic microbes produce anionic compatible solutes. Several

analytical methods are established for the detection of compatible solutes and further quantification using HPLC analysis. Pure compounds can be subsequently crystallized from the product solution and their structure established. The quality can be controlled using NMR measurement as well as isocratic HPLC and FMOC-HPLC techniques. Purification of compatible solutes relies on chromatographic steps (Sauer and Galinski 1998).

For determination of endogenous compatible solute accumulation, cell suspensions were centrifuged; cells were harvested and freeze-dried (Teixidó et al. 2005). Subsequently, cell material was extracted using the method described by Kunte et al. (1993) for quantitative analysis with extraction mixture (methanol/chloroform/water 10:4:4, by volume) by vigorous shaking followed by the addition of equal volumes of chloroform and water. Phase separation was enhanced by centrifugation. The hydrophilic top layer containing compatible solutes was recovered and analysed by HPLC.

For the recovery of ectoines from product solution, the pH of the solution was lowered with HCl to permit ectoines in their cationic form (Sauer and Galinski 1998). Ectoines were purified using cation exchange resin packed in a column and subsequently eluted with NaOH. The ectoine fraction was marked by increased UV absorbance (at 230 nm). The recovered ectoines can be subsequently crystallized from water. At the industrial scale, polar uncharged and zwitter-ionic compatible solutes like ectoine are separated from contaminating salts by crossflow-electrodialysis, followed by chromatographic purification.

Several analytical methods are established for the detection of compatible solutes and further quantification using HPLC analysis. Purification of compatible solutes relies on chromatographic steps. Ectoine and hydroxyectoine concentrations can be determined by HPLC analysis (Severin et al. 1992). The quality of purified ectoines can be controlled using NMR measurement (Frings et al. 1993) as well as isocratic HPLC and FMOC-HPLC techniques. A combination of anion-exchange chromatography and pulse amperometric detection is a very sensitive method that can detect osmolytes such as ectoine after hydrolytic cleavage of the pyrimidine ring (Riis

et al. 2003). Various methods of characterization of organic compatible solutes of halotolerant and halophilic microorganisms by NMR methods have been detailed by Roberts (2005).

2.2.8 Osmoadaptation and agriculture

Adaptation to saline conditions is interesting from an evolutionary standpoint. The brine concentrations during prebiotic evolution, suggest that adaptation of salts by microorganisms may have been among the earliest evolutionary times. Halophilic bacteria constitute excellent models for the molecular study of osmoregulatory mechanisms since many environmental stresses involve or induce osmotic changes in the cell (Ventosa et al. 1998).

Moreover, they can be modeled for the molecular study of osmoregulatory mechanisms that permit them to grow over a wide range of salt concentrations. This has possible application in agriculture to construct salt-resistant plants carrying prokaryotic genes for the synthesis of osmoprotective compounds.

Conventional agricultural species could be made more halotolerant by gene transfer from naturally halotolerant species or by applying treatments developed from an understanding of the mechanisms of halotolerance. *Arabidopsis thaliana* transformed with a choline oxidase gene (which is needed to synthesize betaine) from *Arthrobacter globiformis* has a significantly improved tolerance of salt stress along with improved cold and heat tolerance (Empadinhas and da Costa 2008). This field offers a lot of scope for research and development.

2.3 SALINE HYDROLASES

Enzymes active over a wide range of salinity can be obtained from moderately halophilic species (Shivanand and Jayaraman 2011). These enzymes have potential commercial value. Their production can be further optimized to get substantial yields. Moreover, they constitute an excellent model for structural adaptations that permit them to be active over a wide range of salt concentrations. Most halophilic bacteria producing hydrolases have been assigned to the family *Halomonadaceae* (Setati

2010). This group of bacteria produces industrially relevant enzymes such as amylases, proteases, lipases, cellulases and xylanases (Sánchez-Porro et al. 2002; Govender et al. 2009; Rohban et al. 2009). Cellulases and xylanases are active de-polymerising enzyme systems. These enzymes convert the biomass polysaccharides into fermentable monosachharides. Production of saline hydrolases from halophilic bacteria would be an attractive proposition towards commercialisation.

Halophilic adaptation of enzymes at a molecular level has been reviewed by Madern et al. (2000). Although halophilic enzymes display identical enzymatic functions as their non-halophilic counterparts, they have different properties, including stability and higher activity at different range of salinity (Amoozegar et al. 2007). Extracellular hydrolytic enzymes produced by extremely halophilic microorganisms can be used in many industrial processes where concentrated salt solutions are used. Non-halophilic enzymes would not function under such harsh conditions.

2.3.1 Applications of saline hydrolases

Saline hydrolases find widespread applications in various industrial processes. Some of them are discussed below:

Detergent and Textile industries:

Enzymes derived from halophilic microorganisms are excellent additives for laundry detergents as most of them are often alkalitolerant (Setati 2010). Halophilic enzymes such as amylase from marine *Streptomyces* sp. remain stable in the presence of commercial detergents and hence would be active ingredients in laundry detergents (Chakraborty et al. 2009). Cellulases are mainly used in textile industries for bio-polishing of fabrics and production of stonewashed denims. They are also used in detergents for fabric softening and brightening (Aygan and Arikan, 2008). Several halophilic and halotolerant cellulases with stability at high temperature and alkaline conditions, derived from *Salinivibrio* sp. (Wang et al. 2009) and metagenome library (Voget et al. 2006) have been characterized.

Food industry:

Xylanases find application in food and feed industries (Bedford et al. 1992). Halotolerant proteases are used in saline fermentation processes for protein hydrolysis (Setyorini et al. 2006). Halophilic amylases find extensive use in hydrolysis of starch which is commonly performed at low water activity such as the production of syrups (Margesin and Schinner 2001).

Leather industry:

Hydrolases are used for selective hydrolysis of non-collagenous constituents of the skin and removal of non-fibrillar proteins such as albumins and globulins. Halophilic enzymes are generally believed to be polyextremophilic. These enzymes are not only stable at high salt concentrations but are often also thermotolerant and alkalitolerant (Moreno et al. 2009).

Baking industry:

Endo and exo-proteinases have been used to modify wheat gluten by limited proteolysis. Xylanases are widely used in baking industries to improve the properties like extensibility and strength of dough. The addition of these enzymes reduces the mixing time and results in increased loaf volumes. Halotolerant amylases are used in bread and baking industries to improve volume of dough, color and crumb softness.

Manufacturing of soy products:

Conventional manufacturing of soy sauce is time-consuming. The development of salt tolerant protease system contributes to enhanced proteolytic activity thereby reducing the production time (Su and Lee 2001). Hydrolytic modification of soy proteins helps to improve their functional properties. Treatment of soy proteins with alcalase at pH 8.0 results in soluble hydrolysates with high solubility and low bitterness.

Pharmaceutical industry:

Oral administration of hydrolases has been used as a digestive aid to correct certain lytic enzyme deficiency syndromes. Clostridial collagenase or subtilisin is used in combination with broad-spectrum antibiotics in the treatment of burns and wounds. Several halophilic hydrolytic enzymes exhibiting antimicrobial activity are being used in the preparation of topical ointments. However, inspite of a growing

interest in the use of halophilic enzymes for biotechnological applications, there are relatively few reports in on about their production and characterization (Batnagar et al. 2005).

2.3.2 Xylanases

Xylan, a major component of plant cell wall is a complex polymer of D-Xylose linked by β-1, 4- xylosidic units. Xylan constitutes 35% of dry weight of fully grown plant and is the most abundant hemicelluloses on earth (Reilly 1981). Xylan can be degraded to Xylose and xylo-oligomers by acidic or enzymatic hydrolysis.

Enzymatic hydrolysis of xylan produces free sugars without the by-products formed with the acid (e.g. furans). Hydrolysis of the xylan backbone involves endo-1, 4-β xylanases (EC 3.2.1.8) and b-D-xylosidases (EC 3.3.1.37) (Biely 1985). Endoxylanases act on xylan (arabinoglucuronoxylans, glucuronoxylans) and xylooligosaccharides, producing mainly mixtures of xylooligosaccharides.

β-D Xylosidases, designated exoglycosidase (Puls and Poutanen 1989), exoxylanase (Eriksson and Wood 1985) or xylobiase (Meagher et al. 1988), hydrolyse xylooligosaccharides with a degree of polymerization of 3 to 6 to D-Xylose. The last decade has seen an increase in research on exozymes produced by microorganisms isolated from extreme habitats, in particularly, those of hyperthermophiles and thermophiles (Vieille and Liebl 2001; Sterner and Liebl 2001). Recent studies on exozymes produced by halophilic microorganisms have shown that these enzymes exhibit some unique structural and biochemical characteristics (Fukushima et al. 2005; Hutcheon et al. 2005; Karbalaei- Heidari et al. 2007).

Interest in xylanases and other xylanolytic enzymes has grown markedly during recent years. Till date all research confined to Xylanase production and study is being done in acidic or neutral conditions but very few confined to alkaline high salt tolerant condition. Xylanase production from Halophiles has potential application in increased digestibility of silage (high moisture fodder). Alkaline xylanases finds its applications in bleaching of Kraft pulp, reduces dependency on chlorine being used for bleaching of pulp prior to paper making process, enhance the brightness of bleached pulp, and to increase the freeness of pulp in paper recycling process (Polizeli et al. 2005).

The kraft pulping process creates an alkaline background that requires xylanases that are active and stable at alkaline pH values. Xylanase production using inexpensive agro-residues may be used for production of bio fuel. Alkaline Xylanase can replace the traditional alkaline sulphate cooking of pulp to remove lignin. Enzyme can aid in the removal of lignin from the pulp without any harmful side products. By a limited hydrolysis of xylan a greater release of lignin occur during bleaching process.

Xylanses have been proposed to improve the digestibility of animal feeds by breaking down arabinoxylans (Schäfer et al. 2007; Ullmann's 2003; Gilbert and Hazlewood 1993). Arabinoxylans represent one of the major classes of non-starch polysaccharides present in cereals, particularly wheat and rye.

Biotechnological uses and potential applications of xylanases include bioconversion of lignocelluloses material to useful product e.g. production of whole wheat bread with improved body texture and flavour, clarification of juices, improvement of the consistency of the beer and the digestibility of animal feedstock (Wong and Maringer 1999, Beg et al. 2001). Xylanases have attracted considerable research interest because of their potential industrial applications including hydrolysis of lignocellulose to fermentable sugars for biofuel production, bread making and clarification of beer and juices (Royer and Nakas 1990).

Thermostable cellulase-free alkaline xylanases can be used in the prebleaching of kraft pulps, in order to replace up to 20–30% of the chlorine required to achieve a target pulp brightness (Viikari et al. 1994) and consequently reduce by up to 50% the chloroorganics that are known to form toxic dioxins in the effluent. *Thermomyces lanuginosus*, a thermophilic fungus is known to produce high levels of cellulase-free xylanase in submerged culture using corncobs as carbon source (Gomes et al. 1993; Purkarthofer et al. 1993; Puchart et al. 2001; Singh et al. 2000).

In the search for xylanases from extremophilic sources, acidophiles, alkaliphiles and thermophiles have been studied (Fushinobu et al. 1998; Lopez et al. 1998; Bataillon et al. 2000). However, previous research on xylanases has only dealt with halophilic enzymes to a very limited extent (Johnson et al. 1986). Until recently, the known halophilic bacterial and archaeal isolates had not been reported to degrade

xylan, and thus nothing was known about the microbial degradation of this substrate in hypersaline environments.

Recently, a few xylanolytic halophiles have been isolated and described (Wejse et al. 2003b). Only recently, xylanases from halophilic sources like *Glaciecola mesophila* (Guo et al. 2009), *Nesterenkonia* sp. (Govender et al. 2009) *and Chromohalobacter* sp. (Prakash et al. 2009) have been reported, but few xylanases of halophilic origin have been characterised. From a commercial view point, xylanases are an important group of carbohydrolases, and have a worldwide market of around 200 million dollars (Katapodis et al. 2006).

Xylanase production using xylan rich agro-residues such as wheat bran, wheat straw, rice bran, rice straw, sugarcane bagasse and corn cob has been attempted by several workers (Qinnghe et al. 2004; Bakir et al. 2001; Anthony et al. 2003; Virupakshi et al. 2005; Kohli et al. 2001; Nascimento et al. 2002; Liu et al. 1999). Corn cob is a rich source of both xylan (28%) and xylose (23%) and hence it is a potential substrate for xylanase production.

There is also a distinct possibility of catabolite repression by xylose in the course of xylan (from corn cob) degradation (Lemos and Junior 2002; Kelley et al. 1989). Repression by xylose has also been reported in a strain of *Cryptococcus albidus* (Beily and Petrakova, 1984). Agricultural residues have been reported to induce xylanase synthesis efficiently (Santos et al. 2003). As xylanases are inducible enzymes, various studies on their induction and repression have been reported (Paul and Verma 1990; Xiong et al. 2004).

2.3.3 Cellulases

One of the most important applications of biomass energy systems is reported to be in the fermentation of ethanol from biomass (Lin and Tanaka, 2006). World production of biomass is estimated at 146 billion metric tons a year consisting of mostly wild plant growth. Crops and trees have the ability to generate up to 20 metric tons per acre of biomass a year. It has also been reported that certain types of algae and grasses may produce 50 metric tons per year (Balat and Ayar 2005; Cuff and Young 1980). In addition, agronomic residues raised from human activities, such as

corn stover (corn cobs and stalks), sugarcane waste, wheat or rice straw, forestry, and paper mill discards, the paper portion of municipal waste and dedicated energy crops, also have plentiful cellulose, which can be converted into fuel ethanol.

Cellulose is the most abundant organic compound in the world and this renewable resource can be easily utilised by mankind (Guo et al. 2008). Basically Cellulose is a renewable resource that can be converted into biobased products and bioenergy. But due to inefficient use of this agriculture waste, industrial and municipal cellulose waste has been accumulating due to their high cost of utilization. Therefore, it has become of considerable economic interest to develop processes for the effective treatment and utilization of cellulosic wastes as cheap carbon sources.

CMCase is a key enzyme that functions during the decomposition of plant root-hair walls during symbiosis of bacteria with plants (Chen et al. 2004). Cellulase hydrolysis is accompanied with the aid of cellulase enzyme complex which is made up of three enzymes namely exoglucanase, endoglucanase and β-glucosidase. Characterisation of cellulase enzymes poses special problems to enzymologist – rarely encountered for other enzymes. Kinetic studies are difficult since the natural substrate is both insoluble and structurally variable and thus relatively undefined with respect to concentration and chemical form. Often a multitude of endo- and exo- glucanases act in synergy and in a complex manner still poorly understood (Ghose 1987).

The requirement for enzymes that are active under more extreme conditions of temperature, pH or salinity has meant that microorganisms isolated from ‘extreme’ environments, so-called extremophiles, have proved a valuable resource for the isolation of novel biotechnological products (Rees et al. 2003). A perceived benefit of enzymes from extremophiles for industrial applications is their high stability. Cellulosic wastes yield free celluloses after delignification. Bacterial cellulases can convert cellulose to reducing sugars which on fermentation, is converted to ethanol, a raw material for methane and biogas production. Ethanol can be dehydrated to obtain ethylene gas.

Cellulases have great potential in saccharification of fermentable sugars which can be used for the production of bioethanol, lactic acid and single-cell protein. Complex nitrogen sources such as yeast extract and peptone have been reported to

significantly affect cellulase production (Li et al. 2008). Deka et al. (2011) reported the enzyme activity of *Bacillus subtilis* AS3 in the unoptimised CMC medium to be 0.07 U/ml. By optimising the medium further (CMC, peptone and yeast extract) a six fold increase in enzyme activity was obtained (0.43 U/ml).

Li et al. (2008) reported maximum cellulase activity (0.26 U/ml) of a *Bacillus* sp. when the culture was grown in LB medium supplemented with 1% CMC. It has been reported recently that *Bacillus* sp. (DUSELR 13) and *Brevibacillus* sp. (DUSELG 12) isolated from gold mine produced maximum CMCase activity 0.12 U/ml and 0.02 U/ml, respectively under optimised conditions (Rastogi et al. 2010). In another study, a cellulase activity of 0.0113 U/ml was observed under optimised conditions from *Geobacillus* sp. (Tai et al. 2004).

2.4 LIGNOCELLULOSES

The industrial enzyme production is frequently limited by the costs of substrates for the cultivation of the producer microorganisms. The use of low-cost substrates, such as agricultural wastes, has been suggested as an alternative to reduce the production costs. Biomass can be defined as "all organic material of vegetable or animal origin, which is produced in nature or managed ecosystems (agriculture, aquaculture, forestry) all or not industrially transformed". Agro-residues such as straw, bran, pulp, corn cobs, corn stover, oil cakes, waste wood etc. which are rich in lignocellulosic materials, are either poorly valorized or left to decay on the land (Singh nee' Nigam and Pandey 2009).

However, those residues represent an alternative source for the microbial growth aiming the production of biomass or enzymes. Hemicelluloses and celluloses represent more than 50% of the dry weight of agricultural residues. They can be converted into soluble sugars either by acid or enzymatic hydrolysis. So, they can be used as a plentiful and cheap source of renewable energy in the world.

It has been reported that on a worldwide basis, terrestrial plants produce 1.3×10^{10} metric tons (dry weight basis) of wood per year, which is equivalent to 7×10^{9} metric tons of coal or about two-thirds of the world's energy requirement (Kumar et

al. 2008; Wang et al. 2011). Lignocellulosic biomass account for approximately 70% of plant biomass and represent one of the most abundant organic carbon available on earth (Kukhar 2009). They are composed of strongly intermeshed network of three types of polymers – cellulose, hemiculloses and lignin.

These biopolymers are chemically bonded by both non-covalent forces and covalent cross-linkages (Makkar et al. 2011). Lignin is a non-carbohydrate polyphenolic substance that cements the cells together. The cell wall structures are recalcitrant in nature and offer intrinsic resistance of the lignin complex to enzymatic attack.

Conversion of this cellulosic waste to fermentable sugars is a challenging area of research. Agricultural biomass offers an inexpensive and renewable natural feed-stock supply that can be converted into liquid biofuels and chemicals (Nigam and Singh 2011). In India, ethanol is primarily produced from molasses which is a byproduct from sugar mills. Various other feedstocks need to investigated for the production of alcohol (Uppal 2007; Subramanian et al. 2005)The value of plant biomass content is related to the chemical and physical properties of its molecule (Pérez et al. 2002).

These renewable substrates are advantageous in terms of low capital and energy cost, ease of operation and reduction in environment pollution. Hence utilization of agricultural residues for the production of useful products is a favourable option (Ferreira 2008; Montoneri et al. 2009; Moldes et al. 2007). This in turn adds value to the waste. These residues are mostly burned releasing CO_2 which contributes to the greenhouse effect (Lasco 1998).

2.4.1 Polymer degradation by halophilic microorganisms

In a study on utilization of agricultural residues for poly3-hydroxybutyrate production by *Halomonas boliviensis* LC1 (Van-Thuoc et al. 2008), wheat bran was hydrolysed by a crude enzyme preparation from *Aspergillus oryzae* NM1 to provide a mixture of reducing sugars comprising mainly of glucose, mannose, xylose and arabinose. However, in large scale applications pretreatment with enzymes would

make the process too expensive. Alternate ways of reducing the enzyme cost includes the use of cheap production substrate and using crude enzyme preparation for the target application.

Several different carbon sources can be obtained from hydrolysates of agricultural residues or by-products of food industry including extruded starch, extruded rice bran, corn starch, hydrolyzed whey and sodium valerate. Species of the family *Halomonadaceae* that accumulate polyhydroxy alkanoates were found to assimilate these sources.

The ability of these strains to use different cheap substrates has been exploited for the production of PHB by *Halomonas boliviensis*, the most extensively studied species of the family *Halomonadaceae* regarding the polyester production (Quillaguamán et al. 2010). Xylanase production using inexpensive agricultural wastes from a halophilic *Chromohalobacter* sp. TPSV 101 was reported by Prakash et al. (2009). Various concentrations of lignocellulosic materials like rice bran, wheat bran, sugarcane bagasse, ground nut peel and sawdust were tested. The bacterium was also able to utilize most of the lignocellulosic substrates as raw materials. The highest enzyme production was noted in sugarcane bagasse followed by wheat bran. Xylan at a concentration of 0.2% and/or 1.0% sugar cane bagasse (w/v) supported optimal growth and xylanase production. Among the various organic and inorganic nitrogen sources tested, feather hydrolysate (0.5%) supported maximum bacterial growth and xylanase production.

Two extreme halophilic *Haloferax* strains and one strain each of *Halobacterium* and *Halococcus* capable of degrading crude oil and pure hydrocarbons were isolated from a hypersaline coastal area of the Arabian Gulf (Al-Mailem et al. 2010). Several species belonging to *Halobacterium* are reported to degrade a wide range of n-alkanes and polynuclear aromatic hydrocarbons at about 30% NaCl concentration. *Haloarchaea Haloferax* and *Haloarcula* could degrade aromatic compounds.

Cuadros-Orellana et al. (2006) suggested that the ability to degrade p-hydroxybenzoic acid is a widespread feature among *Halobacteriaceae*. Martínez-Checa et al. (2002) reported *Halomonas eurihalina* strain H-28, a moderately halophilic bacterium that produces an extracellular polysaccharide in media with glucose and also in media supplemented with hydrocarbons (*n*-tetradecane, *n*-hexadecane, *n*-octane, xylene, mineral light oil, mineral heavy oil, petrol, or crude oil).

It is reported that a mixture of anaerobic halophilic bacteria from salt marsh sediments may use chitin as a carbon and hydrogen source for sulphate reduction and methanogenesis (DasSarma and Arora 2001). A halophilic anaerobe with chitinolytic activity, *Haloanaerobacter chitinovorans* was isolated as a novel genus from solar saltern (Liaw and Mah 1991). Two chitin-induced extracellular proteins with molecular weights of 38 x 10^3 and 40 x 10^3 were detected in strain W5C8.

A multifunctional enzyme was found to be produced by *Terendinibacter turnerae* T7902, which is a bacterial symbiont isolated from the wood-boring marine bivalve *Lydrodus pedicellatus*. It binds both cellulose and chitin and possesses cellobiohydrolase and beta-1,4(3) endoglucanase activity allowing it to degrade multiple complex polysaccharides (Maki et al. 2009).

Horikoshi (1999) reported some haloalkaliphiles capable of producing carboxymethyl cellulases including *Bacillus* sp. strain N4, *Bacillus* sp. strain N1139 and *Streptomyces* sp. strain KSM-9. A new halo-alkaliphilic, thermostable endoglucanase from moderately halophilic *Bacillus* sp. C14 was isolated from Van soda lake (Aygan and Arikan 2008). The enzyme was suitable for application in industries like beverage, textile and ethanol production from cellulosic material.

2.4.2 Pretreatment of agricultural residues

Lignin polymer is highly heterogeneous. Ligno-cellulosic biopolymers (agricultural residues) need to be pretreated before use. The purpose is to remove lignin and increase the porosity of the materials. Physical treatments like mechanical comminution and pyrolysis; physico-chemical treatments like autohydrolysis and CO_2

explosion; chemical treatments like acid and alkaline hydrolyses; biological processes using lignin degrading fungi and enzymatic hydrolysis have been used for pretreatment of lignocellulosic materials (Cadoche and Lopez 1989; McMillan 1994; Rensburg et al. 1998).

Application of alkaline chemical treatment (NaOH, $Ca(OH)_2$ or ammonia), possibly in combination with heat is a comparatively economical process to remove lignin and increase accessibility of material. Following this step, the alkaline solution is (partially) neutralized to create the optimum saline environment for polymer degradation. Silverstein et al. (2007) found that sodium hydroxide pretreatment of cotton stalks resulted in the highest level of delignification (65% with 2% NaOH in 90 min at 121°C) and cellulose conversion (60.8%).

Pretreatment can be performed at low temperatures but with a relatively long time and high concentration of the base (Taherzadeh and Karimi, 2008). When soybean straw was soaked in ammonia liquor (10%) for 24 h at room temperature, hemicellulose and lignin decreased by 41.45% and 30.16%, respectively (Xu et al. 2007). However, alkaline pretreatment was shown to be more effective on agricultural residues than on wood materials.

Curreli et al. (1997) suggested two steps, mild alkaline/oxidative pretreatment at low temperature (25–40 °C) and low concentration of chemicals. Alkaline pretreatment (1% NaOH for 24 h) in the first step solubilises hemicellulose and a second alkaline/oxidative step (1% NaOH and 0.3% H_2O_2 for 24 h) in order to solubilise and oxidize lignin. The pretreatment is also useful in removing waxes, silica, and the waterproof cutins that coat plant tissue. Compared with acid or oxidative reagents, alkali treatment appears to be the most effective method in breaking the ester bonds between lignin, hemicellulose and cellulose and avoiding fragmentation of the hemicellulose polymers (Gaspar et al. 2007). For the given reasons alkaline pretreatment of agro-residues is chosen for the present investigation.

2.4.3 Agricultural residues as substrates

The lignocellulosic material consisting of lignin, cellulose and hemicellulose is utilized as feedstock for various bio-refining processes for the production of

bioethanol, biopolymers, enzymes and a range of fine chemicals. The optimization of the process of lignocellulosic digestion and development of effective biocatalyst for the breakdown of cellulose and hemicellulose into simple sugars (hexoses and pentoses) is the pre-requisite for the efficient biotechnological conversion of lignocellulosic materials into valuable products (Koller et al. 2005).

Bertrand et al. (1990) have reported high maintenance energy requirement for microorganism on pentose than hexose. They have reported low specific PHA production rates on pentose sugars than on glucose. In many studies a comparatively good PHA concentration, but with low cell densities have been reported on sugar hydrolysate released from cellulosic and hemi-cellulosic fractions of agricultural residues. It has been reported that feedstock pretreatment is a necessary upstream process to remove lignin and enhance the porosity of the lignocellulosic materials prior to the enzymatic process (Zhu and Pan, 2010; Kumar et al. 2009). The goal of biorefinery approach is the generation of energy and chemicals from different biomass feedstocks, through the combination of different technologies (FitzPatrick et al. 2010).

Various hydrolytic enzymes are believed to control the rate at which various substrates are degraded. Enzymes are the main mediators of various degradation processes (Tiquia et al. 2002). These enzymes help to degrade the non-starch polysaccharides in substrate to reducing sugars. Thus with the decrease in the amount of cellulose, a corresponding increase in reducing sugars is obtained.

In a study on utilization of agricultural residues for poly3-hydroxybutyrate production by *Halomonas boliviensis* LC1, wheat bran was hydrolysed by a crude enzyme preparation from *Aspergillus oryzae* NM1 to provide a mixture of reducing sugars comprising mainly of glucose, mannose, xylose and arabinose (Van-Thuoc et al. 2008).

Hydrolysates of renewable resources like wheat bran and potato residuals are inexpensive carbon and energy sources. The use of cheap and readily available agricultural residues is expected to substantially reduce the cost of the carbon source that can be used in fermentation (Choi and Lee 1999). The use of renewable

agricultural remnants as substrates for fermentation processes involves a hydrolysis step that releases easily metabolizable sugars. This step should be inexpensive and should not result in compounds that inhibit the fermentation process.

2.5 MEDIA OPTIMIZATION STUDIES

Every microorganism has its own special conditions and nutritional requirements for optimum production. The key approaches for achieving improvements in productivity of metabolites from microorganisms are- advancements in fermentation bioprocesses and media design, implementation of innovative bioreactors and overproduction in mesophilic hosts (Chiara and Mario 2002).

Large-scale production of high-value products like compatible solutes, biodegradable plastics, enzymes, pigments and therapeutics is mainly hampered by its high production cost. Cost of the growth medium in industrial fermentations is estimated to account for 30-40 % of the production cost (Huang et al. 2005). The design of fermentation medium is of critical importance for the development of industrial scale fermentation processes. The optimization of the process refers to 'the maximizing or minimizing of a given function possibly subjected to source type of constraints' (Kennedy and Krouse 1999).

2.5.1 Multivariate process optimization

The 'one-variable-at-a-time' strategy of optimization process refers to keep the value of all process variables constant except one. The advantage of this strategy is that it is simple and easy in nature. However, the interaction between variables is ignored in this strategy. It is also time consuming and laborious when applied on a large number of variables. Now-a-days, the development of several mathematical and statistical designs has changed the basic experimental design from 'one-factor-at-a-time' (Kennedy and Krouse 1999; Montgomery 2005).

The improvement in the performance of bio-process and fermentation media uses the 'Design of Experiments (DoE)' approach which integrates both the design and optimization techniques. The design allocates process variables in specific

manner in experiments, while the optimization technique incorporates the mathematical model to predict the improved process performance for medium composition.

Several experimental designs such as full factorial, fractional factorial, Plackett-Burman, Hadamard, orthogonal array, central composite (Box and Wilson) and Box-Behnken have been used to improve the process performance as well as medium compositions (Kennedy and Krouse 1999; Bas and Boyaci 2007; Sin et al. 2006; Bezerra et al. 2008; Nasrabadi and Razavi 2010).

The Plackett-Burman design is usually implemented for screening of variables. Similarly, various optimization strategies such as response surface methodology (RSM), steepest ascent (SA), evolutionary operation (EVOP), canonical analysis (CA), multiple linear regression as well as the artificial intelligence based neural networks and genetic algorithm have been implemented to predict the performance of bioprocess and fermentation systems (Kennedy and Krouse 1999; Gharibzahedi et al. 2012; Nelofer et al. 2012; Abbasi et al. 2013).

2.5.2 Response surface methodology

Response surface methodology (RSM) is the most general and efficient strategic experimental tool among all the recent non-linear optimization techniques, which is used to study the effect and mutual interactions of variables in a multivariable system (De Coninck 2000; Puri et al. 2002; Li et al. 2007). The concentrations of each of the important variables in a production process can be optimized using response surface optimization techniques which were introduced by Box and Wilson (1951). Response surfaces are similar to contour plots or topographical maps. The axes of the contour plot are the experimental variables and the area within the axes is termed the response surface. In this context, response means the result of an experiment carried out at particular values of the variables being investigated.

In its simplest form two variables are examined and the plot is two dimensional. It is important to appreciate that both variables are changed in the

experimental series, rather than one being maintained constant, to ensure that the data are distributed over the response surface (Haaland 1989).

The more sophisticated applications of the response surface technique use mathematical models to analyze the first round of experimental data and to predict the relationship between the response and the variables (Anbu et al. 2009). These calculations then allow predictive contours to be drawn and facilitate a more rapid optimization with fewer experiments. If three or more variables are to be examined then several contour maps will have to be constructed. Statistical methodologies are also generally preferred, due to the variety of recognized advantages to their use (Li et al. 2007).

2.6 MOTIVATION FOR THE PRESENT STUDY

The following observations were made from the survey of literature:

- Surprisingly, little is known about polymer-degrading moderate halophiles, with very few reports available on the usage of renewable agricultural resources for the growth of halophilic bacteria.

- Most of the available literature focuses on the production of cellulases and xylanases from fungal sources with less attempt on production of bacterial hydrolases. The titre of bacterial enzymes obtained is often less compared to that of fungal enzymes.

- Moreover, fewer findings are available on biopolymer degrading *halostable* enzymes. Such extremophilic enzymes have important applications.

- In spite of the widespread applications the present toolbox of solutes and enzymes is not sufficient. The cost of substrates is one the most important factor limiting potential applications.

- Further investigations on media design using agricultural remnants would contribute in downsizing high production costs, thereby improve market competitiveness.

As the study on compatible solute production using agricultural resources has been less attempted, the study would provide more information on the growth of halophiles on biopolymer media and subsequent synthesis of important compatible solutes like ectoine. This would also shed light on the occurrence of novel biopolymer-degrading halophilic bacteria which can be used for bioconversion of lignocellulosic compounds and would further establish the broad spectrum of hydrolytic enzymes synthesized by them.

With these aspects in view, the objectives, mentioned in the section 1.5 of Chapter 1, have been formulated for the present doctoral work.

Chapter 3

Isolation of halophilic bacteria and pretreatment of agricultural residues

3.1 MATERIALS AND METHODS

3.1.1 Chemicals and experimental statistics

All chemicals used were of analytical grade and media components of highest purity grade (Appendix I). The microbiological media used were dehydrated media. Production studies were carried out as batch cultures in 250 ml Erlenmeyer flasks, containing 100 ml of culture media. All the experiments were carried out independently in triplicates and repeated twice. The standard deviation in results was within experimental limits.

3.1.2 Sample collection for isolation of halophilic bacteria

Salt enriched soil and water samples were collected from sea coasts, solar salterns and marine ecosystems of coastal Karnataka. All samples were collected aseptically in sterile bottles and transferred to the lab for further analysis.

3.1.3 Isolation of halophilic bacteria

Bacterial isolation was done by serial dilution plating technique on MM63 medium (Larsen et al. 1987). The medium composition is given in Appendix II. These bacteria were isolated with the following biopolymers as sole carbon source in place of glucose, each at 0.5% w/v – xylan, CMC, inulin (Setati et al. 2010; Allais et al. 1986). Growth was scored according to the color of colonies (Fung and Kraft, 1968). Based on Gram staining and morphology different organisms were isolated, cultured aerobically in MM63 at 30 °C and maintained as pure cultures in agar stabs and glycerol stock.

3.1.4 Procurement of agricultural residues

Crop residues are the most promising non-conventional source for energy generation. Crops like rice, wheat, corn and sugarcane are the widely cultivated crops in India. The amount of crop-residues generated each year increases with the increasing demand for production. These residues are generally burnt in the field as a means of disposal or used as animal feed. However, these organics are rich source of lignocelluloses.

Wheat bran was procured from Jalandhar, Punjab; Siwan, Haryana, Aligarh, U.P. and Bagalkot, Karnataka, India. Rice bran was procured from rice mill, Udupi, Karnataka, India. Sugarcane bagasse was procured from sugar factory, Hubli and Belgaum, Karnataka, India.

Corn cobs were procured from Hubli and Mangalore, Karnataka; Jalandhar, Punjab, India. Groundnut shells and coir pith were procured from local markets in Surathkal, Mangalore, India. For production studies in Bonn, Germany, wheat bran was procured from the provision store Edeka, Bonn.

3.1.5 Alkaline treatment of agricultural residues

Alkaline treatment of agro-residues was carried out according to the method described by Pham et al. (1998) as shown in Fig. 3.4. The procured agricultural residues were ground to particles of 0.5 – 1 cm in a mixer. For de-lignification, 100 g of these agro-residues taken in 1 L Erlenmeyer flasks were soaked in 1% NaOH and autoclaved at 121 ºC for 20 min.

After alkali treatment, the materials were washed with tap water until neutral and oven-dried at 60 °C until a constant weight was obtained. The dried residues were then passed through 0.5 mm screens. These treated residues which had smaller particle size were used as substrates by dissolving the appropriate amount in production media.

3.2 RESULTS AND DISCUSSION

3.2.1 Sample collection for isolation of halophilic bacteria

The salt-enriched soil and water samples were collected from salterns and coastal regions of Karnataka, India (Fig 3.1 and 3.2). The different sites of sample collection are given in Table 3.1. The coastal strip, called Karavali, between the Western Ghats and the Arabian Sea, which is lowland, with moderate to high rainfall levels. This strip is around 320 km in length and 48–64 km wide. Indian coastline consists of various saline environments including lakes, ocean and salt pans, in which the microbial diversity has not been well characterized (Jayachandra et al. 2012).

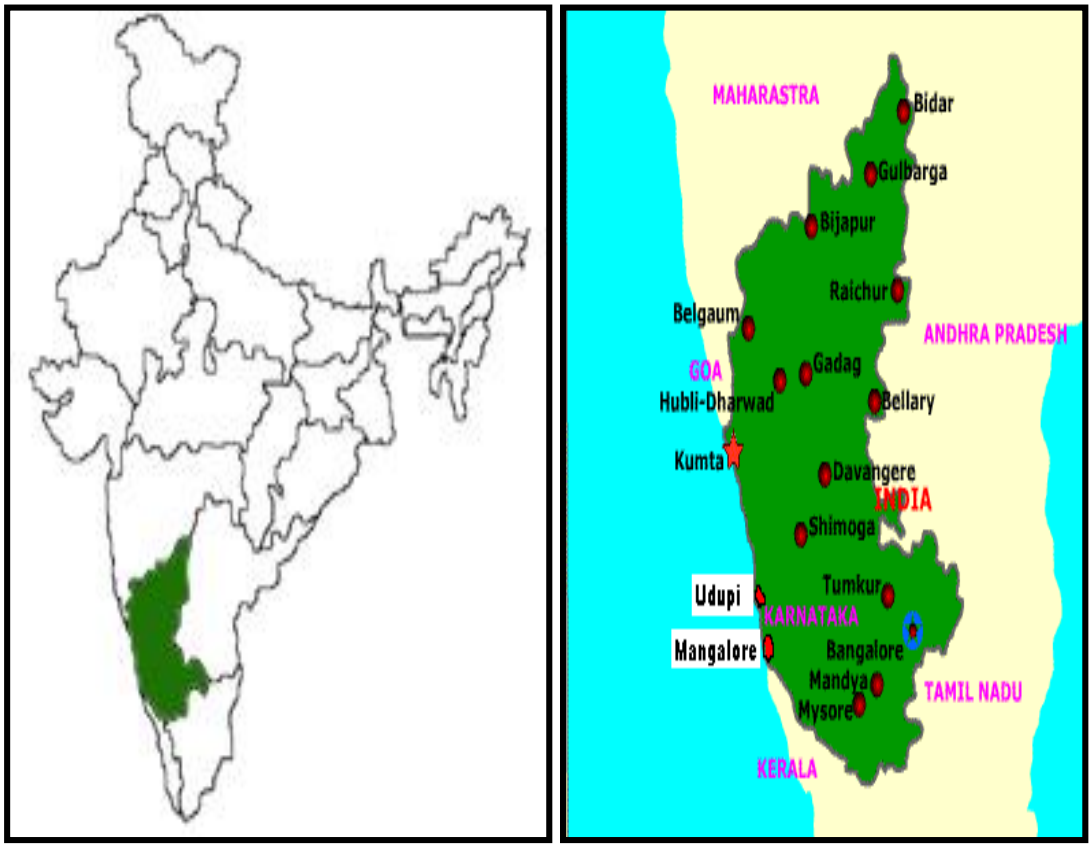

Figure 3.1 Map of India and Karnataka (www.mapzones.com)

Table 3.1 Sites of sample collection in coastal Karnataka, India

Sl. No	Name	Sample	Location
1	**NITK Surathkal beach**	sea water, soil marsh	12.983°N 74.783°E
2	**Panambur beach, Mangalore**	sea water	12.87°N 74.88°E
3	**Sanekatta saltern, Kumta**	water, salt crystals, soil from salt processing unit	14.42°N 74.4°E
4	**Head bunder beach, Kumta**	sea water	14.42°N 74.4°E
5	**Baad salt marsh ecosystem, Kumta**	soil marsh samples	14.42°N 74.4°E
6	**Malpe beach, Udupi**	sea water	13.59°N 74.75°E

Salt deposits around the world show that evaporation of marine salt water and the development of hypersaline habitats is an ongoing process for millions of years. This provides ample time for the evolution of specialized halophilic bacteria and archaea. From the literature it is understood that bacteria are furthermore responsible for degradation of organic matter and conversion of organic nitrogen to ammonia which can be used by the algae and perhaps brine shrimp and brine flies which are common to hypersaline environments (Saju et al. 2011).

Figure 3.2 Sanekatta saltern and salt processing unit, Kumta, Karnataka. The yellow-orange coloration of the salt pans is an indication of the presence of microbial life.

3.2.2 Isolation of polymer degrading bacteria

Forty eight strains of halophilic bacteria were isolated from the coastal regions of Karnataka (Fig. 3.3). Different biopolymers were used as the sole source of carbon in the medium. Bacteria growing on biopolymer medium were expected to harbor the potential enzyme machinery to degrade biopolymers and utilize them for growth and multiplication. Colonies were transferred from agar plates with a sterile loop and inoculated into 5 ml of sterile liquid medium as described above. Once sufficient growth was attained, the cultures were checked for consistent cell morphology. The selected cultures were then grown in 100 ml of sterile media of the same composition. Pure cultures were as stock cultures on agar slants.

Sixteen different strains of halophilic bacteria (PS1-PS16) were isolated on Xylan – a hemicellulose comprising 20-30% dry weight of plants; PS17-PS32 were isolated from Inulin – a polysaccharide belonging to class of fructans, present in roots and rhizomes of many plants; PS33-PS48 were isolated from Cellulose – the most abundant polysaccharide, constituting 50% dw of plant matter. Some of the strains were pigmented – orange, yellow and red colonies. Some of the bacteria formed very minute colonies and were slow growing. The colonies appeared on agar plates only after 72 h. Some of the bacterial strains formed a zone of clearance around the colonies indicating the release of extracellular enzymes.

These isolates were later screened for the production of ectoine, halostable cellulase and halostable xylanase using renewable agricultural residues as medium components. Morphological and physiological characteristics of potential strains were later studied as per the standard protocols (Cappuccino and Sherman 2004; Smibert and Krieg 1994). The potential producers were further identified by 16S rRNA gene amplification and nucleotide sequencing.

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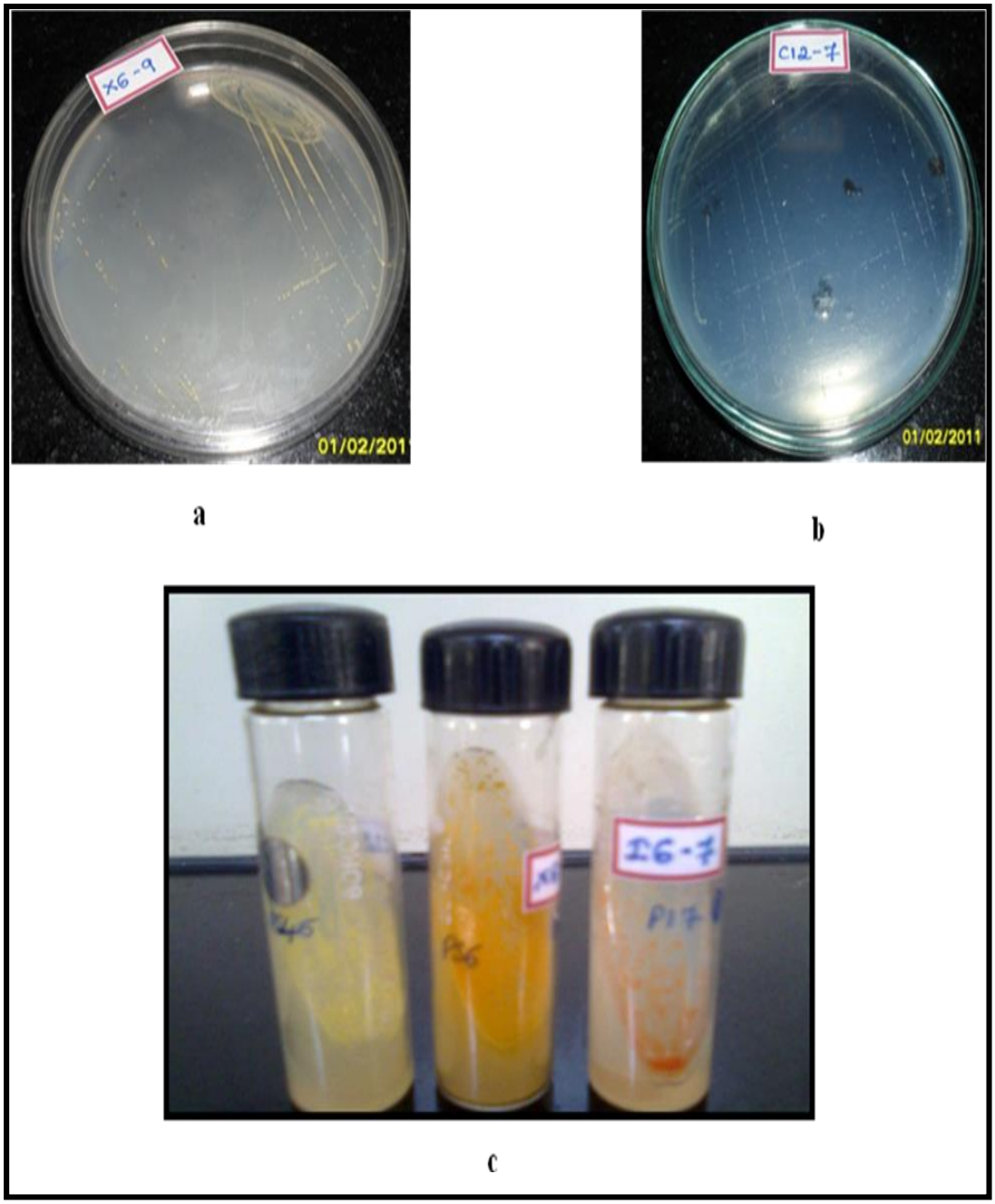


Figure 3.3 Typical morphological characteristics of isolated halophilic bacteria **(a)** Quadrant streak of PS8 **(b)** Pin-point colonies of PS41 **(c)** Pigmented isolates: yellow – PS46, orange PS6 and red PS17.

3.2.3 Pretreatment of agricultural residues

Pretreatment of agro-residues was carried out as shown in Fig. 3.4. Pretreatment of agro-residues helps in the removal of lignin, solubilises hemiculloses and decreases crystallinity of the substrate and increasing the porosity (Dobrev et al. 2007; Kodali and Pogaku 2006).

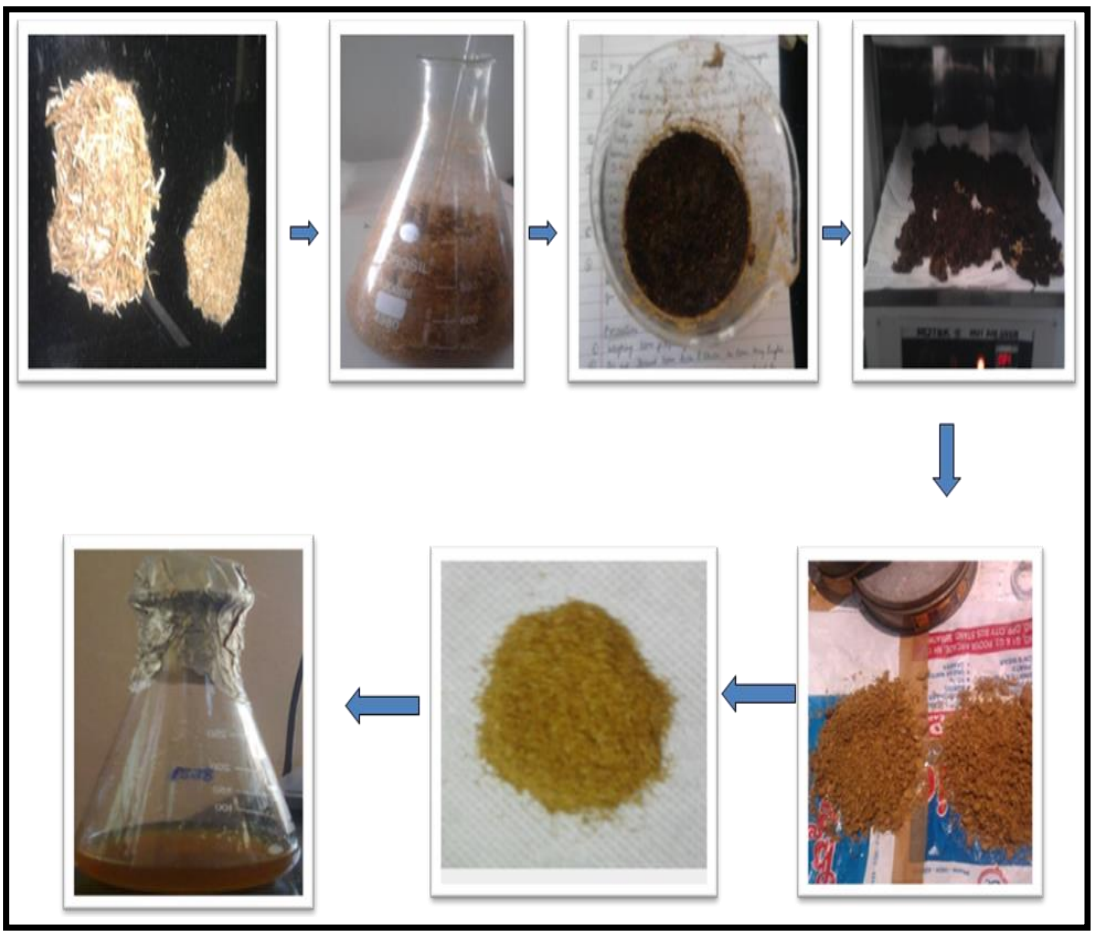


Figure 3.4 Alkaline pretreatment of wheat bran. The residues were ground to particles of 0.5 – 1 cm. 100 g of these agro-residues 1 L Erlenmeyer flasks were soaked in 1% NaOH and autoclaved at 121 ºC for 20 min. After alkali treatment, the materials were washed with tap water until neutral and oven-dried. Treated substrates were passed through 0.5 mm screens for use as medium component.

Cellulose is a linear polymer of anhydro-glucose units with β-1, 4 linkages. The orientation of the linkage (β-1, 4 linkage) and additional hydrogen bonds make the polymer rigid and difficult to break. Hemicellulose is a polymer of short highly branched chains of the various sugars. Hemicelluloses are often polymers of pentoses mainly xylose. Lignin is a large complex polymer of phenyl propane and methoxy groups, a non-carbohydrate polyphenolic substance which encrusts the cell walls and cements the cells together (Lynd et al. 1996).

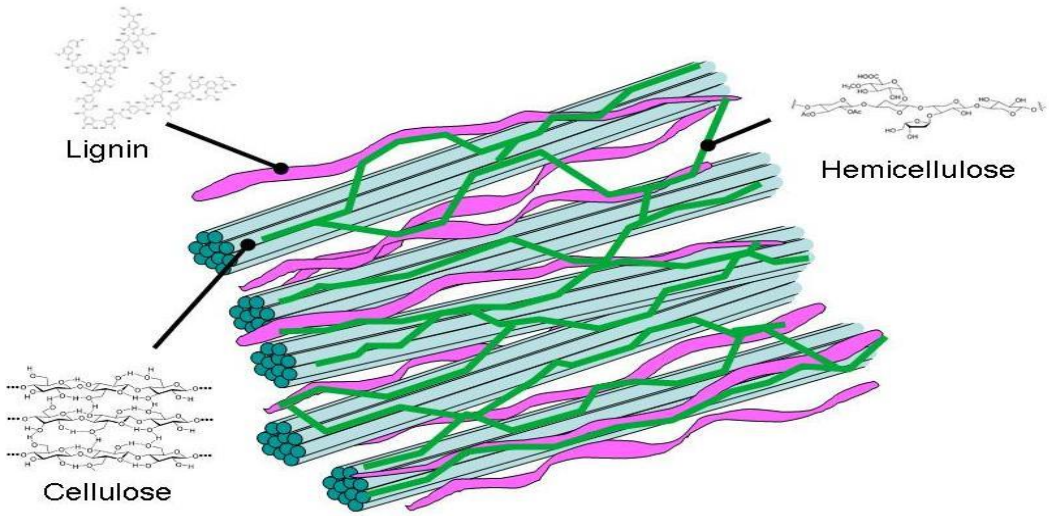


Figure 3.5 Lignin, cellulose and hemicellulose (xylan) in agricultural residues. Lignin acts as a cementing material that binds cellulose together with xylan, confirming the crystallinity (Courtesy: Google images).

The combination of hemicellulose and lignin provides a protective sheath around the cellulose, which must be modified or removed before efficient cellulose hydrolysis can occur, but the crystalline structure of cellulose makes it insoluble and resistant (Fig 3.5). Therefore, pretreatment must be employed for practical cellulose conversion process. Pretreatment is required to alter the structure of cellulosic biomass to make cellulose accessible to the enzymes that convert carbohydrate polymers into fermentable sugars (Lee et al. 1994). The cellulose and hemicellulose, which typically comprise two-thirds of the dry mass, are polysaccharides that can be

hydrolyzed to sugars and eventually to ethanol by fermentation (Hamelinck et al. 2005).

Enzymatic treatment of lignocelluloses is desirable to effectively release monosachharides from polysaccharide biomass. However, the cost of enzyme becomes a bottleneck. Pretreatment involving steam explosion requires electricity for steam generation and hence becomes a coal-dependent approach (Dater et al. 2007). Lignocelluloses can be acid hydrolyzed to obtain fermentable sugars. However, this method is hampered by non-selectivity and byproduct formation like furfural, acetic acid formic acid etc. It has been reported that compared to acid or oxidative reagents, alkali treatment appears to be the most effective method in breaking ester bonds between lignin, hemicellulose and cellulose and avoiding fragmentation of hemicellulose polymers (Gaspar et al. 2007; Vyas et al. 2005). Klinke et al. (2004) indicated that alkali treatment is the most efficient process, producing at least 3.5 times higher ethanol yields compared to steam-blasted or acid-treated biomass. At present, submerged cultivation using agricultural residues is used more widely for bacterial cultures, allowing a higher degree of process intensification and a better level of automation (Gawande and Kamat 2000).

3.3 SUMMARY

Several biopolymer degrading bacterial strains were isolated from sea coasts and solar salterns of coastal Karnataka. Based on Gram staining and morphological studies, forty eight strains of halophilic bacteria were isolated – (PS1-PS16) on xylan, (PS17-PS32) on inulin and (PS33-PS48) on CMC. Bacteria growing on biopolymer medium were expected to harbor the potential enzyme machinery to degrade biopolymers and utilize them for growth and multiplication. The isolates were stored as pure cultures for further screening of production of ectoine, xylanase and cellulase. The agricultural residues were procured from farms, mills and local markets. These residues were pretreated with alkali to remove lignin and increase the porosity of materials to be used medium components. A possible advantage of using halophilic bacteria is that the salt created after neutralization does not need to be removed.

PART ONE

ECTOINE PRODUCTION

Chapter 4

Screening and production of ectoine using biopolymeric substrates

4.1 MATERIALS AND METHODS

4.1.1 Screening of bacterial strains for ectoine production

Halophilic bacteria were cultured in the basal MM63 medium (6% w/v NaCl) with glucose (0.5% w/v) and subsequently screened for the production of ectoine. Growth of the potential strains was carried out in MM63 medium at both neutral and alkaline pH ranges. Growth data were collected from cultures in shaking flasks with appendages for optical density measurements. The cell cultures were harvested for subsequent analysis of ectoine content (Appendix III). The cells were centrifuged at 8,500 rpm for 20 min at 20 ºC. Cell pellets were freeze-dried. Ectoine was extracted from the freeze-dried biomass pellet with chloroform/methanol/water (10:5:4, by vol.) using a modified protocol of Bligh and Dyer (1959) (Appendix IV) as described previously (Galinski and Oren 1991).

4.1.2 Analysis of ectoine

The water-soluble fraction was subsequently analyzed using a HPLC unit in isocratic mode on aminopropyl Nucleosil® 100-3 NH_2 reversed-phase column (Macherey-Nagel, Düren, Germany) (Seip et al. 2011). Solvent acetonitrile (80% v/v) was used as the mobile phase at a flow rate of 1 ml min^{-1} (Galinski and Herzog 1990). The spectrum of compatible solutes present in the sample was determined by comparing the retention time with those of standards. Samples were detected and quantified by UV (210 nm) and RI detectors. The freeze-dried sample containing compatible solutes of PS6 was subjected to NMR analysis and to confirm the structure of ectoine.

4.1.3 Screening for ectoine content on different biopolymers

These bacteria were later grown in MM63 with the following biopolymers as sole carbon source in place of glucose, each at 0.5% w/v –xylan, CMC, inulin, chitin and carob. Growth data were collected at 600 nm in conical flasks with appendages. Ectoine content on these substrates was studied as per the protocol described previously.

4.1.4 Structure elucidation

The freeze-dried sample containing compatible solutes of PS6 was subjected to NMR analysis. Aqueous samples for ^{13}C NMR spectroscopy were supplemented with 1 ml D_2O as an internal lock signal. Spectra were calibrated to the internal standard trimethylsilanepropionic acid (TMSP). The NMR spectra were recorded on a Bruker Avance 300 DPX spectrometer at Institute for Microbiology and Biotechnology, University of Bonn, Germany and the structure was elucidated.

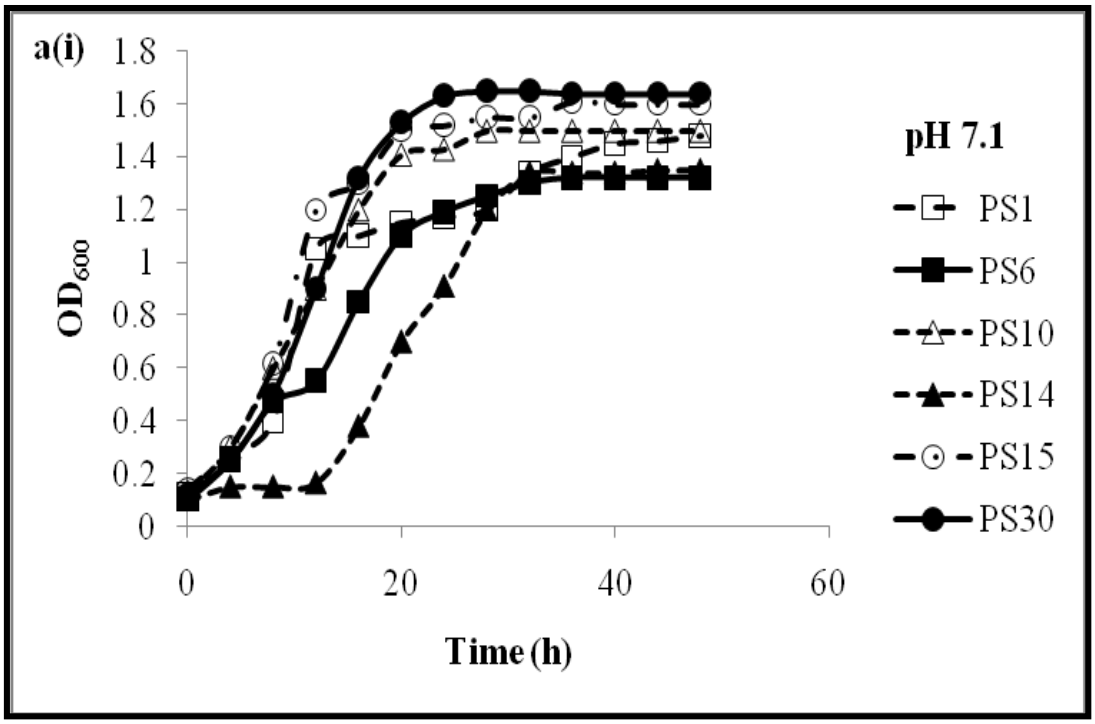
4.1.5 Identification of the potential strain

Genomic DNA of the potential strain PS6 was isolated using the RTP® Bacteria DNA Mini Kit (Invitek GmbH) and amplified using PCR (Appendix V). The amplified product was subjected to sequence analysis (Bonn, Germany). The bacterium was identified by aligning the nucleotide sequence in the NCBI database using BLAST and phylogenetic analysis.

4.2 RESULTS AND DISCUSSION

4.2.1 Selection of the ectoine-producing bacterium

All the isolates were grown in MM63 medium containing glucose as carbon and NaCl at 6% (w/v). The growth pattern of different bacterial strains at neutral and alkaline pH ranges is shown in Fig. 4.1. Those bacterial cultures which showed good growth in the medium were screened for the synthesis of ectoine by HPLC analysis.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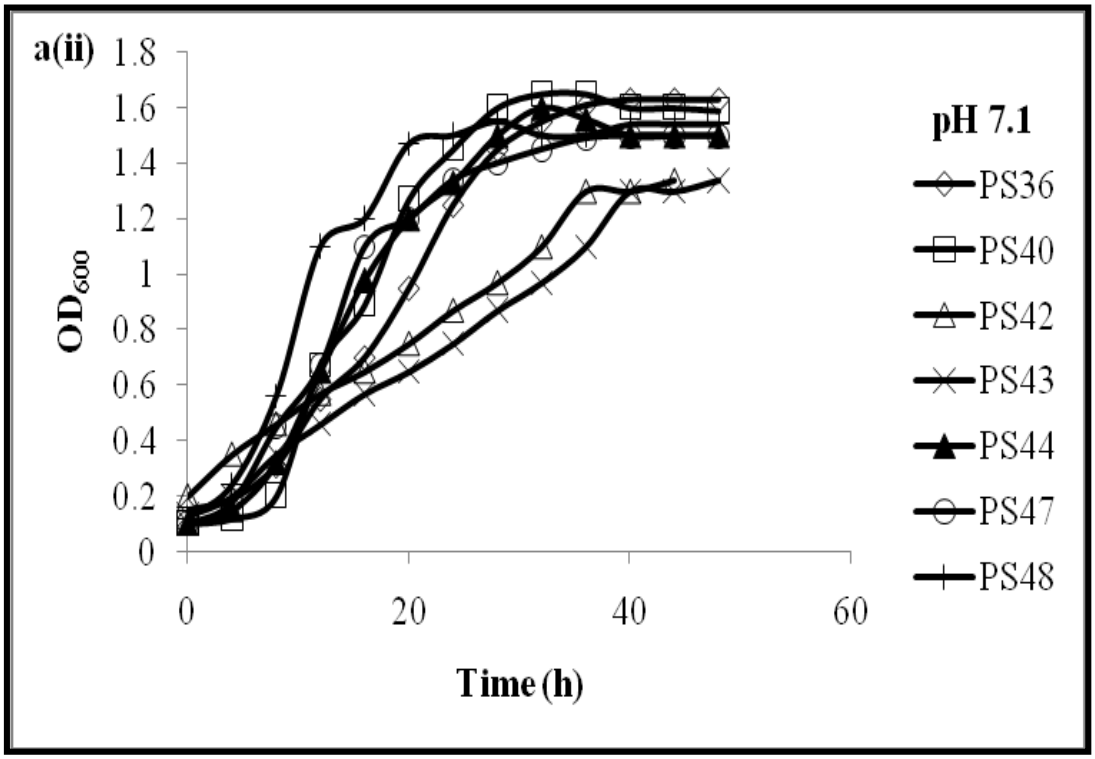
PS10
PS14
PS15
PS30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PS48

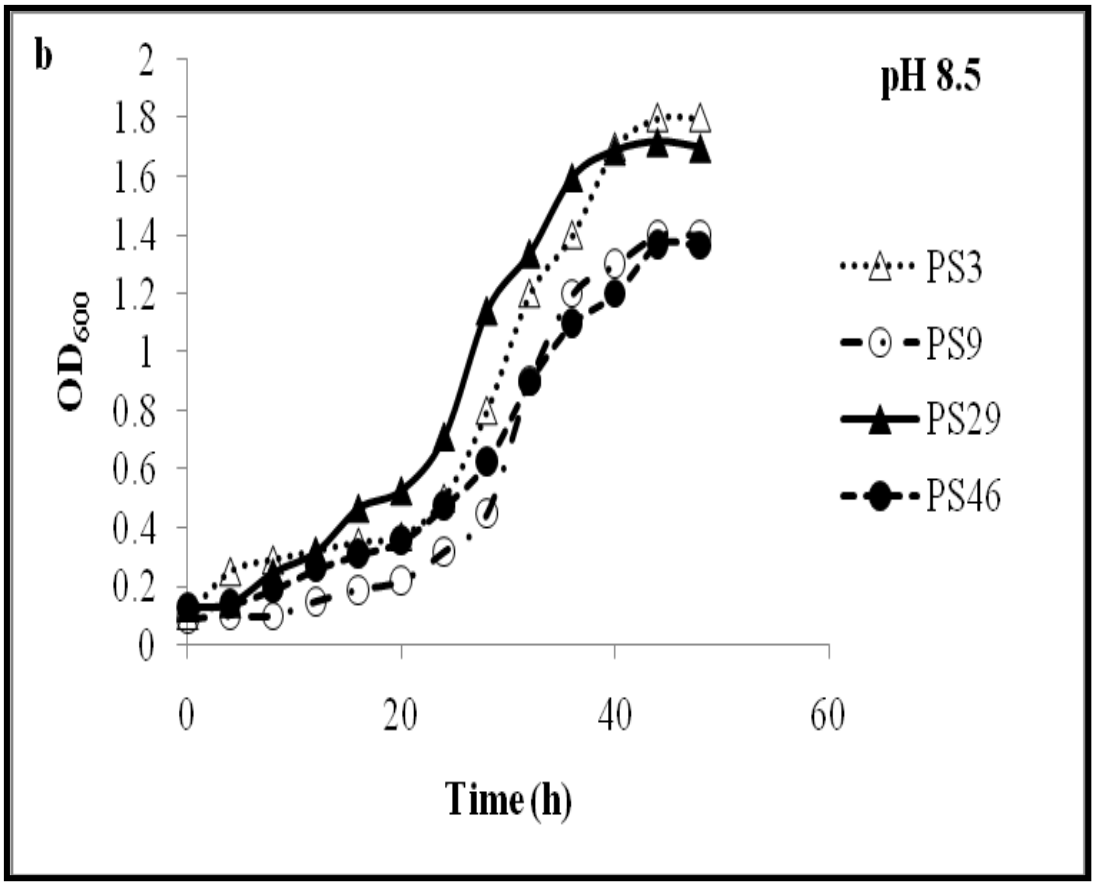

Figure 4.1 Growth studies of bacterial strains in MM63 medium with 6% (w/v) NaCl and containing glucose (0.5% w/v) as the carbon source (a) pH 7.1 (b) pH 8.5. Samples were withdrawn at 4 h interval for the determination of cell growth by OD_{600}.

The spectrum of compatible solutes present in the sample was determined by comparing the retention time with those of standards. The chromatogram for solute of strain PS6 after 48 h growth is shown in Fig. 4.2.

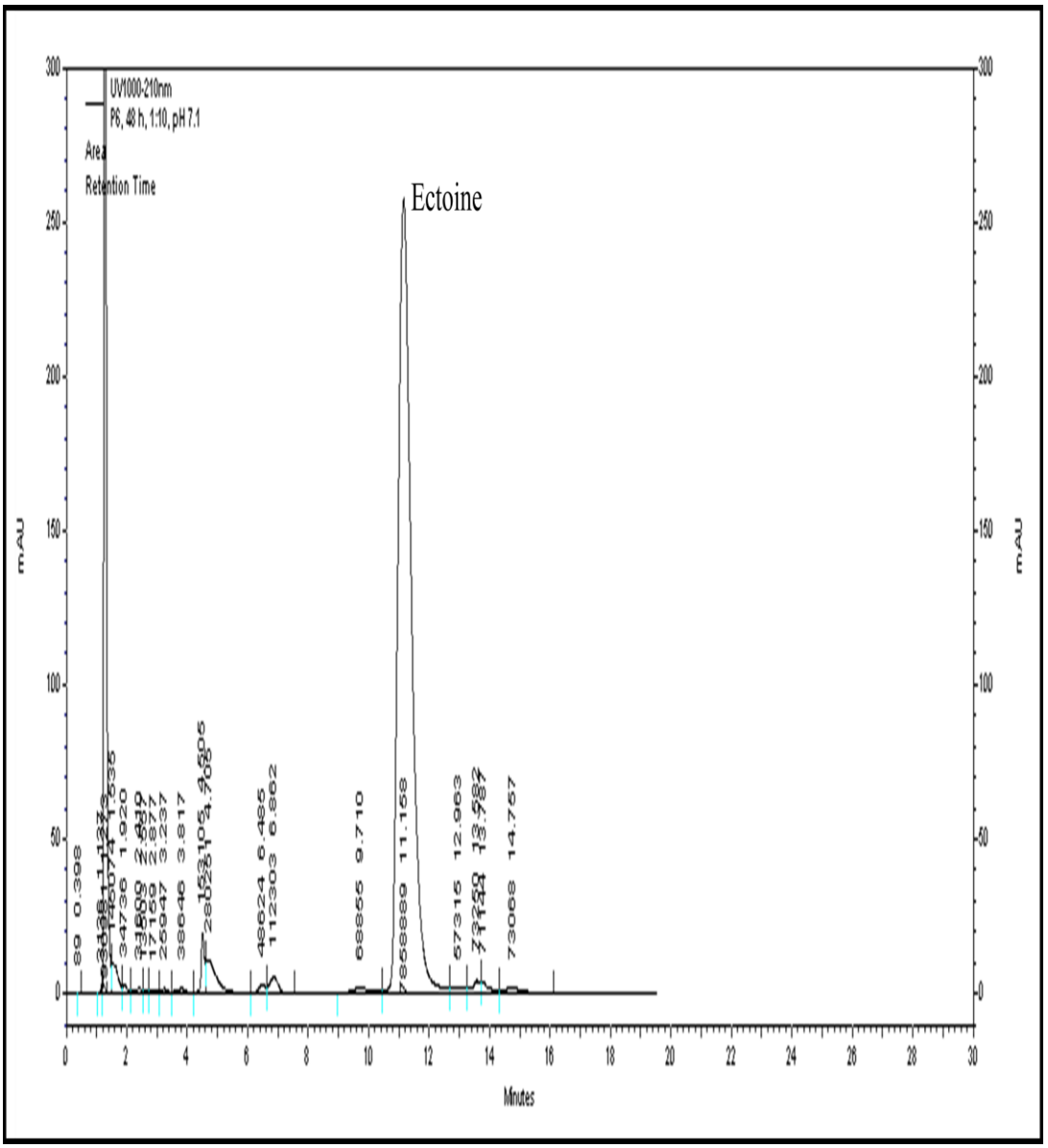

Figure 4.2 HPLC chromatogram of the aqueous extract of strain PS6, revealing a sharp peak of ectoine. Sample was monitored at 210 nm with mobile phase of acetonitrile (80% v/v) at 1 ml min^{-1}.

Ectoine content obtained from different isolates is shown in Fig. 4.3. PS30 was shown to produce highest amount of ectoine (75.2 mg/gcdw) while strain PS6 produced 71.3 mg/gcdw of ectoine. Presence of hydroxyectoine was also observed in most of the isolates. These isolates were further screened for ectoine production in biopolymer media.

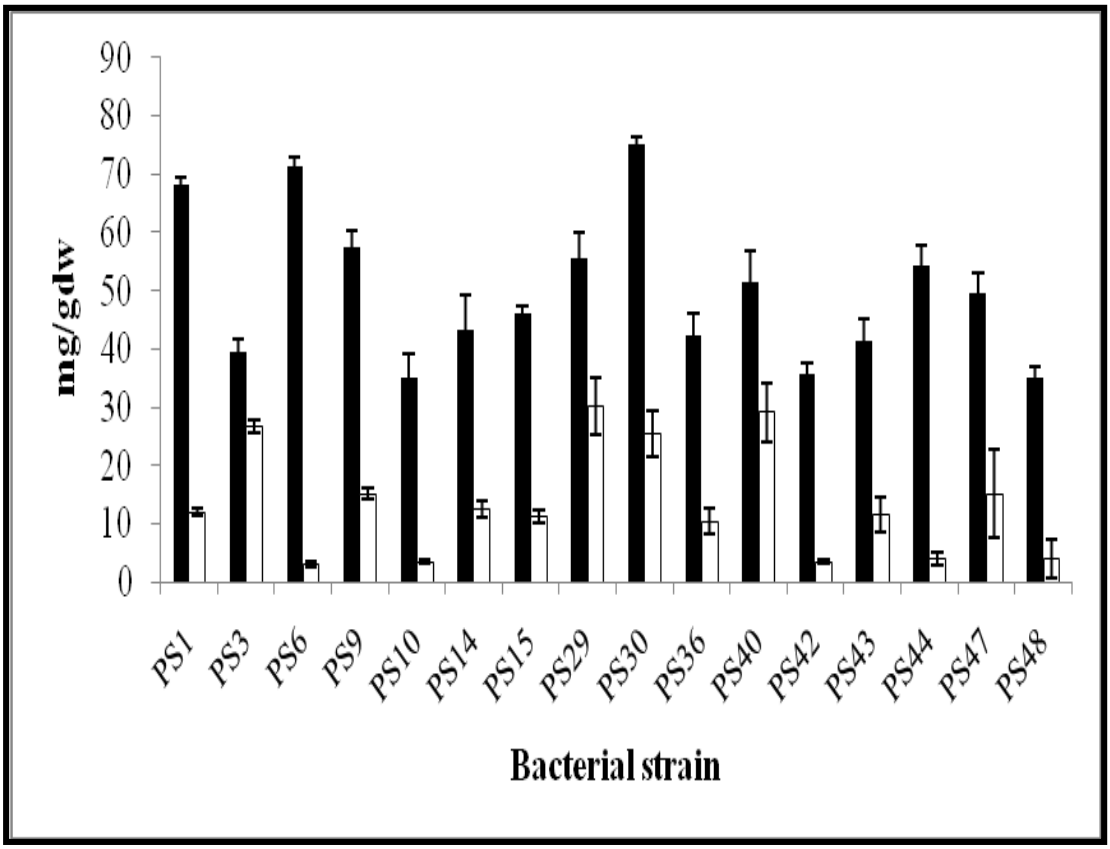

Figure 4.3 Ectoine content in different bacterial strains in MM63 (6% w/v NaCl) medium containing glucose (0.5% w/v) (■) Ectoine and (□) Hydroxyectoine.

4.2.2 Screening for growth and ectoine content on different biopolymers

Growth studies and synthesis of ectoines on different biopolymers are shown in the following figures – Xylan (Fig. 4.4 a and b) and CMC (Fig. 4.5 a and b). In addition, strain PS6 was able to grow and produce ectoine on inulin, chitin and carob as shown in Fig 4.6 (a and b).

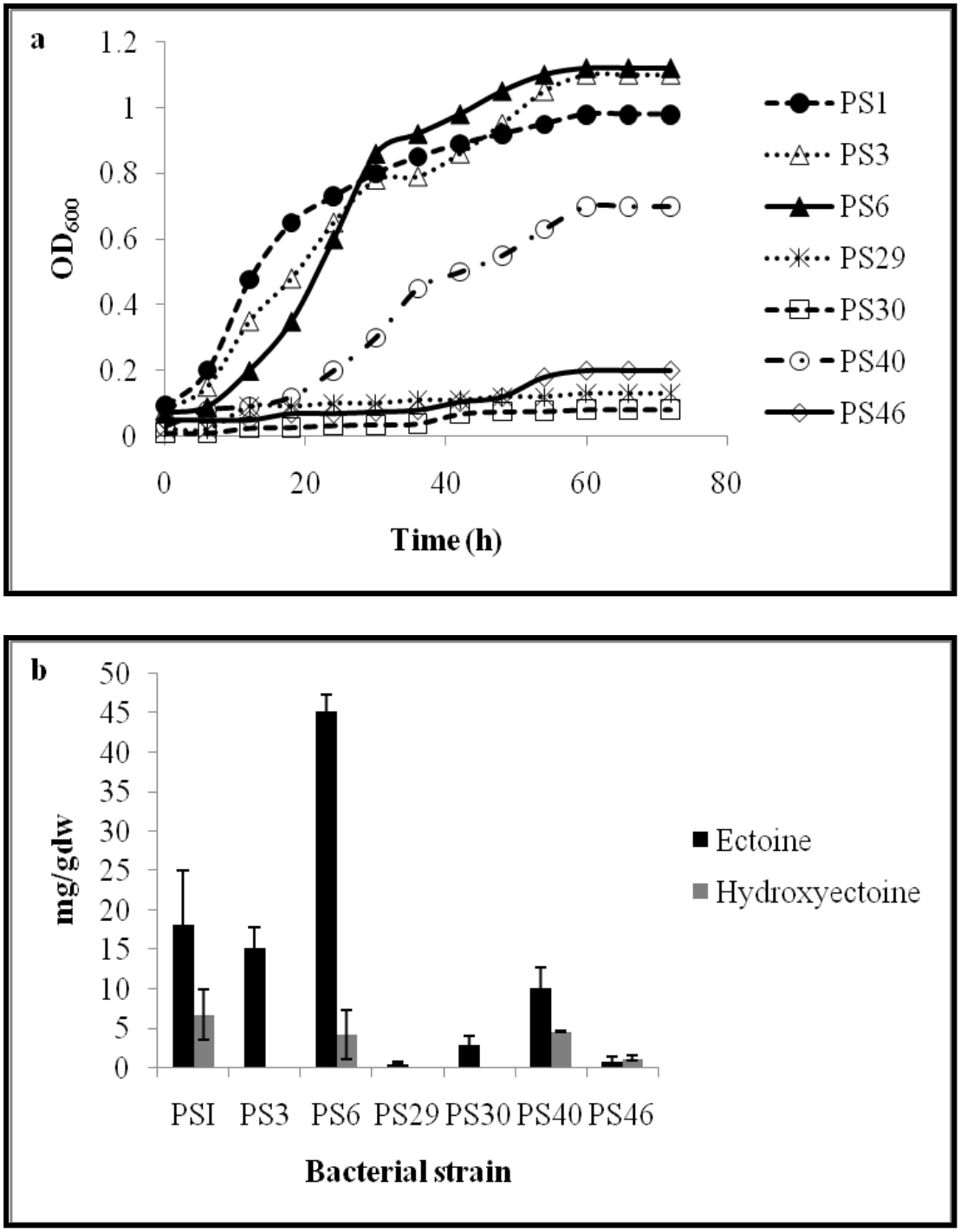

Figure 4.4 (a) Growth studies of bacterial strains in MM63 containing xylan (0.5% w/v). (b) Ectoine production on MM63-xylan medium after 48 h growth.

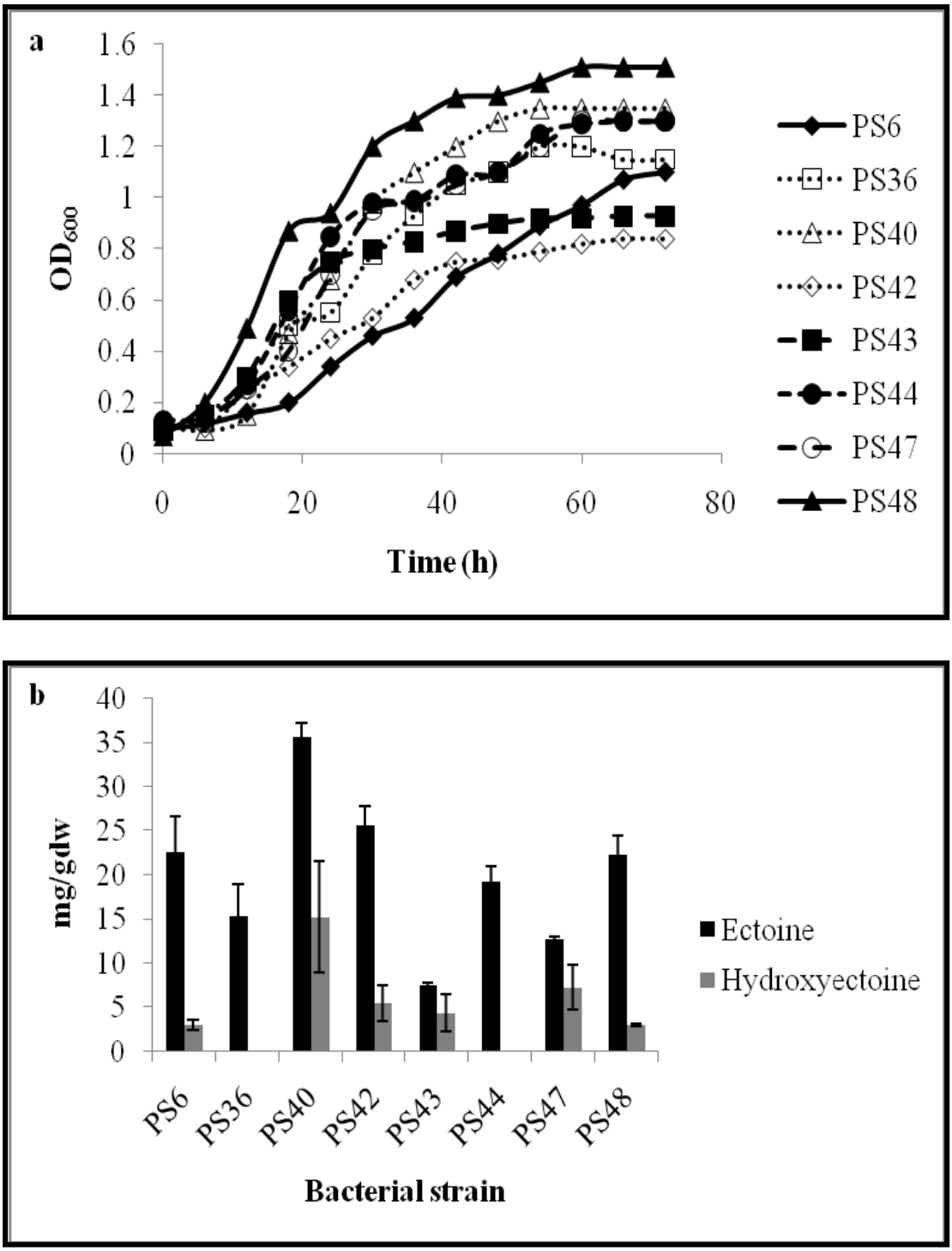

Figure 4.5 (a) Growth studies of bacterial strains in MM63 containing CMC (0.5% w/v). (b) Ectoine production on MM63-CMC medium after 48 h growth.

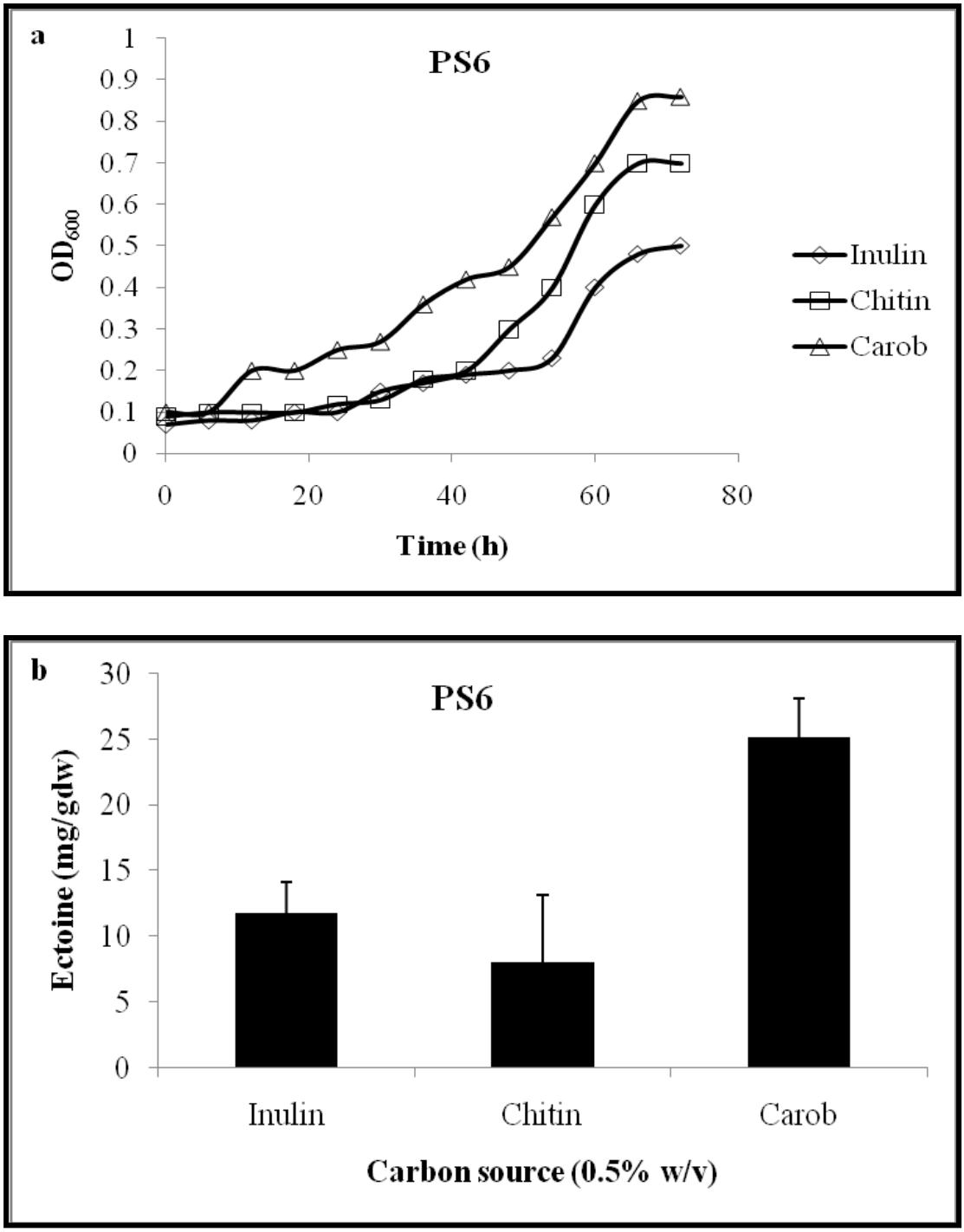

Figure 4.6 (a) Growth studies of strain PS6 in MM63 containing other biopolymeric substrates each at 0.5% (w/v) (b) Ectoine production of strain PS6 after 48 h growth.

Growth of the halophilic bacterial strains on biopolymers was seen to be much slower than on glucose. The yield of solutes, in addition, appears to be lower too. There is a possibility that that the biopolymers are only partially hydrolysed and the sedimented together with the cellular biomass, possibly leading to underestimation of ectoine content. There could also be some additional compounds other than ectoine which the microbes must be producing in the presence of solutes.

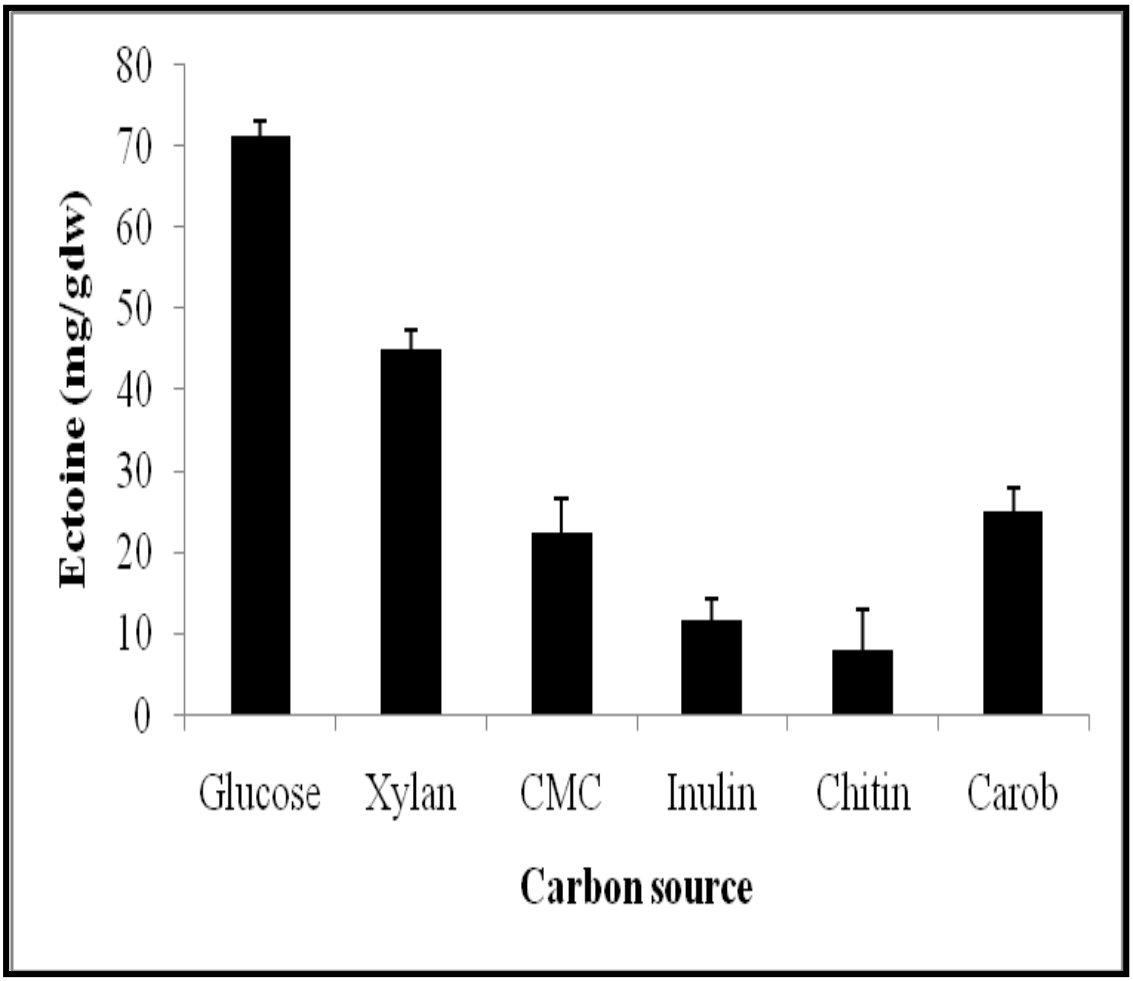

Figure 4.7 Ectoine production of *Halomonas* sp. PS6 in MM63 containing glucose and different biopolymeric substrates each at 0.5% (w/v) after 48 h growth.

Strain PS6 was able to grow and produce ectoine (mg/g dry weight basis) on glucose (71.3) and on the biopolymeric substrates xylan (45.2), CMC (22.6), inulin (11.8), chitin (8.1) and carob (22.7). Hence this strain was used for further studies on agro-residues. The strain was able to produce different enzymes like xylanase, cellulase, and chitinase. Due to the efficient enzyme machinery of biopolymer degradation, strain PS6 was able to grow and produce ectoine on a variety of biopolymers.

4.2.3 Solute analysis of strain PS6

The NMR spectrum confirmed the presence of ectoine (Fig. 4.8). In response to increasing salinity, the organism synthesized ectoine and smaller amounts of hydroxyectoine with traces of trehalose *de novo* as osmolytes. The bacterium may be capable of producing trehalose as an alternative compatible solute which can partly replace glycine betaine when the availability of nitrogen becomes growth limiting (Galinski et al. 1985).

It has been reported that depending on the duration of the osmotic stress, the level of salinity, the availability of substrates and osmolytes in the surroundings or the carbon source used for the growth medium, most bacteria use different solutes for osmotic balance (Roberts 2005; Pastor et al. 2010).

4.2.4 Characteristics of the potential strain

PS6 is a Gram negative rod-shaped bacterium, which formed pale orange-yellow colonies on the agar surface. The cells were motile short rods, predominantly occurring singly. The strain was isolated from Sanekatta saltern, Kumta, Karnataka.

The sequences show that PS6 has 99% identity with *Halomonas* sp. 2MN-1 (DQ659434). The nucleotide sequence (Fig. 4.9) of *Halomonas* sp. PS6 has been submitted to GenBank (KC295600). *Halomonas* sp. is known to be a good producer of the compatible solute ectoine (Sauer and Galinski 1998; Guzmán et al. 2009).

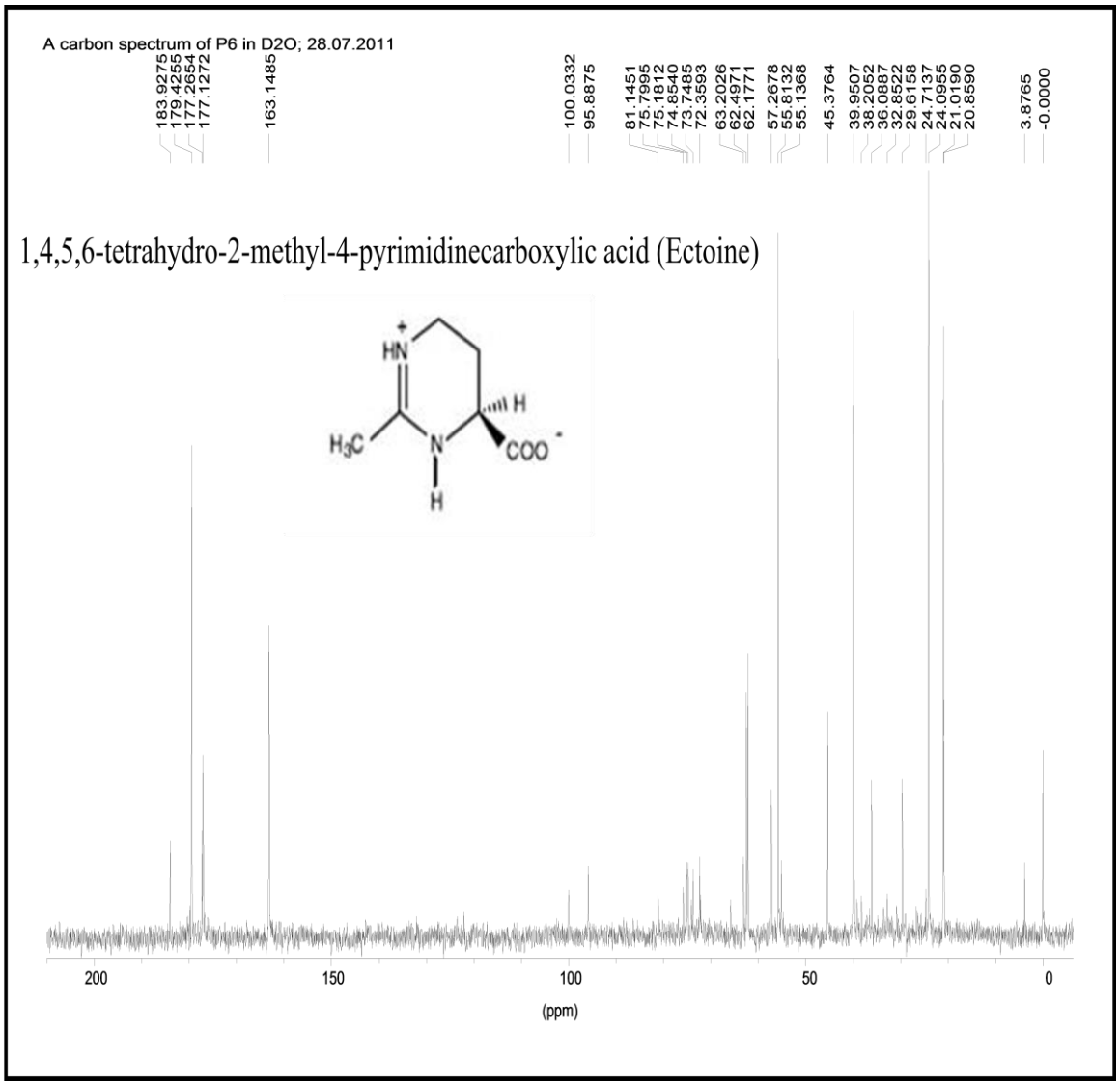

Figure 4.8 Natural abundance ^{13}C NMR spectrum of an aqueous extract from salt-stressed *Halomonas* sp. PS6 cells confirming the structure of ectoine.

NNNNNNNNGNNNNNNNCCTAACACATGCAAGTCGAGCGGCAGCACGGGGAGCTTGCTCCCTGGTGGCGAGCGGCGGACGGGTGAGTAATGCATAGG
AATCTGCCCGGTAGTGGGGGATAACGTGGGGAAACCCACGCTAATACCGCATACGTCCTACGGGAGAAAGCGGAGGATCTTCGGACTTCGCGCTATCGG
ATGAGCCTATGTCGGATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGA
CACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCTTTCGGGT
TGTAAAGCACTTTCAGCGAGGAAGAAAGCCTNTGGGTTAATACCCCAGAGGAAGGACATCACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCC
GCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGCTTGGCACGCCGGTTGTGAAAGCCCCGGGCTCAACCT
GGGAACGGCATCCGGAACGGCCAGGCTAGAGTGCAGGAGAGGAAGGTAGAATTCCCGGTGTAGCGGTGAAATGCGTAGAGATCGGGAGGAATACCAGT
GGCGAAGGCGGCCTTCTGGCCTGACACTGACACTGAGGTGCGAAAGCGTGGGTAGCNAACANGATTAGATACCCNGGNTAGTNCACGCCGTAAACGAT
GTCGACTAGCCGTTGGGACCTTTAAGGACTTAGTGGCGCAGTTAACGCGATAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAAT
TGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACCCTTGACATCCAGAGAAGTTGGCAGAGATGCC
TTCGTGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCT
TATTTGCCAGCGAGTAATGTCGGGAACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGGCCCTTACGGGTA
GGGCTACACACGTGCTACAATGGCCGGTACAAAGGGTTGCGAGACCGCGAGGTGGAGCGAATCCCAGAAAGCCGGCCTCAGTCCGGATCGGAGTCTGCA
ACTCGACTCCGTGAAGTCGGAATCGCTAGTAATCGTGAATCAGAATGTCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAG
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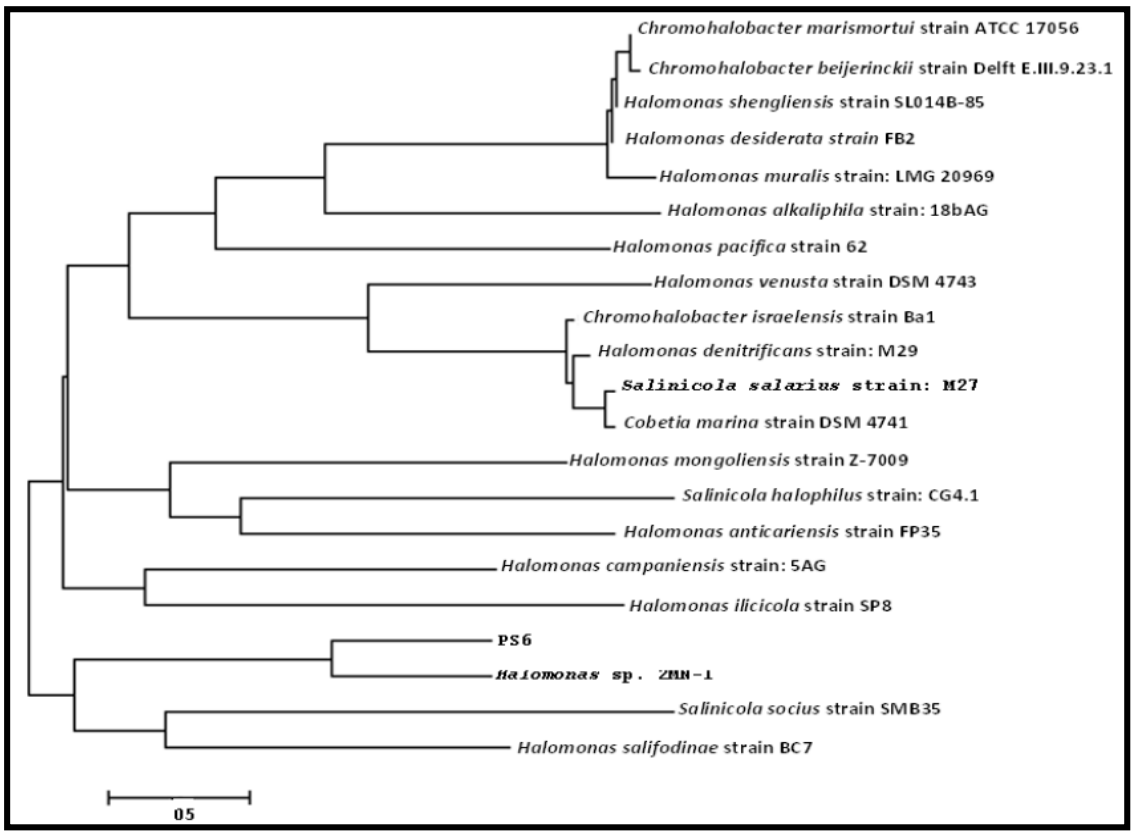


Figure 4.9 16S rRNA gene nucleotide sequence and phylogenetic tree of strain *Halomonas* sp. PS6 (GenBank ID: KC295600).

4.3 SUMMARY

Strain PS6 produced higher amount of ectoine (mg/gdw) - on glucose (71.3) and on a variety of biopolymeric substrates including xylan (45.2), CMC (22.6), inulin (11.8), chitin (8.1) and carob (22.7). The strain utilizes xylan as a favourite polymer showing good growth and better yield of ectoine content. This shows the ability of the halophilic bacterium to utilize *bio-polymers* in the *synthetic medium* for growth and ectoine production. The structure of ectoine was confirmed by HPLC and NMR analysis. The strain was identified as Halomonas sp. 16S rRNA gene sequence analysis and the sequence was submitted to GenBank (ID: KC295600). The study introduces newly isolated halophilic bacterium *Halomonas* sp. PS6, as a potential candidate for producing ectoine using biopolymers and agro-residues.

Chapter 5

Production of ectoine from *Halomonas* sp. PS6 using agricultural resources

5.1 MATERIALS AND METHODS

5.1.1 Screening for ectoine production on different agricultural residues

Halomonas sp. PS6 was grown in MM63 with the following agricultural residues as sole carbon source (each at 1% w/v) – wheat bran, rice bran, corn cobs, sugarcane bagasse, groundnut shells and coir pith. Agro-residues were used both in treated and untreated form. The pretreatment of agricultural residues was carried out as described previously. Ectoine content on these substrates used as medium components was investigated.

5.1.2 Effect of salt concentration on ectoine content

The effect of salt on ectoine content was studied by varying the salt concentration from 3-18% (w/v) in MM63 medium containing the suitable agro-residue as carbon source. Growth and ectoine content of *Halomonas* sp. PS6 was monitored every 6 h, at 30 °C and 150 rpm.

5.1.3 Effect of temperature on ectoine content

Influence of temperature on ectoine content was investigated by varying the medium temperature from 25 °C to 50 °C at pH 7.1, keeping the other parameters constant.

5.1.4 Selection of key determinants for medium optimization

With the results of the preliminary investigations into consideration, rice bran, NaCl and temperature were seen to have significant effect on ectoine production. In order to obtain the optimal concentration of these medium variables leading to maximum ectoine content, response surface methodology was used. The statistical

software 'Design-Expert® Version 8.0.4', Stat-Ease, Inc., (Minneapolis, MN, USA) was used to conduct the experimental design.

5.1.5 Optimisation of ectoine production by response surface methodology

Central composite design was adopted for improving the ectoine content. A total of 20 experiments were conducted. Ectoine content was taken as the response. The trials were run in triplicates and the average value of ectoine content in each trial was reported as the mean observed response. All the variables were taken at a central coded value, which was considered as zero. The range of variables investigated at five levels –α -1, 0, +1 and + α are listed in Table 5.1.

Table 5.1 Experimental range and levels of the three independent variables influencing ectoine content in terms of actual and coded factors.

Variables	**Symbol**	**Range of levels**				
		-α	**-1**	**0**	**+1**	**+α**
Rice bran (g/l)	A	19.8	30	45	60	70.2
NaCl (g/l)	B	79.7	90	105	120	130.2
Temperature (ºC)	C	26.6	30	35	40	43.4

5.1.6 Statistical analysis and modelling

Upon completion of the experiments, the average maximum ectoine content was taken as the response (Y). The data obtained from RSM were subjected to analysis of variance (ANOVA) to evaluate the adequacy of fit. A second order polynomial model was fitted to the response data obtained from the design by the multiple regression procedure. This resulted in an empirical model that related the response to the independent variables of the experiment.

The polynomial equation for a three-variable system is in the following form:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC \text{ ..... } (5.1)$$

With Y, response; β_0, intercept; β_1, β_2, β_3, linear coefficients; β_{11}, β_{22}, β_{33}, squared coefficients and β_{12}, β_{13}, β_{23}, interaction coefficients. The statistical significance of the model equation and model terms was evaluated via the Fisher's test. The quality of fit of the second-order polynomial model equation was expressed via the coefficient of determination (R^2) and the adjusted R^2. The three-dimensional response surface curves were then plotted to understand the interaction of the medium components and the optimum concentration of each component required for maximum ectoine production.

5.2 RESULTS AND DISCUSSION

5.2.1 Ectoine production from *Halomonas* sp. PS6 on different agricultural residues

Among the different agricultural residues tested as medium components, *Halomonas* sp. PS6 was seen to grow well and yield higher ectoine content of 60.2 mg/gcdw on rice bran (Fig. 5.1 a and b). Wheat bran and sugarcane bagasse also yielded substantial amounts of ectoine (36.2 mg/gdw and 34.7 mg/gdw). Lower ectoine amounts of 25.5 mg/gdw, 18.3 mg/gdw and 14.1 mg/gdw were respectively detected when corn cobs, groundnut shells and coir pith were used individually as carbon source. Substantial amount of ectoine was also detected on un-treated agro-residues.

5.2.2 Effect of salt concentration and temperature on ectoine content

Increase in ectoine content was seen with increase in concentration of NaCl and temperature. This could be the result of stress encountered due to increase in salinity of the medium. Higher ectoine was seen at 12% w/v NaCl (85.4 mg/gdw) as shown in Fig. 5.2a and temperature of 37 ºC (70.5 mg/gdw) as shown in Fig 5.2b.

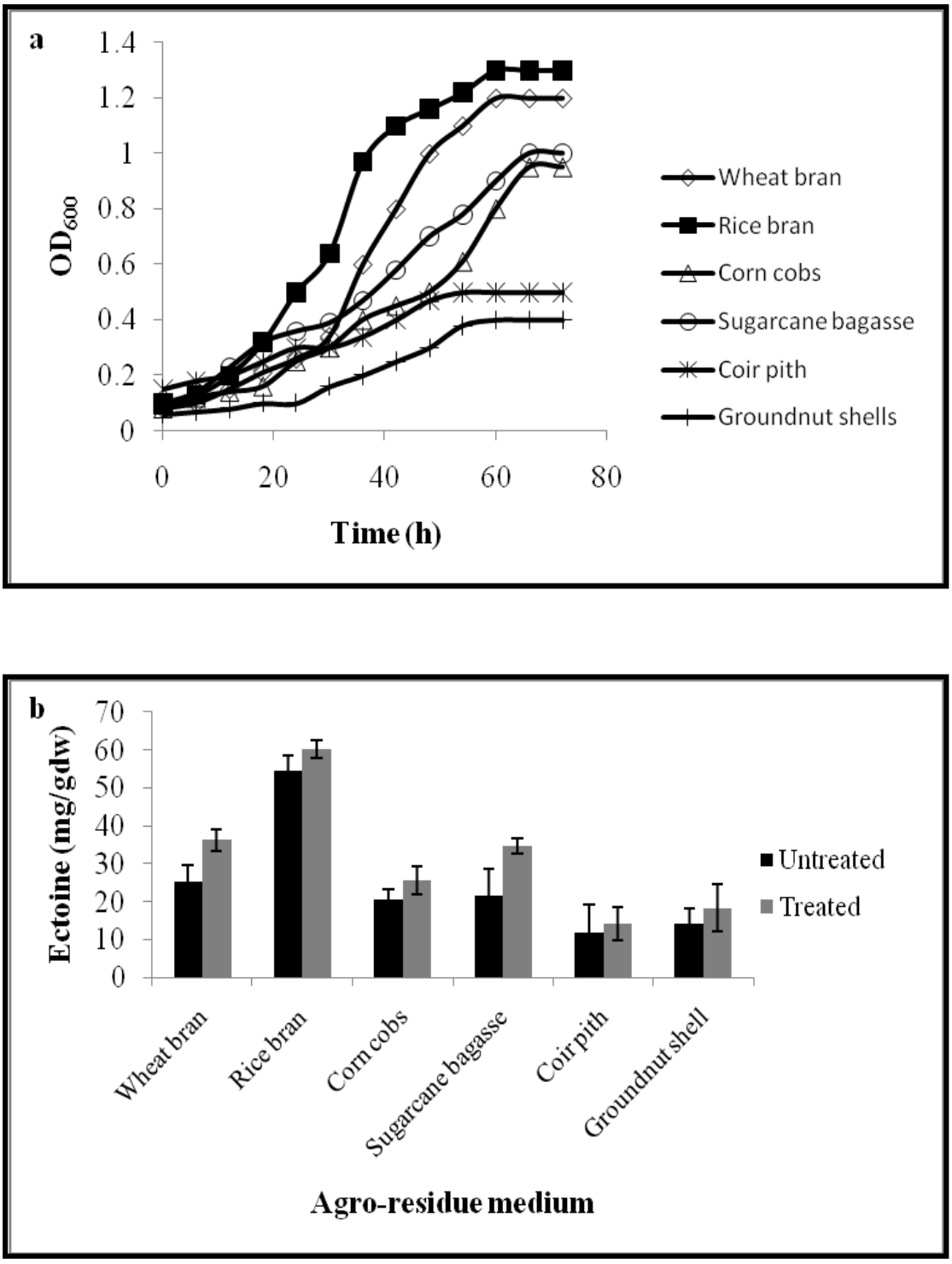
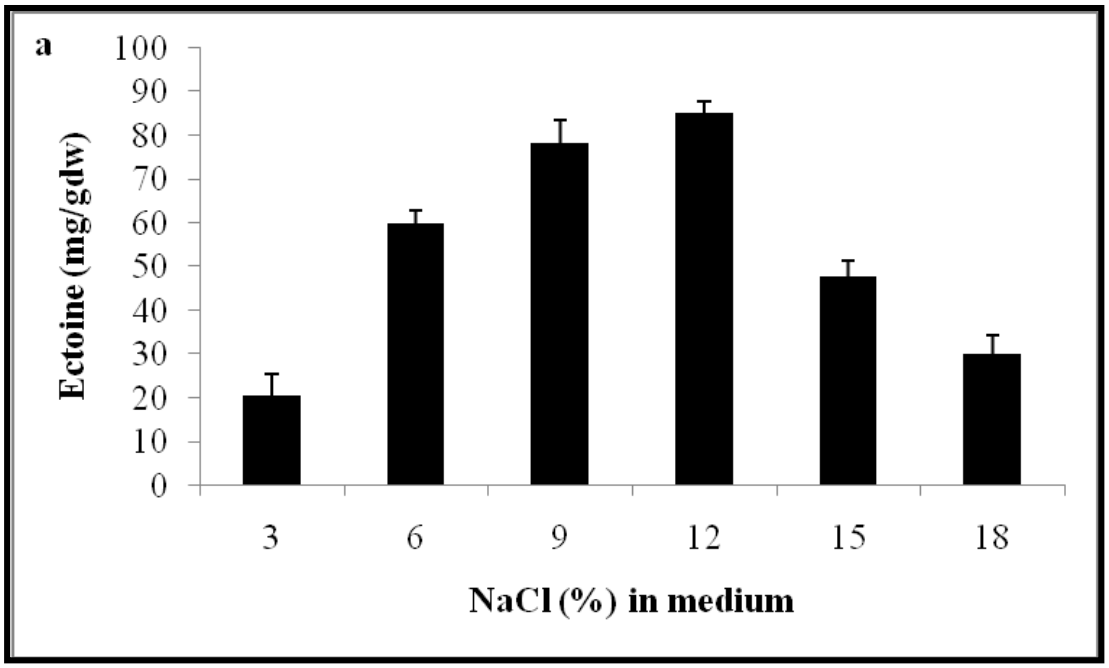


Figure 5.1 (a) Growth studies of *Halomonas* sp. PS6 in MM63 containing agro-residues each at 1% w/v (b) Ectoine content in MM63 containing agro-residue as carbon source. Alkaline pretreatment (1% w/v NaOH) of agro-residues was carried out.



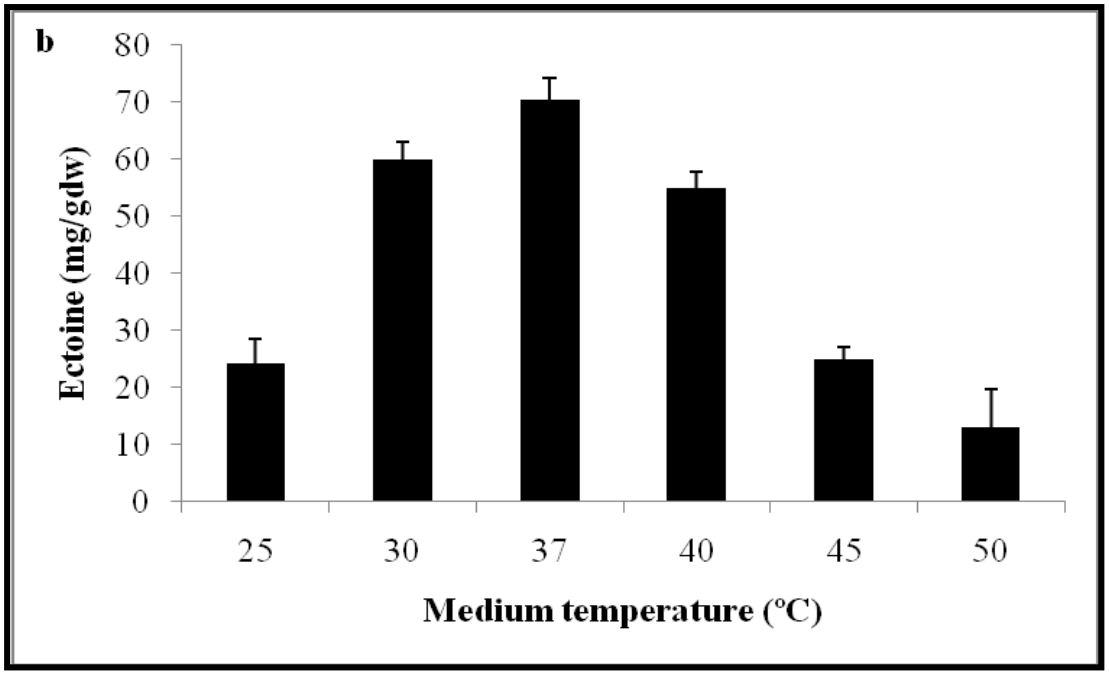


Figure 5.2 (a) Effect of NaCl concentration on ectoine content in *Halomonas* sp. PS6 grown in MM63 at 30 ºC containing rice bran (1% w/v) as carbon source (b) Effect of temperature on ectoine content by *Halomonas* sp. PS6 at 6% (w/v) NaCl.

5.2.3 Optimization of significant variables using response surface methodology

Table 5.2 Central composite design using the three independent variables showing observed and predicted responses for ectoine content

Run order	Factors			Ectoine (mg/gdw)	
	A (Rice bran g/l)	**B (NaCl g/l)**	**C (Temperature ºC)**	**Mean Observed Response**	**Predicted Response**
1	45	105	35	106.5	109.3
2	45	105	26.6	73.7	75.5
3	30	90	40	79.5	83.8
4	60	90	40	77.8	74.2
5	45	105	35	107.4	109.3
6	30	90	30	91.7	89.2
7	60	120	30	93.7	90.3
8	45	105	35	110.1	109.3
9	60	120	40	110.6	112.9
10	45	105	43.4	92.7	90.1
11	45	79.7	35	73.5	73.2
12	45	130.2	35	91.7	92.1
13	45	105	35	110.2	109.3
14	30	120	30	70.3	72.9
15	60	90	30	72.3	74.4
16	70.2	105	35	101.4	99.9
17	30	120	40	92.74	90.5
18	19.8	105	35	89.74	93.4
19	45	105	35	107.9	109.3
20	45	105	35	109.5	109.3

The results of the twenty-trial, central composite design for studying the effects of the three independent variables, viz., rice bran, NaCl and temperature on ectoine production are presented in Table 5.2 along with the predicted and mean observed responses.

The data obtained from RSM on ectoine production were subjected to analysis of variance (ANOVA) as shown in Table 5.3. The results were further analysed to estimate the aptness and applicability of the model for ectoine production.

The regression equation indicated the R^2 (determination coefficient) value of 0. 0.9807 (a value >0.75 indicates fitness of the model), which ensured a satisfactory adjustment of the quadratic model to the experimental data and indicated that 98.07% of the variability in the response could be explained by the model. The 'adjusted R^2' is 0.9724 and the 'predicted R^2' is 0.9361, which are in reasonable agreement. This indicates a high significance of the model (for a good statistical model, the R^2 value should be in the range of 0-1.0, and nearer to 1.0 the value is, the more fit the model is deemed to be (Khuri and Cornell 1987).

The 'adequate precision value' of 29.4 indicates an adequate signal and that the model can be used to navigate the design space as it measures the signal-to-noise ratio (values higher than 4 are essential prerequisites for a model to be a good fit). At the same time, a relatively lower value of the coefficient of variation (CV%: 2.06) indicates improved precision and reliability of the conducted experiments (Box et al. 1987).

The high *F*-value (118.67) and low *p*-value (<0.0001) indicated that the obtained experimental data was a good fit with the model (Akhnazarova and Kafarov 1982). The coefficients of the regression equation were calculated using Design Expert® and the data was fitted to a second order polynomial equation. Ectoine production by *Halomonas* sp. PS6 can be expressed in terms of the following regression equation:

$Y = +109.4 +1.93A +5.6B +4.3 +8AB +1.27AC +5.7BC -4.5A^2 -9.4B^2 - 9.4C^2$.....(5.2)

with, Y, ectoine content (response); A, rice bran; B, NaCl and C, temperature. ANOVA confirms a satisfactory adjustment of the reduced quadratic model to the experimental data.

Table 5.3 Analysis of variance for the response surface quadratic equation on ectoine production in *Halomonas* sp. PS6

Term	**F-value**	**p-value**
Model	118.6711	< 0.0001 (Significant)
A-Rice bran	13.71007	0.0041
B-NaCl	116.2306	< 0.0001
C-Temperature	68.74838	< 0.0001
AB	139.0574	< 0.0001
AC	3.486616	0.0914
BC	71.02866	< 0.0001
A^2	77.97657	< 0.0001
B^2	344.8122	< 0.0001
C^2	342.4447	< 0.0001
Lack of Fit	4.36	0.0654

5.2.4 Response surface plots

The three-dimensional response surface curves and contour plots were then constructed to understand the interaction effects and optimum levels of the variables. This is done by plotting the response (ectoine content) on the Z-axis against any two independent variables, while maintaining the other variables at fixed levels (zero, for instance).

Fig. 5.3a shows the response surface plot obtained as function of rice bran *vs.* NaCl, while temperature was maintained at zero level. Higher amount of ectoine was observed at rice bran 45 g/l and NaCl 105 g/l. Fig. 5.3b shows the response surface plot obtained as function of NaCl *vs.* temperature, while rice bran was maintained at zero level. Higher cellulase activity was observed at NaCl 105 g/l and temperature 35 °C. Fig. 5.3c shows the response surface plot obtained as function of rice bran *vs.* temperature, while NaCl was maintained at zero level. An increase in ectoine production was observed at rice bran 45 g/l and temperature 35 °C. The response surfaces had a curvature indicating existence of optimum point. The shapes of the contour plots indicate the nature and extent of the interactions. The graphs reveal that the optimal values of variables lie within the ranges chosen initially. These plots are helpful in studying the effects of the factor variation in the studied field and consequently, in determining the optimal experimental conditions.

5.2.5 Validation of the model

The model predicted a maximum ectoine production of 113.6 mg/gdw at rice bran 50 g/l, NaCl 110 g/l and temperature 37 ºC. Trials were conducted in triplicates at this medium composition. Ectoine content of 112.3 mg/gdw was observed at this optimized composition with a validity of 98%. Two-fold increase in ectoine content was observed by optimizing the levels of rice bran, NaCl and temperature as compared to that in the unoptimised medium.

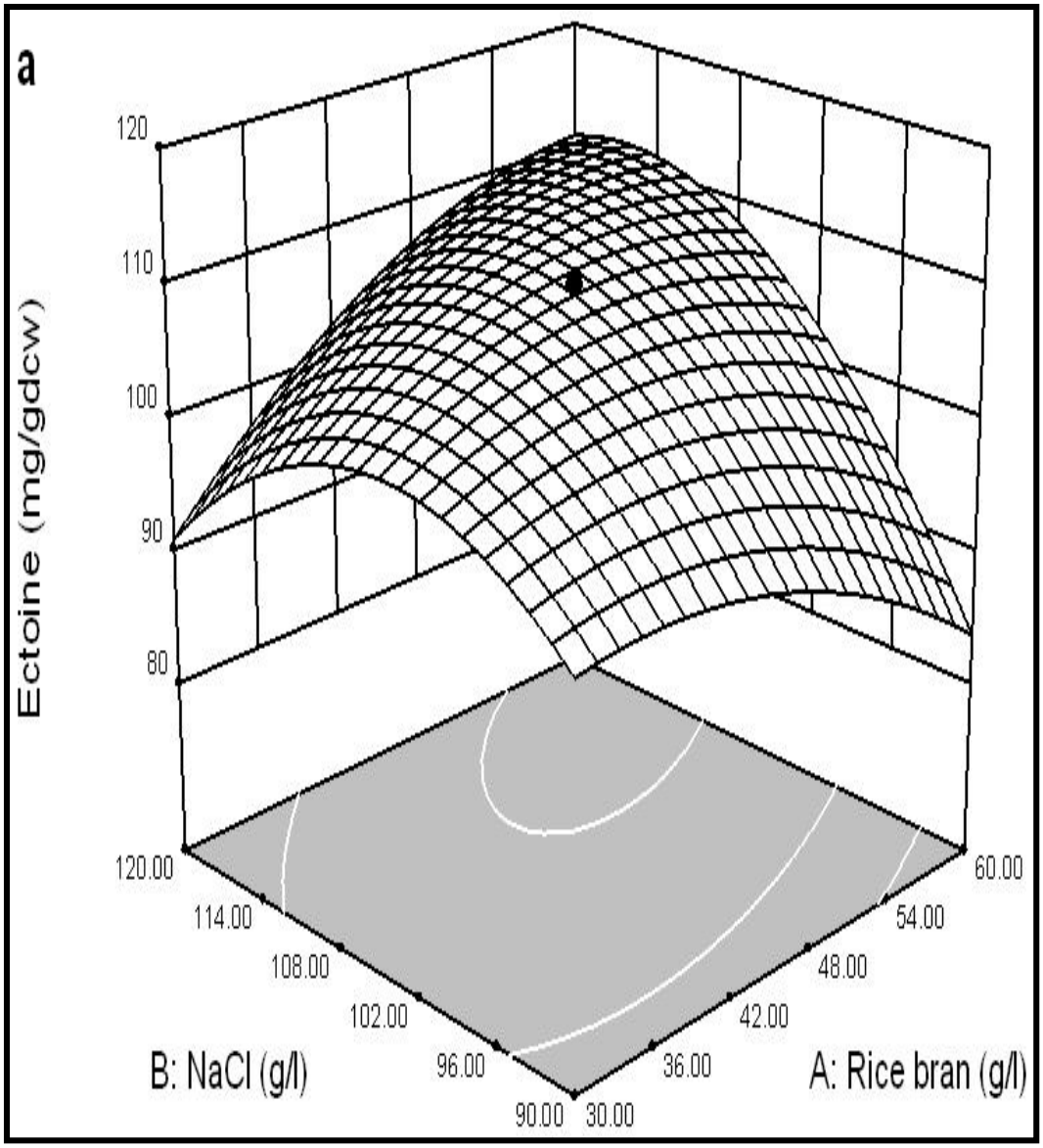
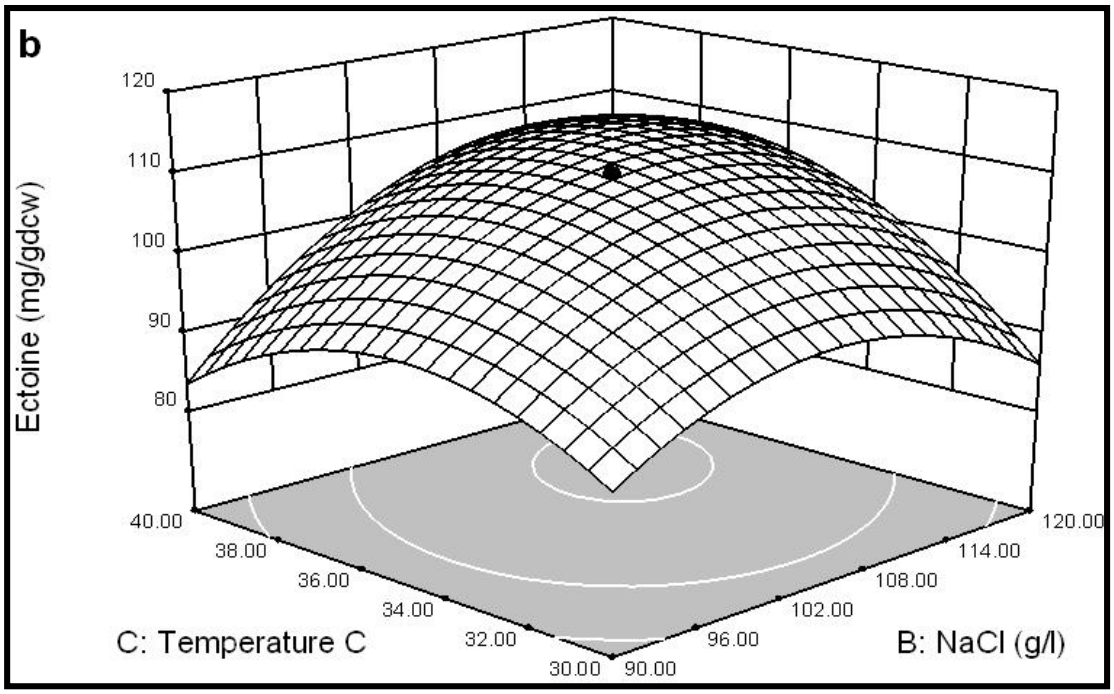
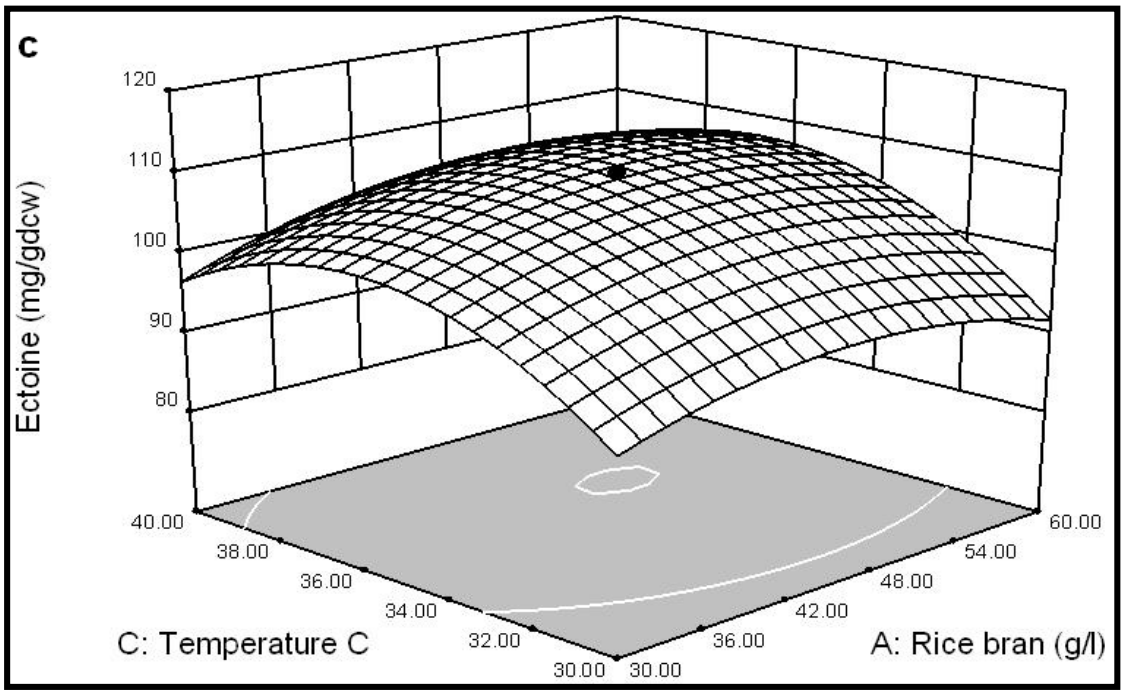


Figure 5.3 Response surface curves of ectoine content from *Halomonas* sp. PS6 showing interaction between (a) rice bran vs. NaCl



(b) NaCl vs. temperature



(c) rice bran vs. temperature.

5.3 SUMMARY

Strain PS6 produced substantial amounts of ectoine on treated agro-residues (mg/gdw) - rice bran (60.2), wheat bran (36.2), sugarcane bagasse (34.7), corn cobs (25.5), groundnut shells (18.3) and coir pith (14.1). Higher amount of ectoine was seen at increased NaCl concentration (85.4 mg/gdw ectoine at 12% w/v NaCl) and higher temperature (70.5 mg/gdw ectoine at 37 ºC). Higher amount of ectoine was also detected on un-treated agro-residues. Strain PS6 has an efficient machinery of biopolymer-degrading enzymes. This is significant from industrial viewpoint since pretreatment of agro-wastes, a costly step, can be avoided. As observed by the experimental results, the growth of microorganisms in medium containing agricultural residues as sole carbon source is not as high as compared to those on pure carbon sources like glucose. This has subsequently decreased the amount of ectoine produced. Secondly, the 'Bacterial milking' process is a controlled production process carried out in a fermentor, using glucose as carbon source, where the mixing of nutrients is more homogeneous than using agro-residues in shake-flasks which would have contributed to comparatively lower yields. Using response surface methodology studies, a maximum ectoine content of 113.6 mg/gdw at rice bran 50 g/l, NaCl 110 g/l and temperature 37 ºC was predicted. Ectoine content of 112.3 mg/gdw was observed with validity of 98% and two-fold increase over the unoptimised medium.

PART TWO

XYLANASE PRODUCTION

Chapter 6

Screening and production of halostable xylanase

6.1 MATERIALS AND METHODS

6.1.1 Screening of xylanase producing bacteria

The strains on MM63 containing xylan as the sole carbon source (PS1-PS16) were screened for extracellular xylanolytic activity. After incubation of the plates at 30 °C for 48 h., zones of hydrolysis were visualized by staining the plates with aqueous solution of 0.1% (w/v) Congo red for 15 min, and then destained with 1 M NaCl (Teather and Wood 1982).

Morphological and physiological characteristics of the potential strain were studied. The strain was further identified by 16S rRNA gene amplification and nucleotide sequencing.

6.1.2 Inoculum preparation and enzyme assay

A 1% inoculum from an overnight grown culture in log phase was added to 100 ml MM63 medium taken in 250 ml Erlenmeyer flasks. After incubation for 48 h, at 30 °C, under shaking condition of 150 rpm, the culture was harvested and growth was measured as OD_{600}, spectrophotometrically. The cultures were centrifuged at 10,000 rpm for 10 min at 4 °C. The cell free extract was used as crude preparation to measure xylanase activity.

For enzyme assay xylan was used as the substrate. Enzyme activity was determined by measuring the release of reducing sugars during the enzyme substrate reaction using dinitrosalicylic acid method (Ghose 1987; Miller 1959). The values were determined from xylose standard curve (Appendix VI). One unit (IU) of activity was defined as the amount of enzyme required to liberate 1 μmol of xylose per minute under given assay conditions.

6.1.3 Xylanase production in MM63 medium

Xylanase secretion was investigated in MM63 medium by replacing the carbon source glucose with xylan and nitrogen source $(NH_4)_2SO_4$ with organic sources like yeast extract and peptone. Different agro-residues like wheat bran, rice bran, corn cobs, sugarcane bagasse, groundnut shells, coir pith and sunflower seeds were checked for the source of carbon in the medium at 1% (w/v) for xylanase production. The culture was grown at pH 8.5, 30 ℃ at 150 rpm for 48 h before estimation of enzyme activity. Xylanase activity was checked in both treated and untreated agro-residue media. The activity was also checked in media supplemented with yeast extract and peptone as nitrogen sources.

6.1.4 Growth and xylanase production in media with different salt concentration

The effect of salt on growth and xylanase secretion was studied by varying the NaCl concentration from 0 – 15% (w/v) in the wheat bran medium. The initial pH of the medium was adjusted to 8.5. Growth and xylanase activity of PS3 were monitored every 4 h, at 30 °C and 150 rpm for a period of 72 h.

6.1.5 Effect of pH and temperature of the medium for xylanase production

In order to investigate the influence of pH on growth and xylanase production, the isolate, PS3 was grown in wheat bran medium containing 6% (w/v) NaCl at different pH (3.0 – 13.0) and constant temperature of 30 °C. After 48 h, xylanase activity was quantified. Similarly, influence of temperature was investigated by varying the growth temperature (25 – 50 °C) at optimum pH, keeping the other parameters constant.

6.1.6 Properties of the extracellular xylanase

The optimum pH for xylanase activity was determined with xylan as substrate dissolved in the following buffer systems: potassium phosphate buffer (pH 6.0-8.0) and glycine-NaOH buffer (pH 9.0-12.0). The optimum temperature was determined for the xylanase at different temperatures (10-70 °C). For the study of halostability,

the enzyme was pre-incubated with NaCl (0-4 M) at 30 °C for 1 h and the enzyme activity was determined.

6.2 RESULTS AND DISCUSSION

6.2.1 Selection of the xylanase-producing bacterium

Moderately halophilic bacteria were isolated from the coastal regions and salt marsh ecosystems of west coast of Karnataka, India, on MM63 medium containing 6% (w/v) NaCl and xylan as the sole source of carbon. Bacteria growing on this biopolymer medium were expected to harbor the potential enzyme machinery to degrade xylan and utilize the polymer for growth and multiplication.

Based on colony characteristics sixteen different organisms (PS1-PS16, numbered serially) were isolated, cultured aerobically in the same medium at 30 °C and maintained as pure cultures in agar stabs and glycerol stock. Out of these, six strains showed prominent zone of clearance on the xylan agar indicating extracellular xylanolytic activity. The following enzyme activities were recorded (per ml): PS1 (0.95 U), PS3 (1.4 U), PS6 (0.62), PS8 (0.65), PS9 (0.83) and PS13 (1.1 U). Therefore, based on the highest enzyme activity, PS3 was selected for further investigation (Fig. 6.1). The strain was isolated from Panambur beach area in Mangalore region, Karnataka, India.

6.2.2 Characteristics of the potential strain

Strain PS3 is a Gram-positive, coccoid shaped aerobic bacterium which formed small circular, convex, pale yellow-coloured colonies on the agar surface. Cells were non-motile and did not form endospores. Growth occurred with 0.5-18 % (w/v) NaCl (optimally at 9%, w/v), at pH 7.5-10.0 (optimally at pH 8.5) with an optimal growth temperature of 30 °C. On the basis of 16S rRNA gene analysis and morphological studies, strain PS3 has been identified as *Brachybacterium* sp. (Fig. 6.2). The ribosomal RNA gene sequence has been submitted to GenBank (ID: JQ425852). *Brachybacterium* sp. strain PS3 is a relatively novel marine bacterium. The study of growth kinetics of *Brachybacterium* sp. strain PS3 with reference to

xylanase production in MM63 medium indicated that the lag phase of the organism was small up to 3 h with an extended exponential phase up to 56 h followed by stationary phase. Xylanase secretion corresponded with growth and was maximal in the late exponential phase (30 – 60 h) with the highest activity recorded at 42 - 50 h (4.2 U/ml). Zone of clearance in the screen-plate and optimum levels of xylanase secretion in the basal MM63 medium indicate xylanolytic activity as an inherent property of *Brachybacterium* sp. strain PS3.

Figure 6.1 Congo Red dye-stained plate of strain PS3, containing xylan as the sole carbon source. Zone of clearance around the colonies indicate xylanase activity.

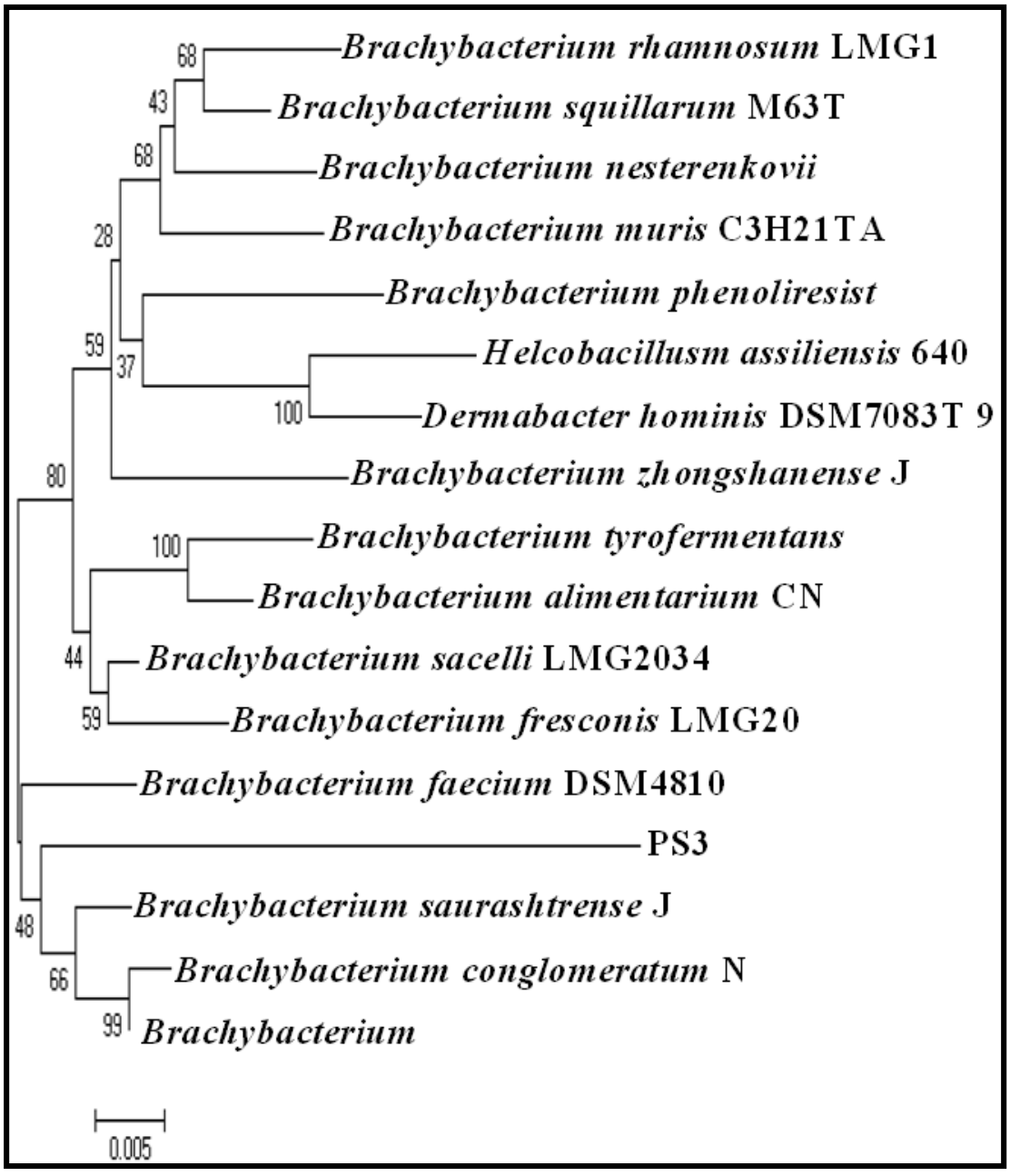


Figure 6.2 Neighbour-joining tree showing the position of isolate *Brachybacterium* sp. PS3 to a selected number of members of halophilic bacteria.

6.2.3 Xylanase production in MM63 medium

Xylanase production was investigated in the basal MM63 medium in the presence of xylan, yeast extract and peptone (Fig. 6.3a). The enzyme activity increased to 1.8 U/ml when the medium containing glucose, was supplemented with complex nitrogen sources like yeast extract and peptone in place of inorganic nitrogen source $(NH_4)_2SO_4$.

When xylan was used as carbon source instead of glucose and $(NH_4)_2SO_4$ as the nitrogen source in the basal medium the enzyme activity was found to be 3.5 U/ml, 2.5 times the original value.

The xylanase biosynthesis is reported to be induced by its substrate xylan (Kulkarni et al., 1999; Subramanyan and Prema 2002). However, only yeast extract and peptone in the absence of any other carbon source failed to induce substantial enzyme production (0.36 U/ml). The highest activity (4.2 U/ml) was seen when glucose in MM63 medium was replaced by xylan and $(NH_4)_2SO_4$ was replaced by a combination of yeast extract and peptone as the organic nitrogen sources. The activity was 3 fold higher than the original value.

The enzyme secretion corresponded with growth and was higher in late exponential phases of growth (Fig. 6.3b). This shows that xylan acts as an inducer while yeast extract and peptone enhance the xylanase enzyme production by *Brachybacterium* sp. PS3.

Yeast extract and peptone have been reported to have a significant effect on xylanase production. Yeast extract is known to be an effective medium component for the growth of halophilic bacteria (Shivanand and Jayaraman 2009). Hence these organic nitrogen sources stimulate the buildup of bacterial biomass and subsequently the bacterium can synthesize the sufficient enzyme machinery to degrade xylan.

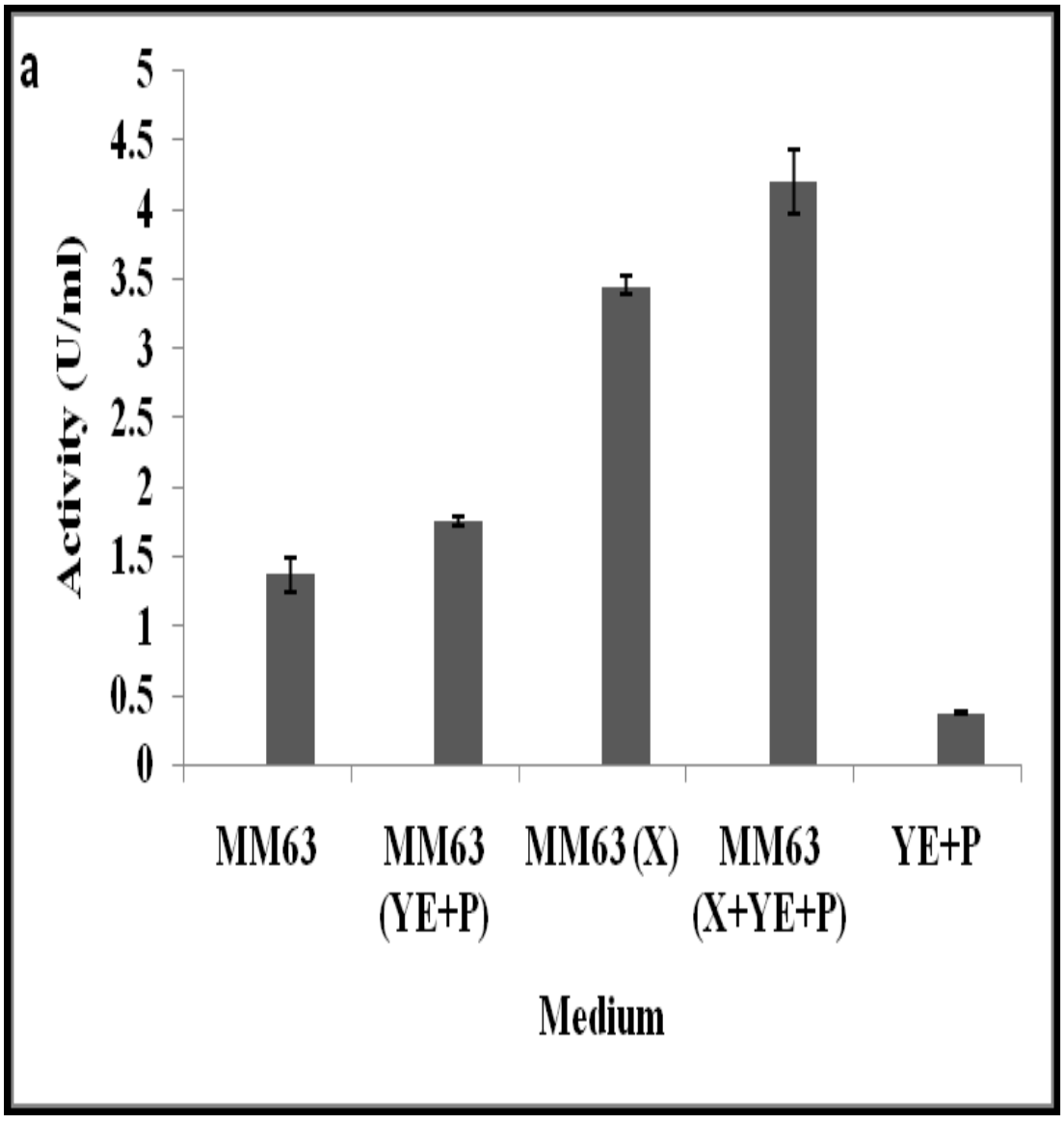


Figure 6.3 (a) Xylanase production of *Brachybacterium* sp. PS3 in MM63. X-xylan; P-peptone; YE-yeast extract. Enzyme activity was determined after 48 h of bacterial growth in MM63 with 6% (w/v) NaCl

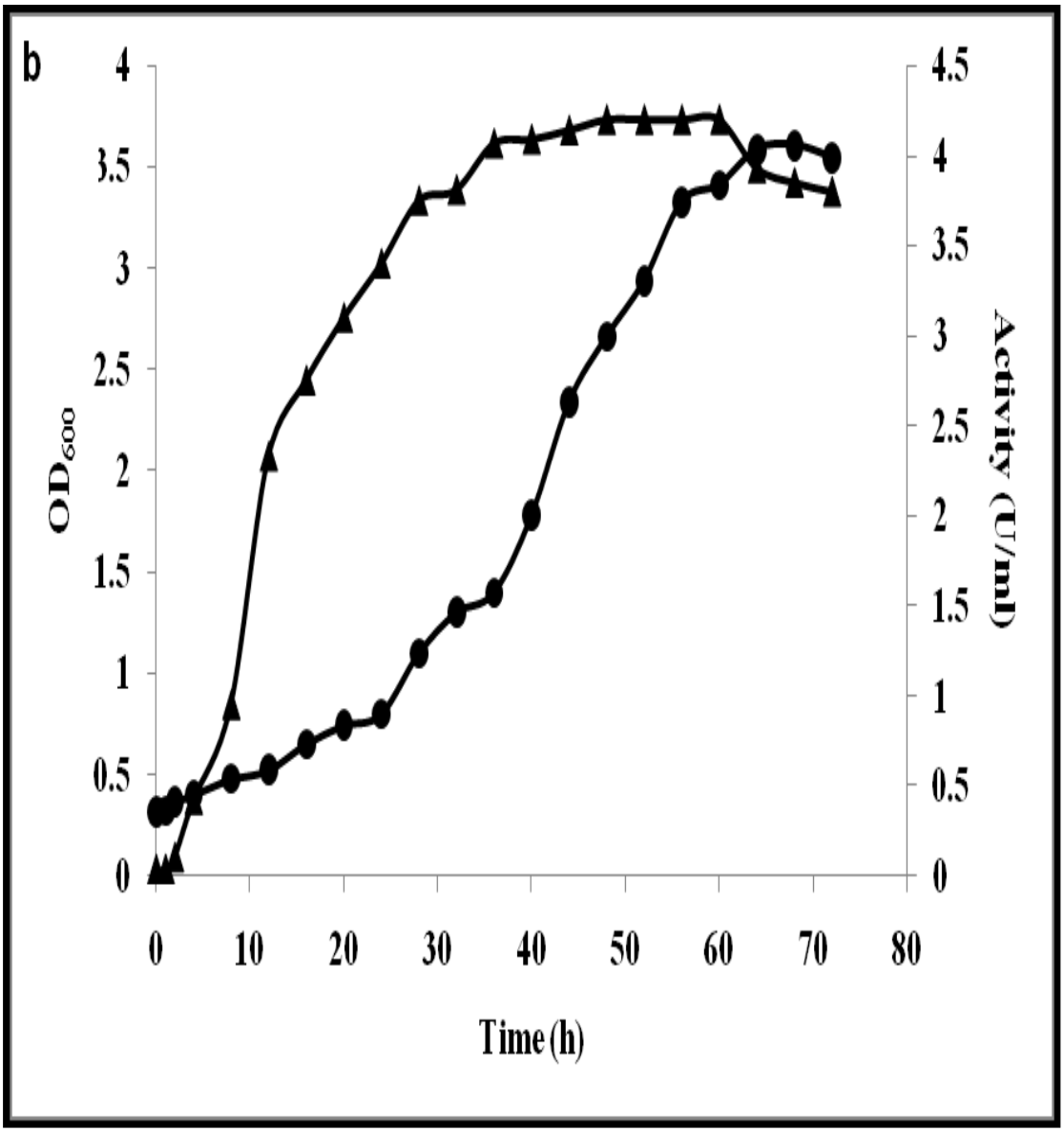


Figure 6.3 (b) Growth curve and xylanase secretion of *Brachybacterium* sp. PS3 in MM63 containing xylan. Samples were withdrawn at 4 h interval for the determining cell growth by OD_{600} (●) and xylanase activity (▲).

Xylanase secretion is associated with growth since xylan is the sole source of carbon. Higher xylanase activity is seen in the exponential phase and decreases in the stationary phase of growth.

6.2.4 Xylanase production on different agricultural residues

Among the different agricultural residues used, wheat bran and corn cobs were found to yield higher xylanase activities of 0.9 U/ml and 0.75 U/ml, respectively (Fig. 6.4a). Rice bran could also yielded substantial amounts of xylanase (0.7 U/ml). Lower xylanase activities of 0.44 U/ml, 0.48 U/ml and 0.4 U/ml were respectively detected when sugarcane bagasse, groundnut shells and coir pith were used individually as carbon source. Very low enzyme units (0.24 U/ml) were recorded when sunflower seeds were used in the medium.

It has been reported that among the agricultural residues, wheat bran and corn cobs are most often used in nutrient media for microbial xylanase production. These agro-residues can induce multiple xylanases which act in synergy for xylan hydrolysis (Dobrev et al. 2007; Xu et al. 2005).

The addition of yeast extract and peptone to the pre-treated wheat bran medium resulted in over two-fold increase in activity to 1.91 U/ml. The enzyme secretion in the wheat bran medium was higher in the late exponential and stationary phases of growth (Fig. 6.4b). Higher production of enzyme requires the presence of complex nitrogen sources (Singh et al. 2001).

Utilization of cellulosic wastes as cheap carbon and energy sources can serve as alternative to our rapidly depleting finite sources of fossil fuels. Halophilic bacteria capable of utilizing cheap renewable agricultural wastes could be exploited as an economic alternative to existing production processes.

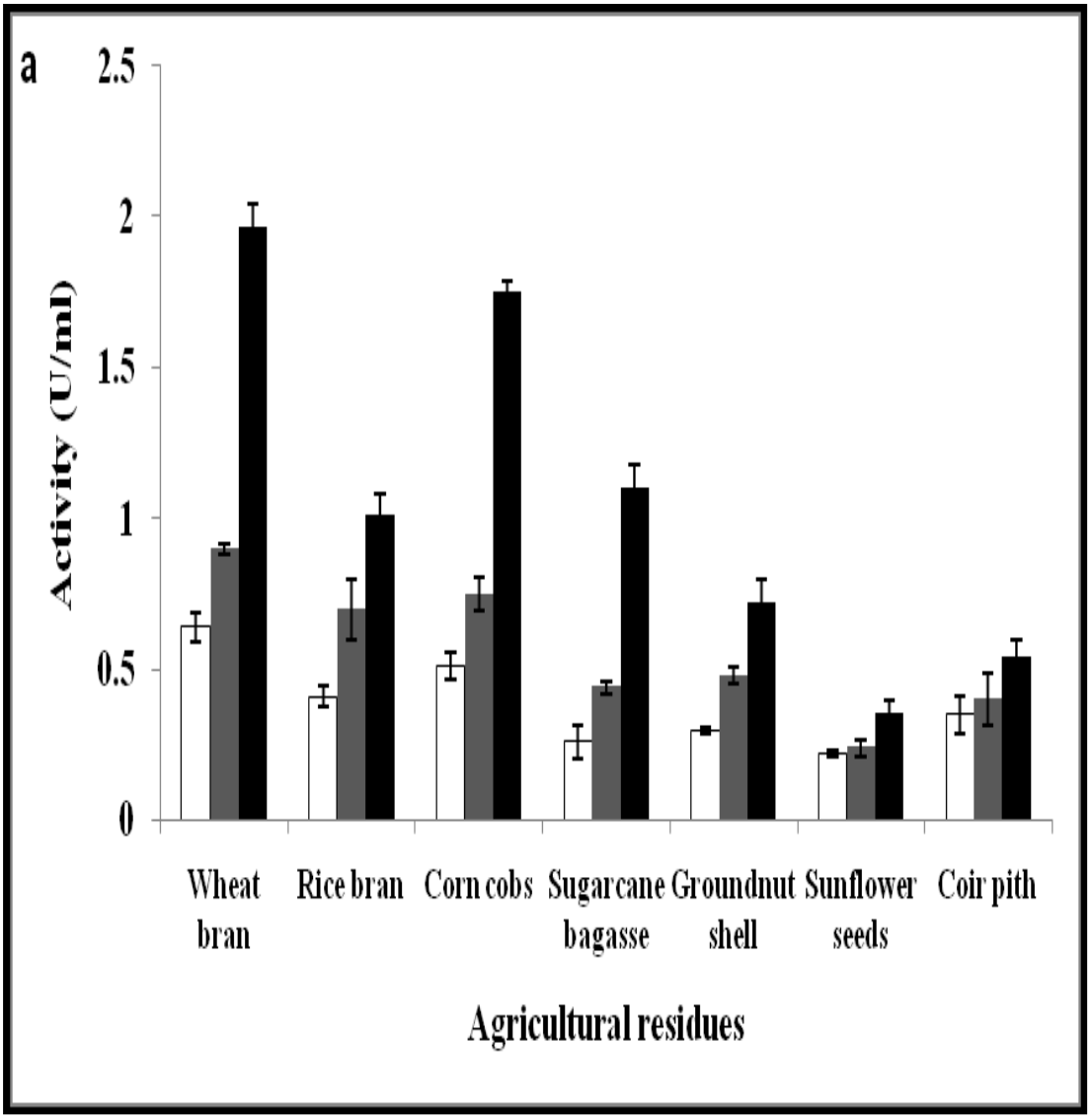


Figure 6.4 (a) Xylanase production of *Brachybacterium* sp. PS3 in MM63 containing (□) – untreated agro-residue; (■) – treated agro-residue and (■) – treated agro-residue along with yeast extract and peptone. Alkaline pretreatment (1% w/v NaOH) of agro-residues is carried out.

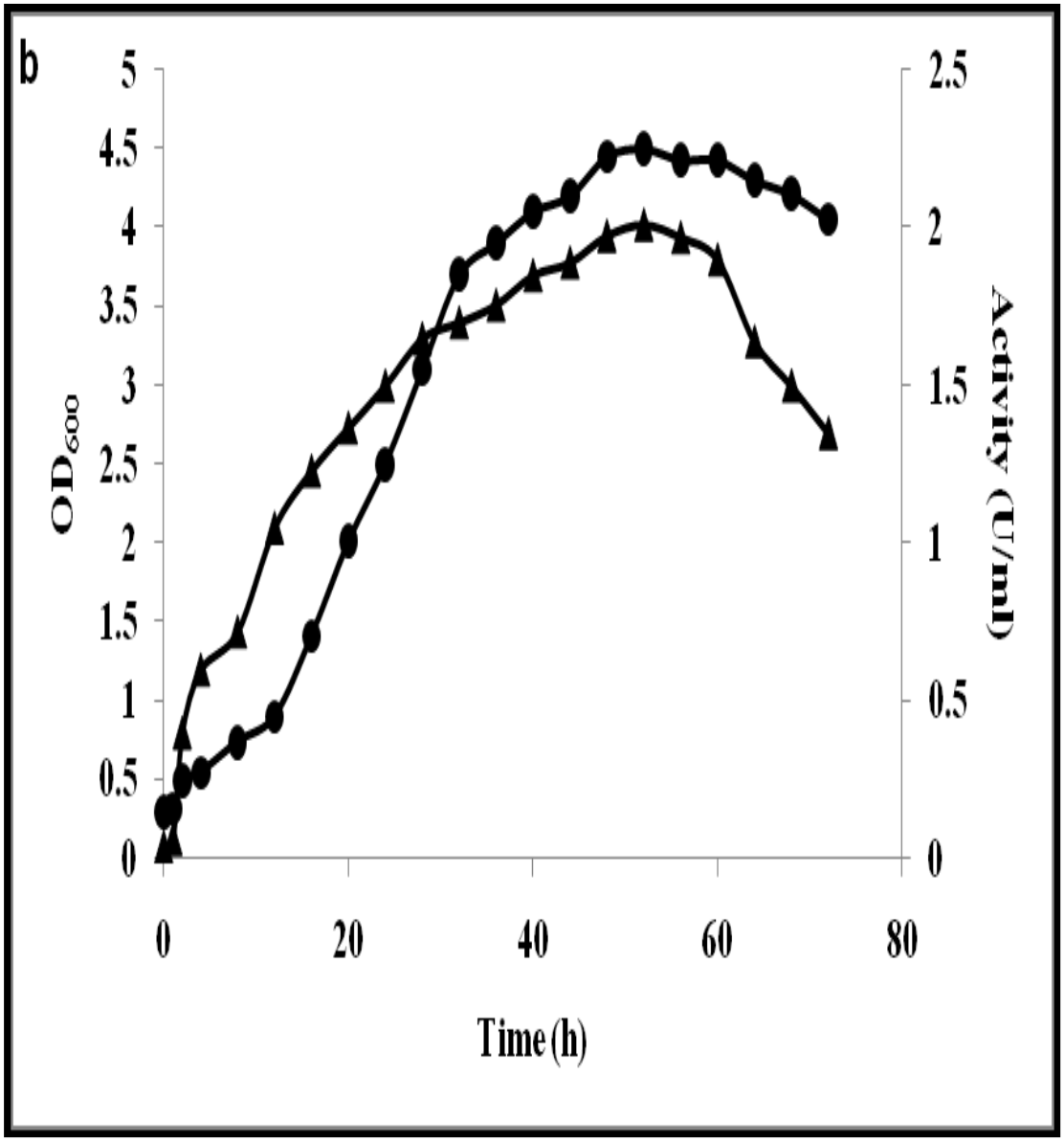


Figure 6.4 (b) Growth curve and xylanase secretion of *Brachybacterium* sp. PS3 in MM63 containing wheat bran. Samples were withdrawn at 4 h interval for the determination of cell growth by OD_{600} (●) and xylanase activity (▲).

6.2.5 Growth and xylanase production in media with different salt concentration

Growth pattern of *Brachybacterium* sp. PS3 in media with different concentrations of NaCl is shown in Fig. 6.5a. Growth was found to increase with increasing concentrations of salt. The bacterium was able to grow well up to NaCl concentration of 18% (w/v). Efficient growth was not seen at lower concentrations of NaCl. Growth was optimal at NaCl concentrations in the range of 9% - 15% (w/v) where the bacterium followed similar growth patterns.

Xylanase production (Fig. 6.5b) was also higher in this range of NaCl (9 - 15%, w/v), the optimum being at 12% (w/v). Though growth was much stunted at 3% and 6% (w/v) NaCl, the bacterium was able to produce extracellular xylanase. However, above 15% (w/v) NaCl concentrations, the bacterium had an extended lag phase and the growth was much higher. These studies confirm the moderately halophilic nature of the bacterium and its versatility in adaptations to increasing levels of salinity.

6.2.6 Effect of pH and temperature of the medium

Brachybacterium sp. PS3 could grow and produce substantial amounts of extracellular xylanase over an alkaline pH range of 8.0 - 10.0. Higher activity was seen in the pH range of 8.0 – 9.0 of the medium. Maximum xylanase production was obtained at pH 8.5 (Fig. 6.6a). The optimum temperature for xylanase production was 30 °C (Fig. 6.6b). Production was significantly reduced above 40 °C, which was not favourable for the bacterial growth.

The range of parameters like salt concentrations, temperature and pH to which a microorganism adapts to generally determines the range at which the enzymes produced by the organism are active and stable (Ventosa et al. 1998). Hence the xylanase produced by *Brachybacterium* sp. PS3 can be expected to retain activity and remain stable at varying conditions of temperature, pH and salinity.

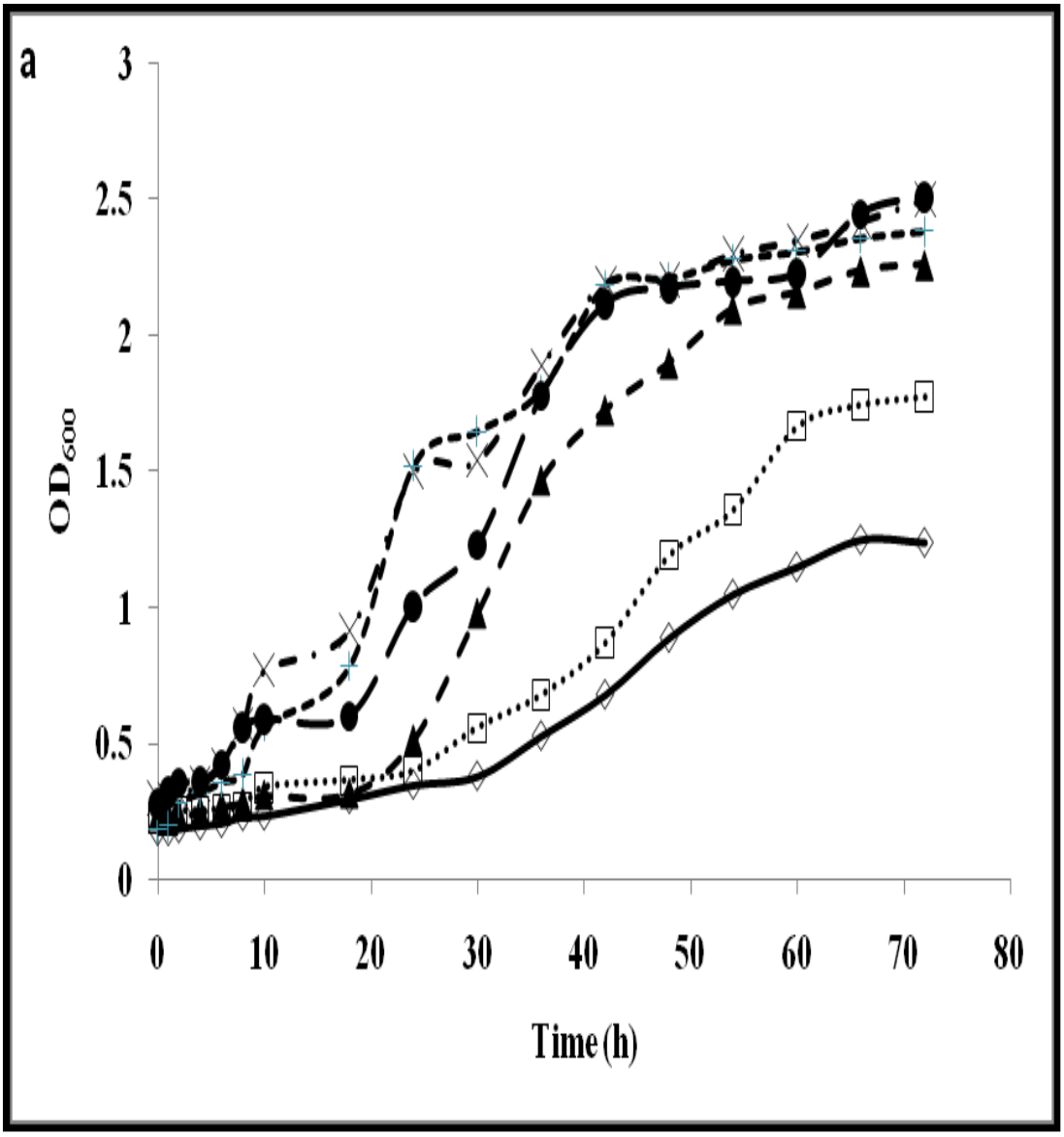


Figure 6.5 (a) Growth curves of *Brachybacterium* sp. PS3 in media with different salt concentrations, % (w/v) - 0 (◊); 3 M (□); 6 M (▲); 9 (×); 12 (+) and 15 (●). Samples were withdrawn at every 6 h interval for the determination of cell growth (OD_{600}).

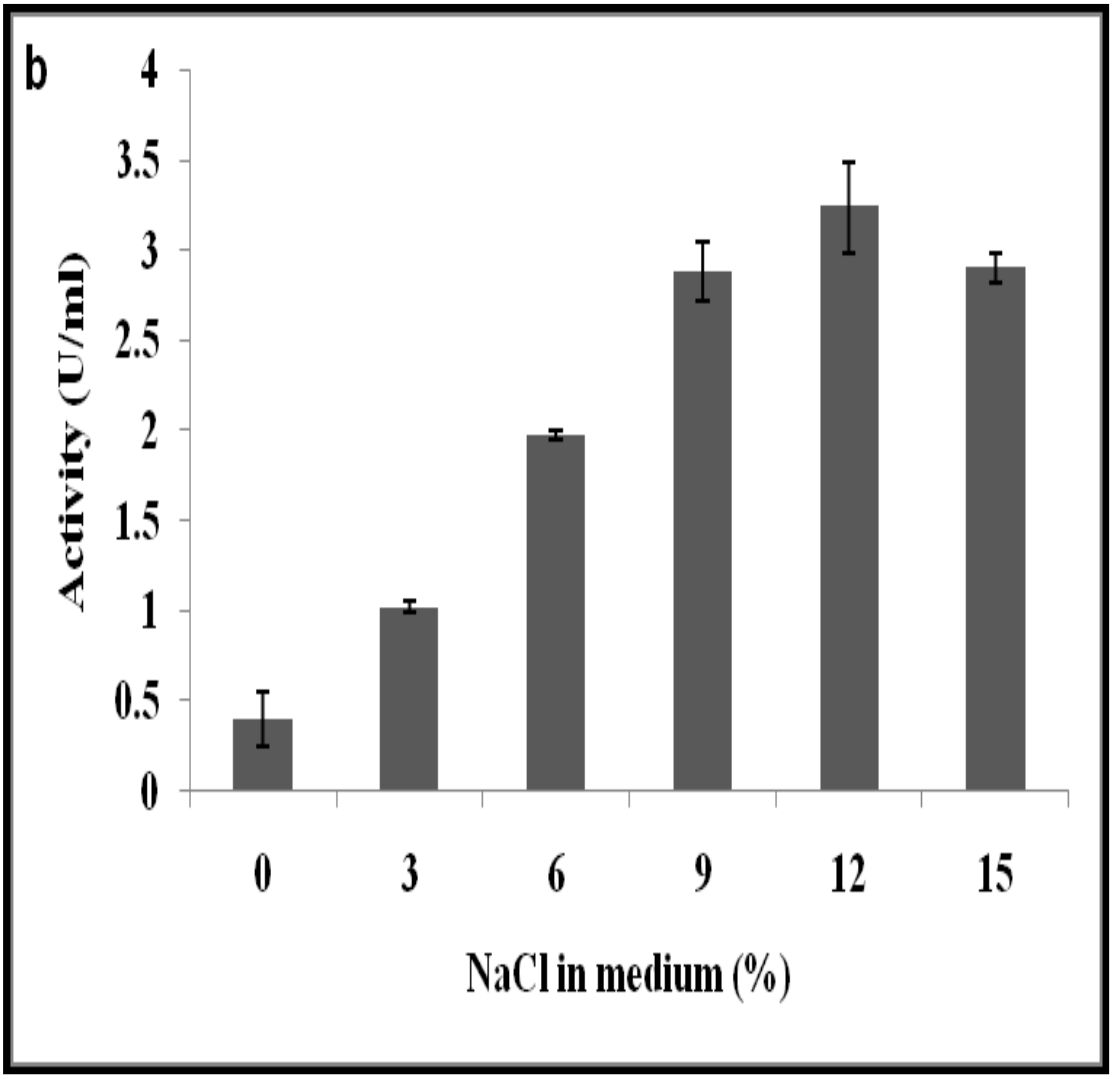


Figure 6.5 (b) Xylanase activity of *Brachybacterium* sp. PS3 in media with different salt concentrations. The highest activity in 12% (w/v) NaCl medium is taken as 100%.

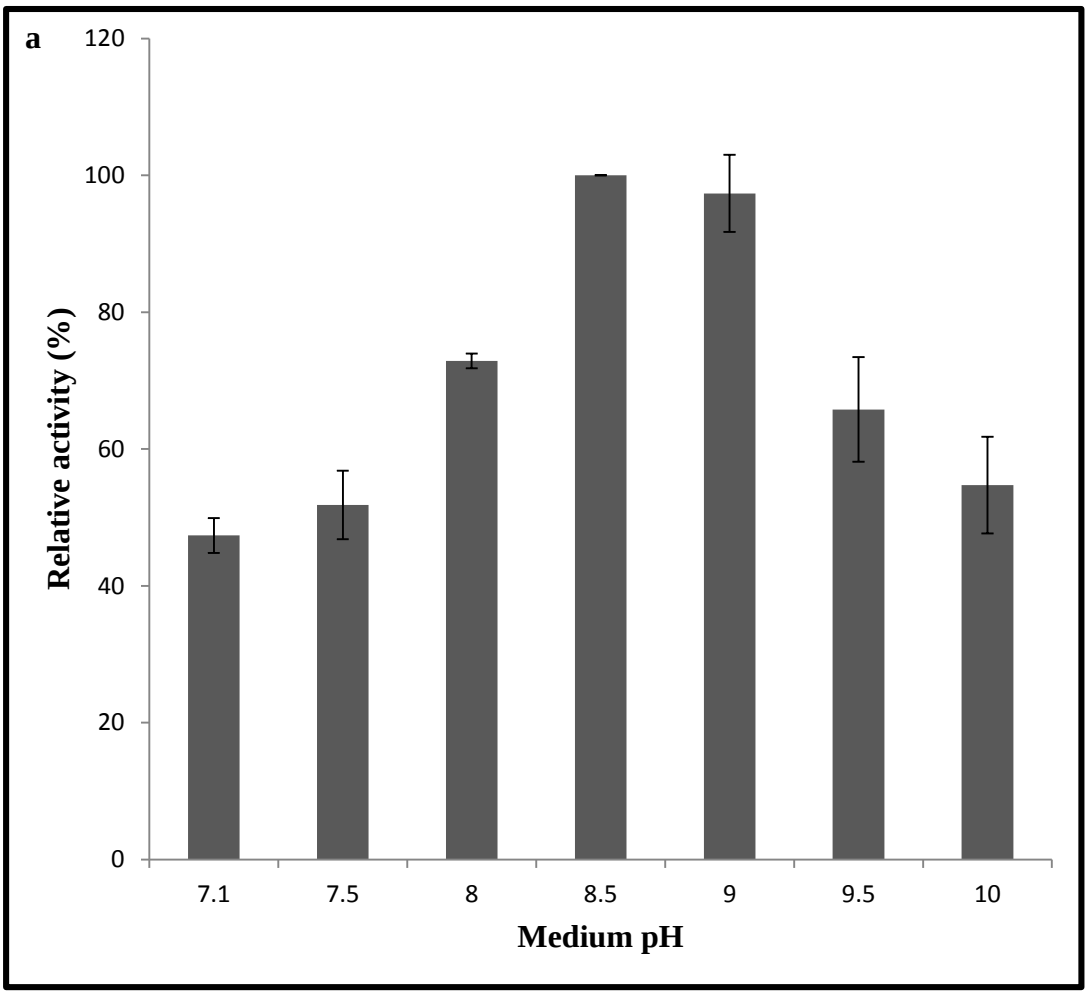


Figure 6.6 (a) Effect of medium pH on xylanase production by *Brachybacterium* sp. PS3. Samples were taken after incubation of 48 h at 30 °C, for the determination of xylanase activity (U/ml). Highest production at pH 8.5 is taken as 100%.

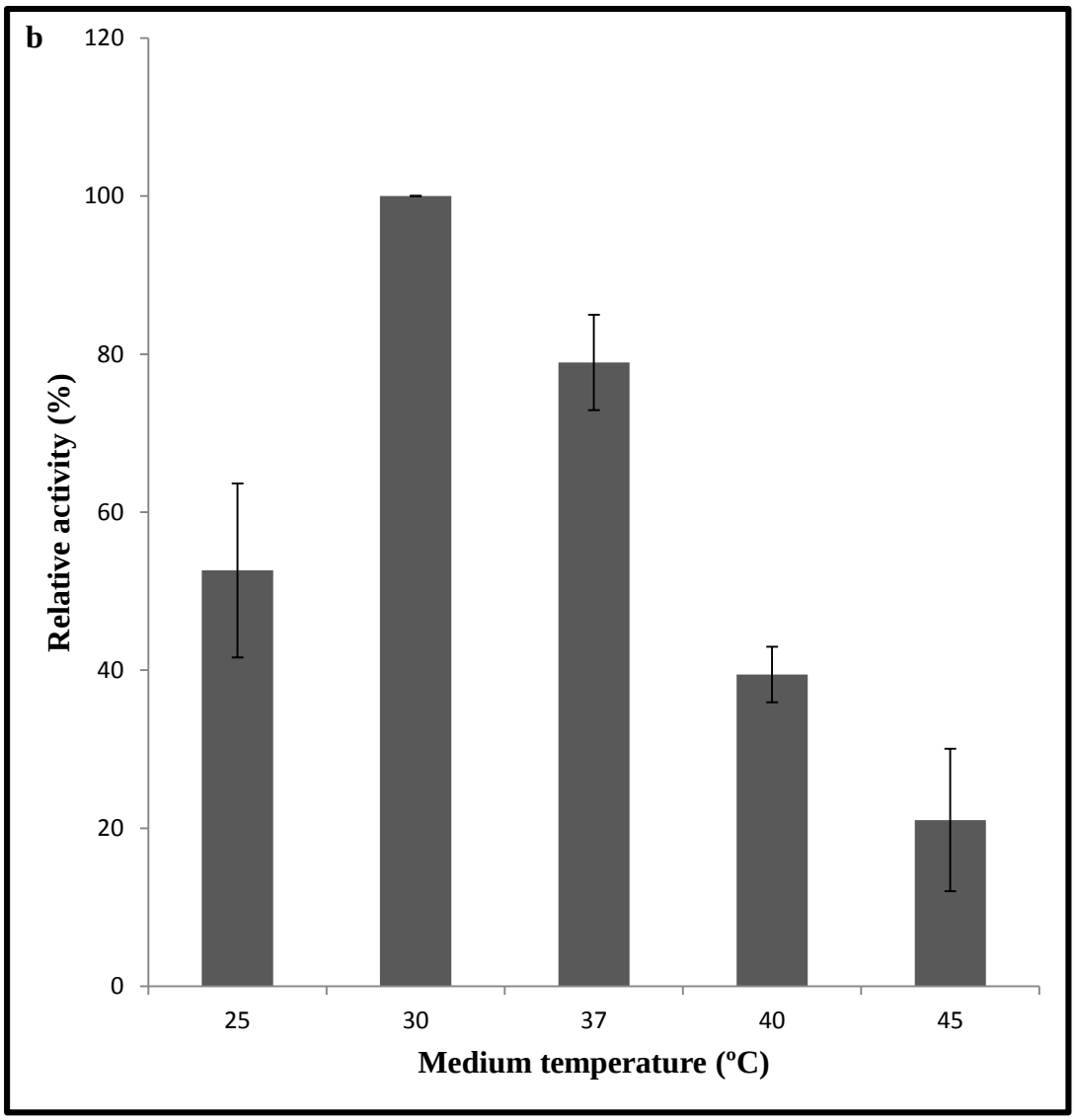


Figure 6.6 (b) Effect of medium temperature on xylanase production by *Brachybacterium* sp. PS3. Samples were withdrawn after incubation of 48 h at pH 7.5, for the determination of xylanase activity (U/ml). Highest production at 30 °C is taken as 100%.

6.2.7 Properties of the extracellular xylanase

The xylanase was active in the pH range of 7.5 -10.0, with an optimum at pH 9.0 (Table 6.1). The enzyme had maximum xylanolytic activity at 55 °C, but is active over a wide range of temperature (40-65 °C). The enzyme exhibited appreciable activity in the presence of NaCl up to a concentration of 4 M, although the highest activity was seen in the presence of 0 – 1 M NaCl. The activity was reduced to 52% of the original value at 3 M NaCl and to 37% at 4 M NaCl.

In addition to being halostable, enzymes obtained from halophilic microorganisms are also adapted to high temperature and pH conditions and thus polyextremophilic. Hence these enzymes find varied applications in industries where harsh conditions are used (Adams et al. 1995; Wainø and Ingvorsen 2003; Amoozegar et al. 2007).

Table 6.1 Properties of the extracellular cellulase produced by *Brachybacterium* sp. strain PS3.

pH opt.	9.0
T opt.	55 °C
NaCl stability	
0 M	2.1±0.05 U/ml
0.5 M	1.96±0.03 U/ml
1 M	1.96±0.1 U/ml
1.5 M	1.41±0.02 U/ml
2 M	1.41±0.2 U/ml
3 M	1.1±0.5 U/ml
4 M	0.79±0.5 U/ml

6.3 SUMMARY

Brachybacterium sp. PS3 (GenBank ID: JQ425852) produced xylanase (1.37 U/ml) on basal MM63 medium. Higher activity (4.2 U/ml) was seen when glucose in MM63 medium was replaced by xylan and $(NH_4)_2SO_4$ was replaced by a combination of yeast extract and peptone. Strain PS3 produced higher xylanase activities (U/ml) - on wheat bran (0.9), corn cobs (0.75), rice bran (0.7 U/ml), sugarcane bagasse (0.44), groundnut shells (0.48) and coir pith (0.4) Addition of yeast extract and peptone to the pre-treated wheat bran medium resulted in over two-fold increase in activity to 1.91 U/ml. PS3 xylanase exhibited highest activity at pH 9.0 and 55 °C. Enzyme exhibited stability up to 4 M NaCl and higher activity at 0 – 1 M NaCl. The activity was reduced to 52% of the original value at 3 M NaCl and to 37% at 4 M NaCl.

The present study assumes significance in the production of extracellular halostable xylanase from newly isolated halophilic bacterium, *Brachybacterium* sp. strain PS3, using renewable agricultural residues like wheat bran, corn cobs and rice bran. The bacterium is able to utilise different biopolymers for xylanase production. Moreover, growth and production are favourable over a wide range of NaCl concentrations. The thermo-stable, salt and pH-tolerant xylanase is a promising candidate for industrial applications. Few reports are available on the usage of renewable agricultural resources for the growth of halophiles. Thus, halophilic bacteria capable of utilizing cheap agricultural wastes could be exploited as an economic alternative to existing production processes.

Chapter 7

Optimisation of halostable xylanase production from *Brachybacterium* sp. PS3

7.1 MATERIALS AND METHODS

7.1.1 Screening of significant factors by Plackett-Burman design

Medium components and conditions for xylanase production were screened and significant factors identified by the Plackett-Burman design using statistical software package 'Design-Expert® Version 8.0.4', Stat-Ease, Inc., and (Minneapolis, MN, USA). A total of eleven parameters were considered at two levels: −1 (low level) and +1 (high level) namely A- wheat bran (10-40 g/l), B- NaCl (30-90 g/l), C- yeast extract (2-5 g/l), D- peptone (2-5 g/l), E- tris (8-12 g/l), F- $MgSO_4 \cdot 7H_2O$ (0.25-0.5 g/l), G- K_2HPO_4 (0.5-2.5 g/l), H- pH (8.0-10.0), J- corn cobs (10-40 g/l), K- $(NH_4)_2SO_4$ (2-5 g/l) and L- temperature (25-40 ºC). Xylanase activity was taken as the response. All experiments were carried out in triplicates.

The design is based on the first-order polynomial model: $Y = \beta_0 + \sum \beta_i X_i$, where Y is the response (enzyme activity), β_0 is the model intercept, β_i is the linear coefficient, and X_i is the level of the independent variable. From the regression analysis the variables, which were significant at or above 95% level (*p-value* < .05), were considered to have greater impact on xylanase activity and were further optimized by central composite design.

7.1.2 Optimization of key determinants and statistical analysis

The next stage in the medium formulation was to determine the optimum levels of the significant variables, viz. A – pH (8.0-11), B - wheat bran (20-50 g/l), C - NaCl (60-120 g/l) and D – corn cobs (20-50 g/l) and study the interaction between them. For this purpose, central composite design was adopted for improving xylanase activity using the Design Expert software. A total of 30 experiments were conducted. All the variables were taken at a central coded value, which was considered as zero.

The average maximum xylanase yield was taken as the independent variable or response (Y). The data obtained were subjected to analysis of variance (ANOVA). Once the xylanase activity (U/ml) was determined, a second order polynomial model was fitted to the response data obtained from the design by the multiple regression procedure. This resulted in an empirical model that related the response measured to the independent variables of the experiment. The polynomial equation for four-variable system is in the following form:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2 + \beta_{12} AB + \beta_{13} AC + \beta_{14} AD + \beta_{23} BC + \beta_{24} BD + \beta_{34} CD \quad \text{.....(7.1)}$$

with, Y, predicted response; A, B, C, D, variables; β_0, intercept; β_1, β_2, β_3, β_4, linear coefficients; β_{11}, β_{22}, β_{33}, β_{44}, squared coefficients; β_{12}, β_{13}, β_{14}, β_{23}, β_{24}, β_{34}, interaction coefficients. The statistical significance of the model equation and model terms was evaluated via the Fisher's test. The quality of fit of the second-order polynomial model equation was expressed via the coefficient of determination (R^2) and the adjusted R^2. The three-dimensional response surface curves were then plotted to understand the interaction of the medium components and the optimum concentration of each component required for maximum xylanase production. The combination of different optimized variables, which yielded the maximum response, was determined to verify the validity of the model.

7.2. RESULTS AND DISCUSSION

7.2.1 Screening of significant factors by Plackett-Burman design

The effects of eleven variables were investigated for xylanase production in 12 runs using Plackett-Burman design. The variation in xylanase activity (Table 7.1) reflected the significance of factors on enzyme activity. Among the variables screened, factors with high level of significance were in the order of pH, wheat bran, NaCl and corn cobs as shown in Fig. 7.1. Hence these variables were chosen for further optimization studies (Table 7.2). It has been widely reported that pH of the

fermentation medium has a significant influence on xylanase production (Smith and Wood 1991).

Table 7.1 Plackett-Burman design eleven variables influencing xylanase production by *Brachybacterium* sp. PS3

Run	A g/l	B g/l	C g/l	D g/l	E g/l	F g/l	G g/l	H	J g/l	K g/l	L ºC	R U/ml
1	40	30	2	2	12	0.25	2.5	10	10	5	40	2.17
2	10	30	2	5	8	0.5	2.5	8	40	5	40	0.14
3	40	90	2	2	8	0.5	0.5	10	40	2	40	3.22
4	40	90	5	2	8	0.25	2.5	8	40	5	25	2.12
5	10	90	2	5	12	0.25	2.5	10	40	2	25	2.62
6	40	30	5	5	8	0.5	2.5	10	10	2	25	2.12
7	10	30	2	2	8	0.25	0.5	8	10	2	25	0.71
8	10	90	5	2	12	0.5	2.5	8	10	2	40	0.14
9	10	30	5	2	12	0.5	0.5	10	40	5	25	2.01
10	40	30	5	5	12	0.25	0.5	8	40	2	40	1.81
11	40	90	2	5	12	0.5	0.5	8	10	5	25	1.83
12	10	90	5	5	8	0.25	0.5	10	10	5	40	2.05

A-Wheat bran, B-NaCl, C-Yeast extract, D-Peptone, E-Tris, F-$MgSO_4.7H_2O$, G-K_2HPO_4, H- pH, J-Corn cobs, K-$(NH_4)_2SO_4$, L-Temperature, R-Xylanase activity

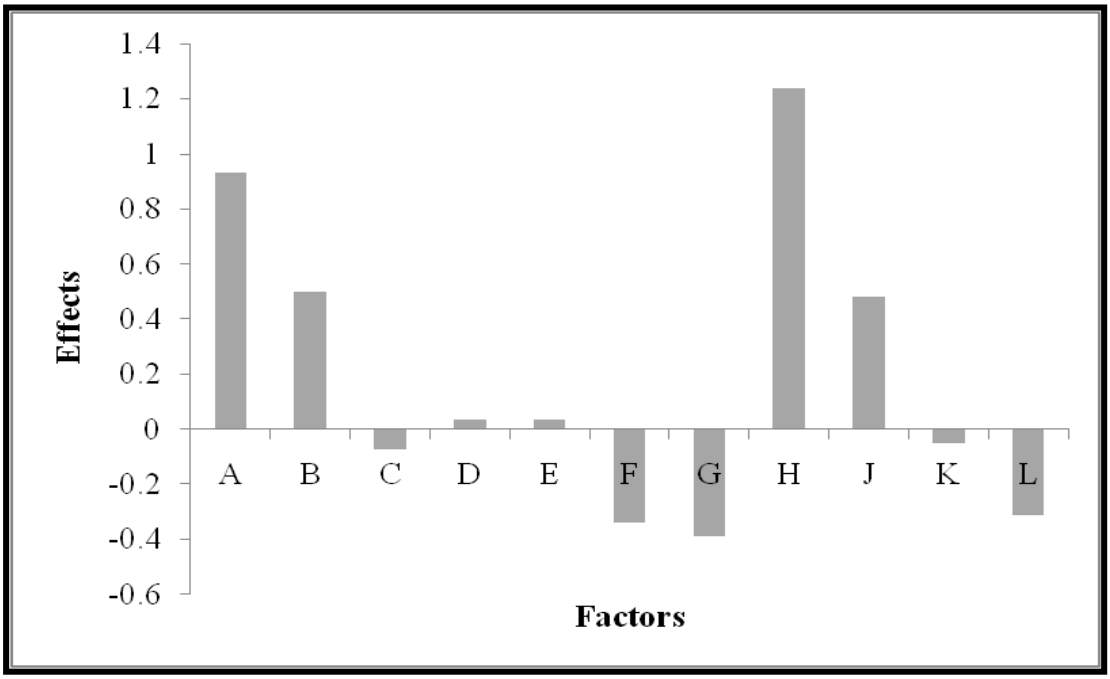


Figure 7.1 Main effects of different variables on xylanase production in Plackett-Burman design. A-Wheat bran, B-NaCl, C-Yeast extract, D-Peptone, E-Tris, F-$MgSO_4.7H_2O$, G- K_2HPO_4, H- pH, J-Corn cobs, K-$(NH_4)_2SO_4$, L-Temperature

Table 7.2 Range of variables for central composite design of xylanase production

Variables	Symbol	Range of levels				
		-α	**-1**	**0**	**+1**	**+α**
pH	A	6.5	8	9.5	11	12.5
Wheat bran (g/l)	B	5	20	35	50	65
NaCl (g/l)	C	30	60	90	120	150
Corn cobs (g/l)	D	5	20	35	50	65

7.2.2 Optimization of significant variables using central composite design

Higher enzyme activity was observed with increased concentrations of NaCl. Quadratic regression models allow object description in a comparatively wide area of input variable change and hence are widely used (Vuchkov and Stoyanov 1980). The results of the thirty-trial design for studying the effects of the four independent variables, viz., pH (A), wheat bran (B) NaCl (C) and corn cobs (D) on xylanase production are presented in Table 7.3 along with the predicted and mean observed responses.

7.2.3 Analysis of the model terms

The adequacy of the model was checked using analysis of variance (ANOVA) which confirms a satisfactory adjustment of the reduced quadratic model to the experimental data. It showed that this regression is statistically significant 95% confidence level. The regression equation indicated the R^2 value of 0.9669 (a value >0.75 indicates fitness of the model), which ensured a satisfactory adjustment of the quadratic model to the experimental data and indicated that 96.69% of the variability in the response could be explained by the model. The 'adjusted R^2' is 0.9339 and the 'predicted R^2' is 0.9373, which are in reasonable agreement. This indicates a high significance of the model as nearer the value of R^2 to 1.0, the more fit the model is deemed to be.

The '*adequate precision* value' of 55.792 indicates an adequate signal. At the same time, a relatively lower value of the coefficient of variation (CV%: 0.57) indicates improved precision and reliability of the conducted experiments. The high *F*-value (339.97) and low *p*-value (<0.0001) indicated that the experimental model is significant. The *p*-value denotes significance of coefficients and also important in understanding the pattern of mutual interactions among variables. The *Lack of Fit* of the model at *p*-value of 0.6753 is not significant suggesting that the obtained experimental data were in good fit.

Table 7.3 Central composite design for three variables influencing xylanase activity along with response

Run	A pH	B Wheat bran g/l	C NaCl g/l	D Corn cobs g/l	Xylanase Activity U/ml	
					Observed U/ml	Predicted U/ml
1	9.5	35	90	35	8.41	8.46
2	8	20	120	50	5..43	6.81
3	6.5	35	90	35	7.1	7.43
4	8	20	120	20	7.2	6.95
5	9.5	65	90	35	7.8	7.92
6	9.5	35	150	35	6.7	7.03
7	11	50	60	50	7.2	7.42
8	11	20	120	50	7.1	7.280
9	8	20	60	20	7.32	6.97
10	11	20	120	20	7.2	7.25
11	12.5	35	90	35	5.15	7.18
12	11	50	120	20	7.45	7.79
13	11	20	60	50	6.45	7.39
14	11	50	60	20	7.15	7.52
15	9.5	35	30	35	7.1	7.12

Run	A pH	B Wheat bran g/l	C NaCl g/l	D Corn cobs g/l	Xylanase Activity U/ml	
					Observed U/ml	Predicted U/ml
16	9.5	35	90	5	6.98	7.27
17	8	20	60	50	6.75	6.79
18	9.5	35	90	35	8.4	8.46
19	9.5	35	90	35	8.45	8.46
20	9.5	5	90	35	7.3	7.21
21	9.5	35	90	65	6.96	7.17
22	9.5	35	90	35	8.41	8.46
23	9.5	35	90	35	8.43	8.46
24	8	50	120	20	7.97	7.93
25	11	50	120	50	7.1	6.93
26	9.5	35	90	35	8.49	8.46
27	8	50	60	50	7.2	7.1825
28	8	50	120	50	6.45	7.21
29	11	20	60	20	7.35	7.5
30	8	50	60	20	7.67	7.1

The coefficients of the regression equation were calculated using the Design Expert software and the data was fitted to a second order polynomial equation. The xylanase production by *Brachybacterium* sp. PS3 can be expressed in terms of the following regression equation:

$$Y = + 8.46 + 0.023A + 0.15B - 0.019C - 0.16D - 0.15AB - 0.091AC + 0.053AD + 0.047BC - 0.047BD - 0.0031CD - 0.33A^2 - 0.24B^2 - 0.35C^2 - 0.27D^2 \quad (7.2)$$

Where Y is xylanase activity (response); A, pH; B, wheat bran; C, NaCl and D, corncobs. The above equation indicates that pH and wheat bran have higher contribution and significant interactions are observed between pH and corn cobs; wheat bran and corn cobs and NaCl and corn cobs. The increase in concentration of one of the factors requires a higher concentration from the other factor.

7.2.4 Response surface plots

The three-dimensional response surface curves were then plotted to understand the interaction effects and optimum levels of the variables. This is done by plotting the response on the Z-axis against any two independent variables, while maintaining the other variables at fixed levels (zero, for instance).

Fig. 7.2a shows the response surface plot obtained as function of pH *vs.* wheat bran, while NaCl and corn cobs were maintained at zero levels. Fig. 7.2b shows the contour plot obtained as function of pH *vs.* NaCl, while wheat bran and corn cobs were maintained at zero levels. Fig. 7.2c shows the response surface plot obtained as function of pH *vs.* corn cobs, while wheat bran and NaCl were maintained at zero levels. Fig. 7.2d shows the contour plot obtained as function of wheat bran *vs.* NaCl, while pH and corn cobs were maintained at zero levels. Fig. 7.2e shows the response surface plot obtained as function of wheat bran *vs.* corn cobs, while pH and NaCl were maintained at zero level. Fig. 7.2f shows the contour plot obtained as function of NaCl *vs.* corn cobs, while pH and wheat bran were maintained at zero level.

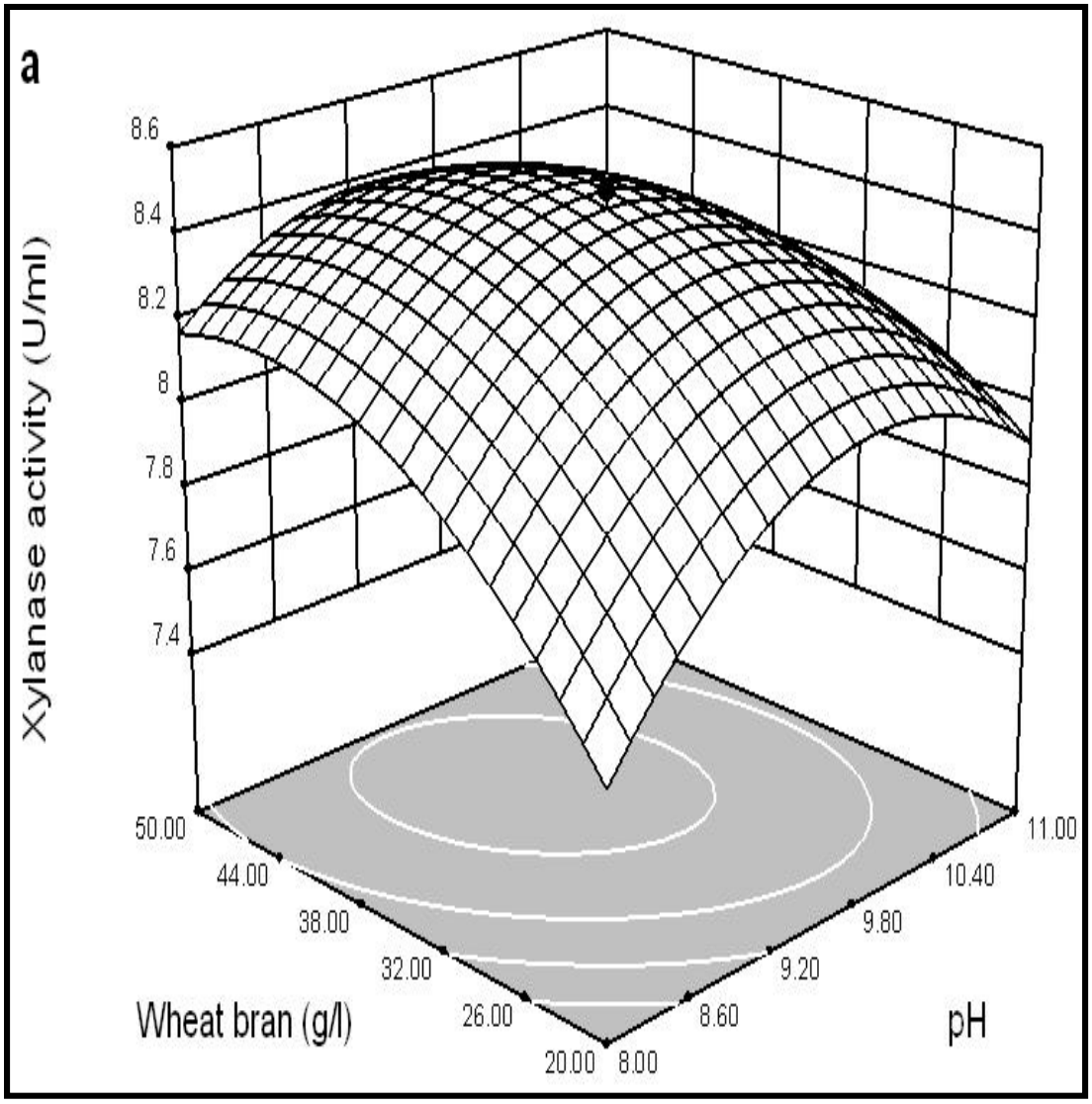
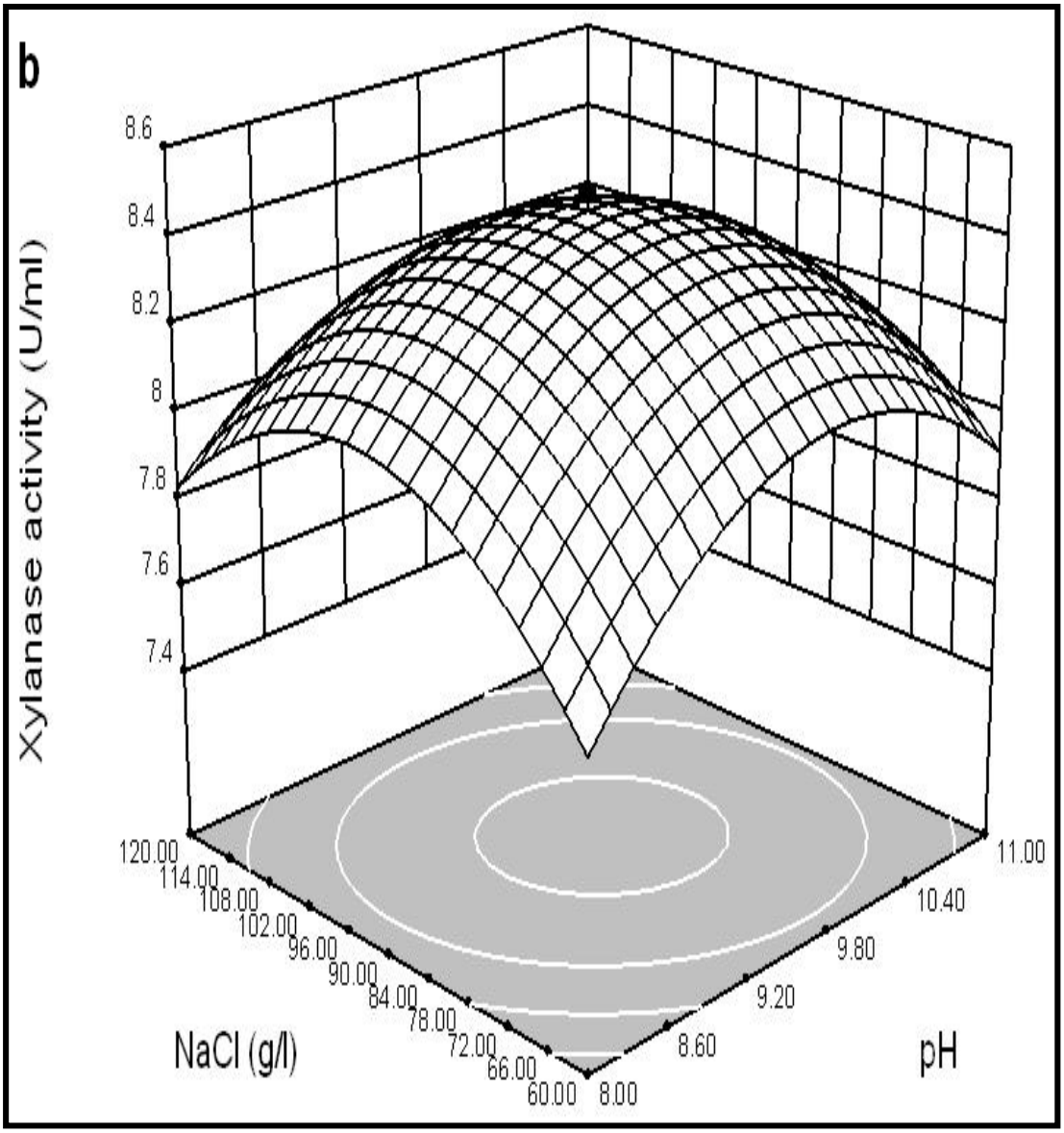
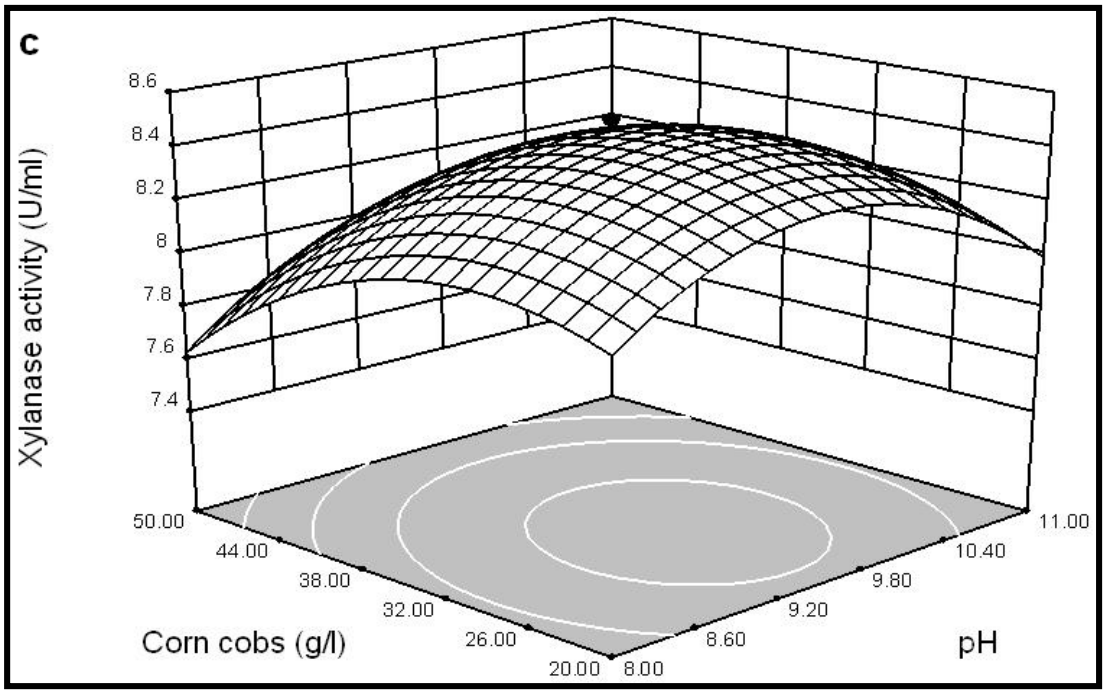
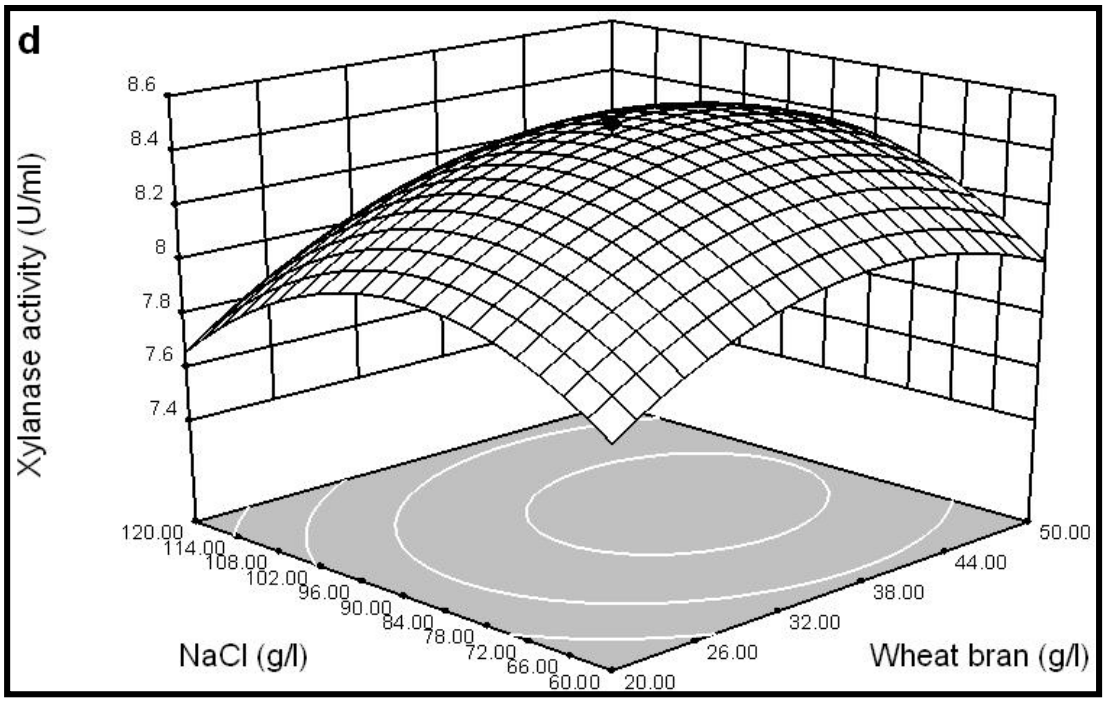


Figure 7.2 Three-dimensional response surface and contour plots for xylanase production showing the interactive effects of variables: (a) pH vs. wheat bran

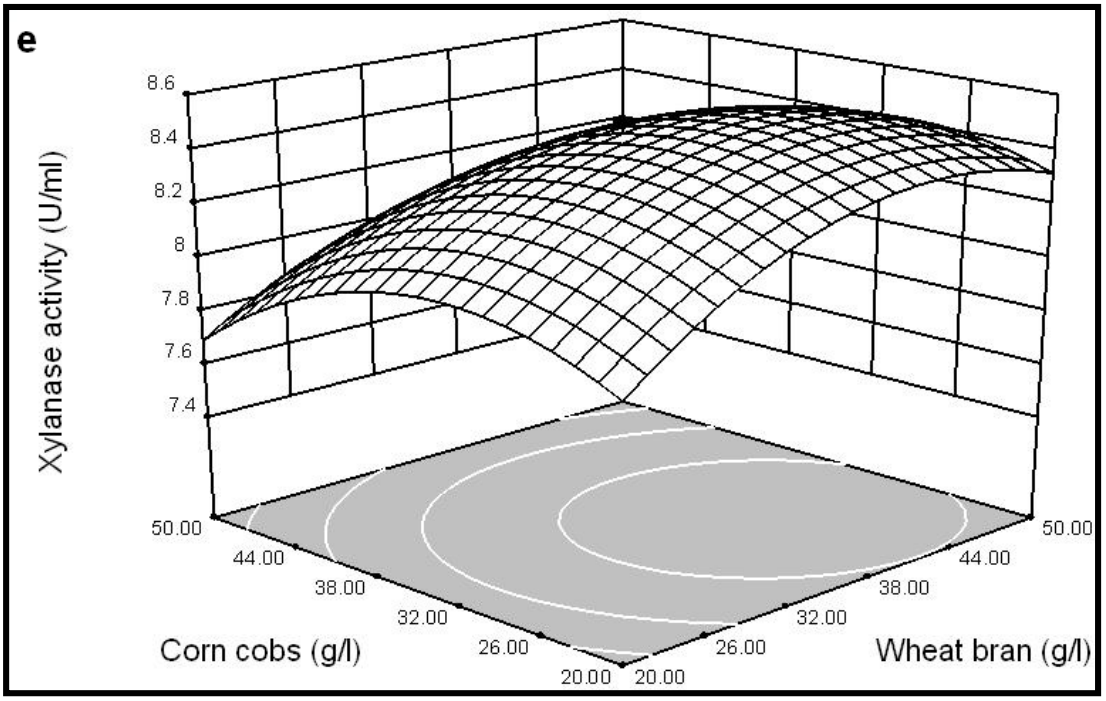

b
Xylanase activity (U/ml)
8.6
8.4
8.2
8
7.8
7.6
7.4
120.00
114.00
108.00
102.00
96.00
90.00
84.00
78.00
72.00
66.00
60.00
NaCl (g/l)
8.00
8.60
9.20
9.80
10.40
11.00
pH

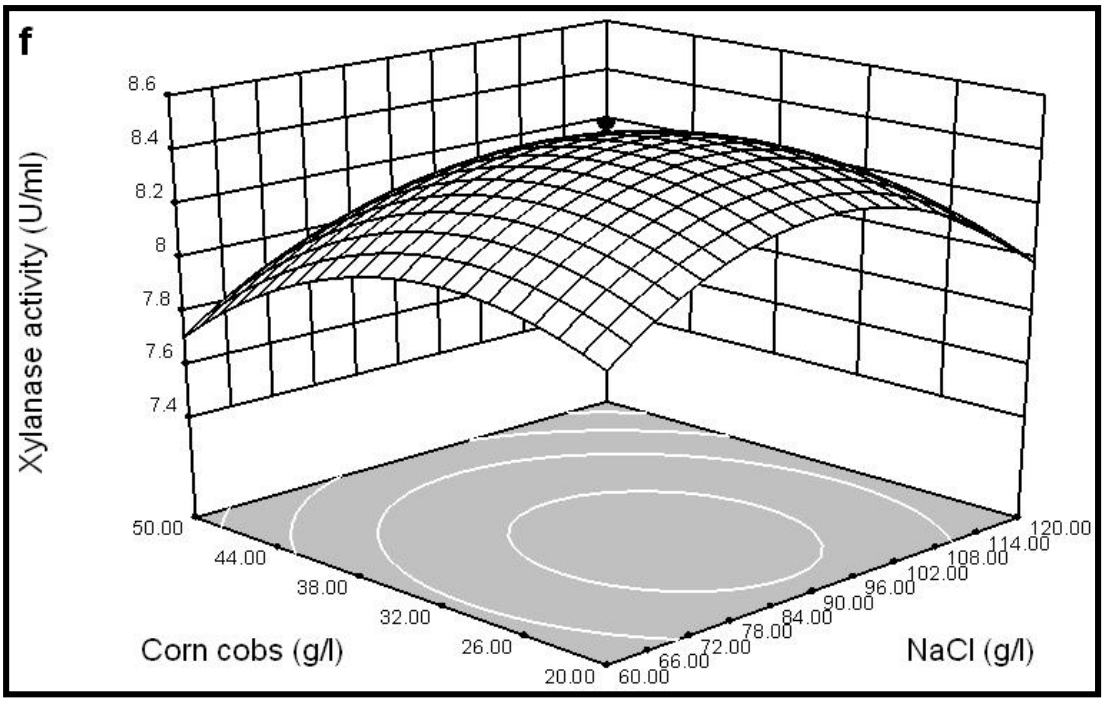
(b) pH vs. NaCl





(c) pH vs. corn cobs (d) wheat bran vs. NaCl





(e) wheat bran vs. corn cobs (f) NaCl vs. corn cobs

7.2.5 Validation of the model

The model predicted a maximum xylanase activity of 8.65 U/ml at pH (9.0), wheat bran (40 g/l), NaCl (90 g/l) and corn cobs (30 g/l). The close relationship between the predicted and observed response values from the experimental design demonstrated the acceptability of the statistical model for the optimization of medium nutrients, allowing for maximum yields. To validate the predicted model an experiment was conducted in triplicate using the optimum medium composition. Xylanase activity of 8.23 U/ml was observed at this optimized medium composition against the predicted response of 8.65 U/ml, with a validity of 95%. The correlation between predicted and experimental values justifies the validity of the model and the existence of an optimum point.

Halostable xylanase can be used in high-salt and high osmotic pressure environments thereby reducing water consumption. The xylanase biosynthesis is induced by its substrate xylan (Subramanyan and Prema 2002), and hence, some of the agro-residues like corn cobs and wheat bran, with high xylan content can be used as cheap sources of inducers. Wheat bran accounts for 15-20% of the weight of the grain and contains 20-30% arabinoxylan. The bran remains loose even under high moisture conditions to provide a large surface area thereby making it a suitable substrate for microbial growth and enzyme production (Archana and Satyanarayana 1997). Lequart et al. (1999) have reported that wheat bran consists of soluble sugars (% w/w) such as glucose (42.5), xylose (15.4), arabinose (3.1) and galactose (2.7%). Corn cobs are shown to contain sugars (g/l) such as glucose (6.6), xylose (27.2) and arabinose (5.5) (Beall and Ingram 1992). It is indicated that except for starch content, corn cobs do not vary greatly in chemical composition (Hespell 1998). These sugars serve as nutrients for the growth of microorganism and subsequent production of enzyme. It is possible that multiple xylanases can be induced from different xylans in corn cobs and wheat bran, which act synergistically in xylan hydrolysis. The use of plant material as carbon in production media has been reported to be a cost effective strategy for producing microbial enzymes (El-Helow and El-Ahawany 1999).

7.3 SUMMARY

Using Plackett-Burman design and response surface methodology, a maximum xylanase activity of 8.5 U/ml at pH 9.0, wheat bran 40 g/l, NaCl 90 g/l and corn cobs 30 g/l was predicted. Xylanase activity of 8.23 U/ml was observed with validity of 95.2% and over four-fold increase over the unoptimised medium. Response surface plots were very useful in visualizing the main effects and interaction of significant variables. The existence of optimum point was observed and established. The optimum culture medium obtained gives a basis for further studies on scale-up experiments. Thus, halophilic bacteria capable of utilizing cheap agricultural wastes could be exploited as an economic alternative to the existing production processes. The present study establishes an optimized medium with an improvement in xylanase production for *Brachybacterium* sp. strain PS3 which is able to utilise different renewable agro-residues for xylanase production.

PART THREE

CELLULASE PRODUCTION

Chapter 8

Screening and production of halostable cellulase

8.1 MATERIALS AND METHODS

8.1.1 Bacterial isolation and screening for cellulase activity

Bacterial strains isolated from MM63 containing CMC as the sole carbon source (PS33-PS48), were screened for cellulase activity. The isolates were grown at 30 ℃ for 48 h. Supernatants from these cultures were incubated in the wells of CMC (1%, w/v) enriched agar plates. After incubation of the plates for 24 h, zones of hydrolysis were visualized by staining the plates with aqueous solution of 0.1% (w/v) Congo red for 15 min, and then destained with 1 M NaCl (Teather and Wood 1982). Morphological and physiological characteristics of the potential strain PS47 were studied. The strain was further identified by 16S rRNA gene amplification and nucleotide sequencing from the Agharkar Research Institute, Pune, India.

8.1.2 Inoculum preparation and enzyme assay

A 1% inoculum from an overnight grown culture in log phase was added to 100 ml MM63 medium taken in 250 ml Erlenmeyer flasks. After incubation for 48 h, at 30 °C, under shaking condition of 150 rpm, the culture was harvested and growth was measured as OD_{600}, spectrophotometrically. The cultures were centrifuged at 10,000 rpm for 10 min at 4 °C. The cell free extract was used as crude preparation to measure cellulase activity.

For enzyme assay CMC was used as the substrate. Enzyme activity was determined by measuring the release of reducing sugars during the enzyme substrate reaction using dinitrosalicylic acid method (Miller 1959; Ghose 1987). The values were determined from glucose standard curve (Appendix VI). One unit (IU) of activity was defined as the amount of enzyme required to liberate 1 μmol of glucose per minute under given assay conditions.

8.1.3 Cellulase production in MM63 medium

Cellulase production was investigated in MM63 medium by replacing the carbon source glucose with CMC and nitrogen source $(NH_4)_2SO_4$ with organic sources like yeast extract and peptone. Different agro-residues like wheat bran, rice bran, corn cobs, sugarcane bagasse, groundnut shells and sunflower seeds were checked for the source of carbon in the medium at 1% (w/v) for cellulase production. The culture was grown at pH 7.1 and temperature 30 ℃ at 150 rpm for 48 h before estimation of enzyme activity. Cellulase activity was checked in both treated and untreated agro-residue media. The activity was also checked in media supplemented with yeast extract and peptone as nitrogen sources.

8.1.4 Growth and cellulase production in media with different salt concentration

The effect of salt on growth and cellulase secretion was studied by varying the NaCl concentration from 0 – 15% (w/v) in the wheat bran medium. The initial pH of the medium was adjusted to 7.1. Growth and cellulase activity of PS47 were monitored every 4 h, at 30 °C and 150 rpm for a period of 72 h.

8.1.5 Effect of pH and temperature of the medium for cellulase production

In order to investigate the influence of pH on growth and cellulase production, the isolate, PS47 was grown in MM63-wheat bran medium containing 6% (w/v) NaCl at different pH (3.0 – 13.0) and constant temperature of 30 °C. After 48 h, cellulase activity was quantified. Similarly, influence of temperature was investigated by varying the growth temperature (25 – 50 °C) at optimum pH, keeping the other parameters constant.

8.1.6 Properties of the extracellular cellulase

The optimum pH for cellulase activity was determined with CMC as substrate dissolved in the following buffer systems: potassium phosphate buffer (pH 6.0-8.0) and glycine-NaOH buffer (pH 9.0-12.0). The optimum temperature was determined for the cellulase at different temperatures (10-70 °C). For the study of halostability,

the enzyme was pre-incubated with NaCl (0-4 M) at 30 °C for 1 h and the enzyme activity was determined.

8.2 RESULTS AND DISCUSSION

8.2.1 Selection of the cellulase-producing bacterium

Moderately halophilic bacteria were isolated from the coastal regions and salt marsh ecosystems of west coast of Karnataka, India, on MM63 medium containing 6% (w/v) NaCl and CMC as the sole source of carbon. Bacteria growing on this biopolymer medium were expected to harbor the potential enzyme machinery to degrade CMC and utilize the polymer for growth and multiplication. Based on colony characteristics sixteen different organisms (PS33-PS48, numbered serially) were isolated, cultured aerobically in the same medium at 30 °C and maintained as pure cultures in agar stabs and glycerol stock.

Out of these, six strains showed prominent zone of clearance on the CMC agar indicating extracellular cellulolytic activity. The following enzyme activities were recorded (per ml): PS33 (0.075 U), PS36 (0.089 U), PS37 (0.087), PS41 (0.0049), PS43 (0.003 U), PS44 (0.07 U) and PS47 (0.14 U). Therefore, based on the highest enzyme activity, PS47 was selected for further investigation (Fig. 8.1). The strain was isolated from the Baad - Gudeangadi salt marsh ecosystem in Kumta region, Karnataka, India.

8.2.2 Characteristics of the potential strain

Strain PS47 is a Gram-negative, short rod, which formed circular, convex, yellow-coloured mucoid colonies on the agar surface. Cells were encapsulated and motile occurring singly or in pairs. Growth occurred with 0.5-15 % (w/v) NaCl (optimally with 6 %, w/v), at pH 7.0-10.0 (optimally at pH 7.1) with an optimal growth temperature of 30 °C. According to *The Bergey's Manual of Determinative Bacteriology* and on the basis of 16S rRNA gene studies, strain PS47 has been identified as *Halomonas* sp. having 86.6% identity to *Halomonas koreensis*

(AY382579) shown in Fig 8.2. The ribosomal RNA gene sequence has been submitted to GenBank (ID: JQ425853).

The study of growth kinetics of *Halomonas* sp. PS47 with reference to cellulase production in MM63 medium indicated that the lag phase of the organism was small up to 3 h, after which the growth was exponential up to 24 h followed by stationary phase. Cellulase secretion was maximal in the late exponential and early stationary phase (30 – 54 h) with the highest activity recorded at 48 h (0.0076 U/ml) in the basal medium. Zone of clearance in the screen-plate and optimum levels of cellulase secretion in the basal MM63 medium indicate cellulolytic activity as an inherent property of *Halomonas* sp. PS47.

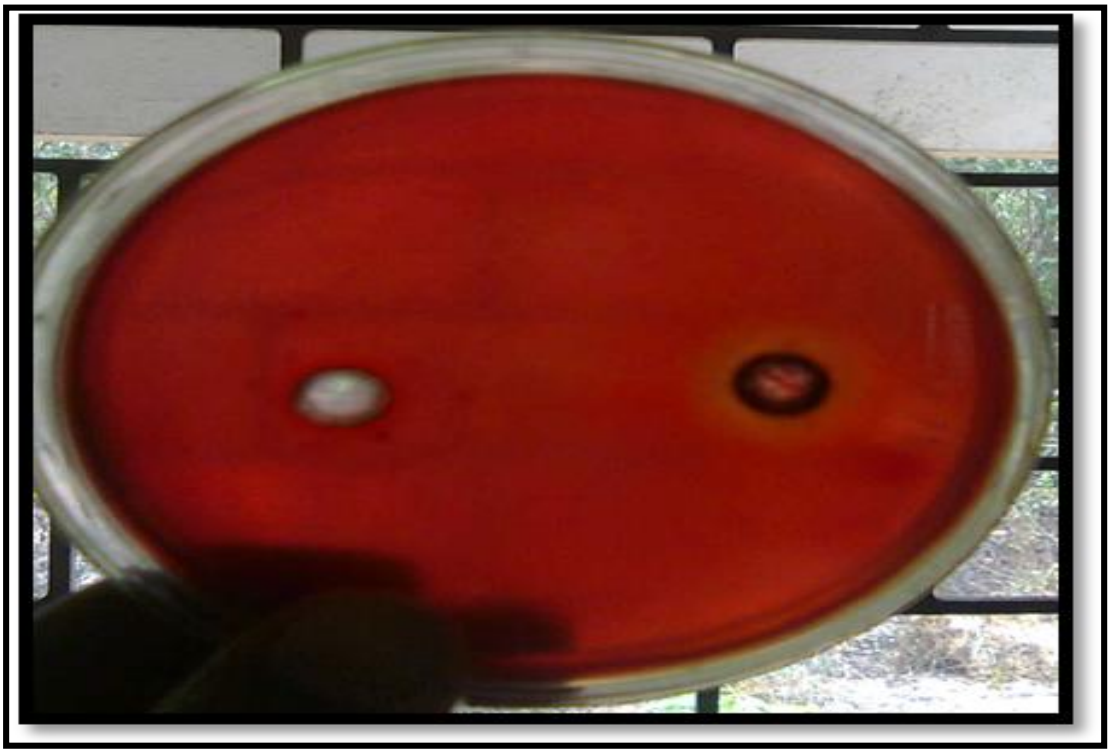


Figure 8.1 MM63 plate containing CMC as the sole carbon source was incubated with the supernatant of PS47 in the right well against distilled water blank in the left well. Congo Red dye-staining shows zone of clearance around the supernatant well on the right side indicating cellulase activity.

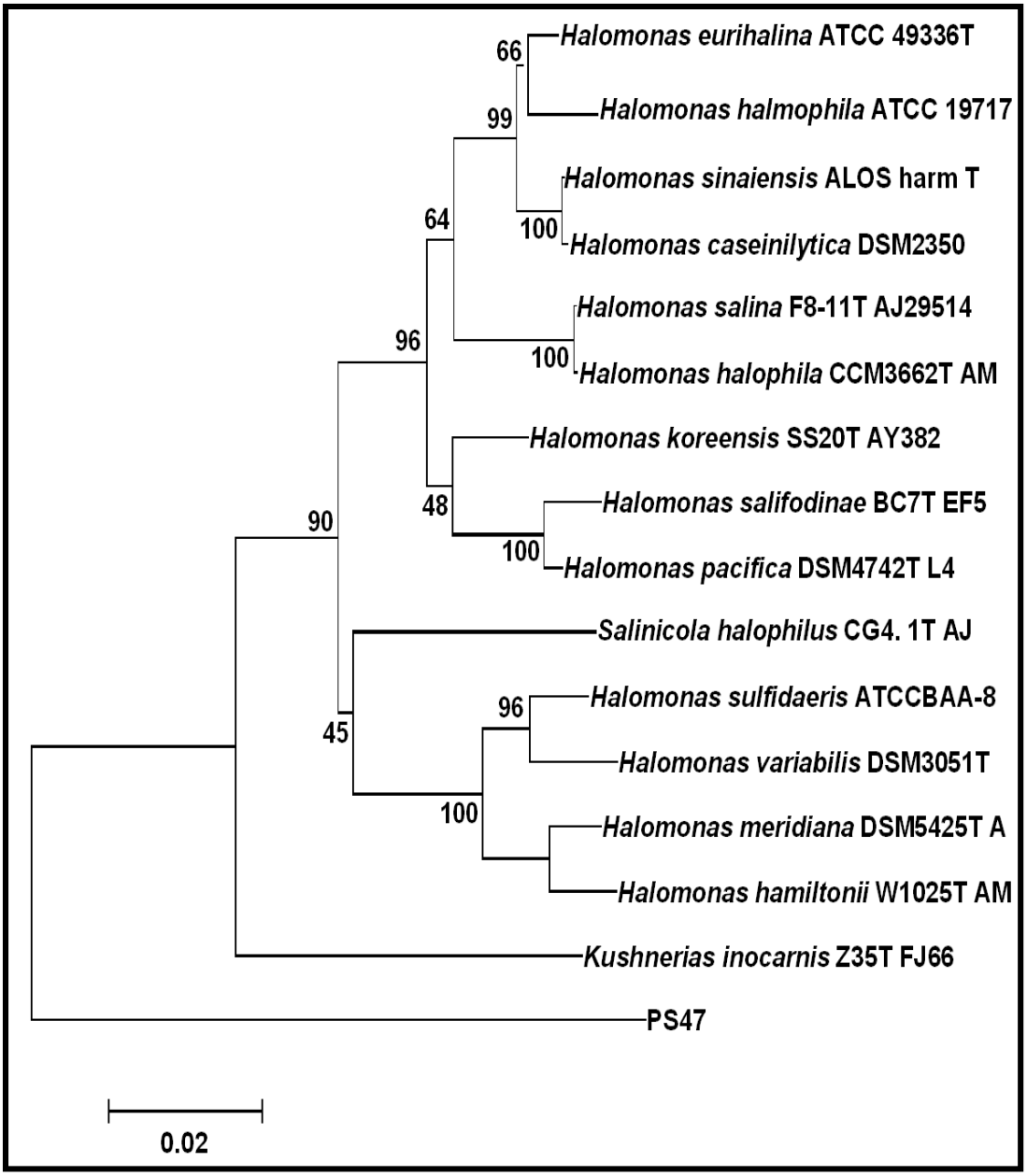


Figure 8.2 Neighbour-joining tree showing the position of isolate *Halomonas* sp. PS47 to a selected number of members of the halophilic bacteria. Values are the branch lengths reflecting the actual distances between the sequences.

8.2.3 Cellulase production in MM63 medium

Cellulase production was investigated in the basal MM63 medium in the presence of CMC, yeast extract and peptone (Fig. 8.3a). The enzyme activity increased around 5 times to 0.036 U/ml when the medium containing glucose was supplemented with complex nitrogen sources like yeast extract and peptone in place of inorganic nitrogen source $(NH_4)_2SO_4$. When CMC was used as carbon source instead of glucose and $(NH_4)_2SO_4$ as the nitrogen source in the basal medium the enzyme activity was found to be 0.03 U/ml, 4 times the original value. However, only yeast extract and peptone in the absence of any other carbon source failed to induce substantial enzyme production (0.0056 U/ml). The highest activity (0.14 U/ml) was seen when glucose in MM63 medium was replaced by CMC and $(NH_4)_2SO_4$ was replaced by a combination of yeast extract and peptone as the organic nitrogen sources. The activity was 18 fold higher than the original value. The enzyme secretion corresponded with growth and was higher in late exponential and stationary phases of growth (Fig. 8.3b).

This shows that CMC acts as an inducer while yeast extract and peptone enhance the cellulase enzyme production by *Halomonas* sp. PS47. Cellulase production is controlled by catabolite repression and induction, as reported for the alkaline cellulases from *Bacillus* sp. KSM-19, KAM-64 and KSM-520 (Shikata et al. 1990). The production of cellulases is induced in the presence of substrate and is repressed in the presence of easily utilisable sugars in the medium.

The cellulolytic capabilities of different microorganisms are highly variable. Although the reported activity is lower compared to the activities of some fungal strains, it is substantial when compared to those reported from bacterial species. Every microorganism has its own special condition for growth and metabolite production. Optimization of the medium components and growth conditions using statistical methodologies would result in enhanced production of cellulase.

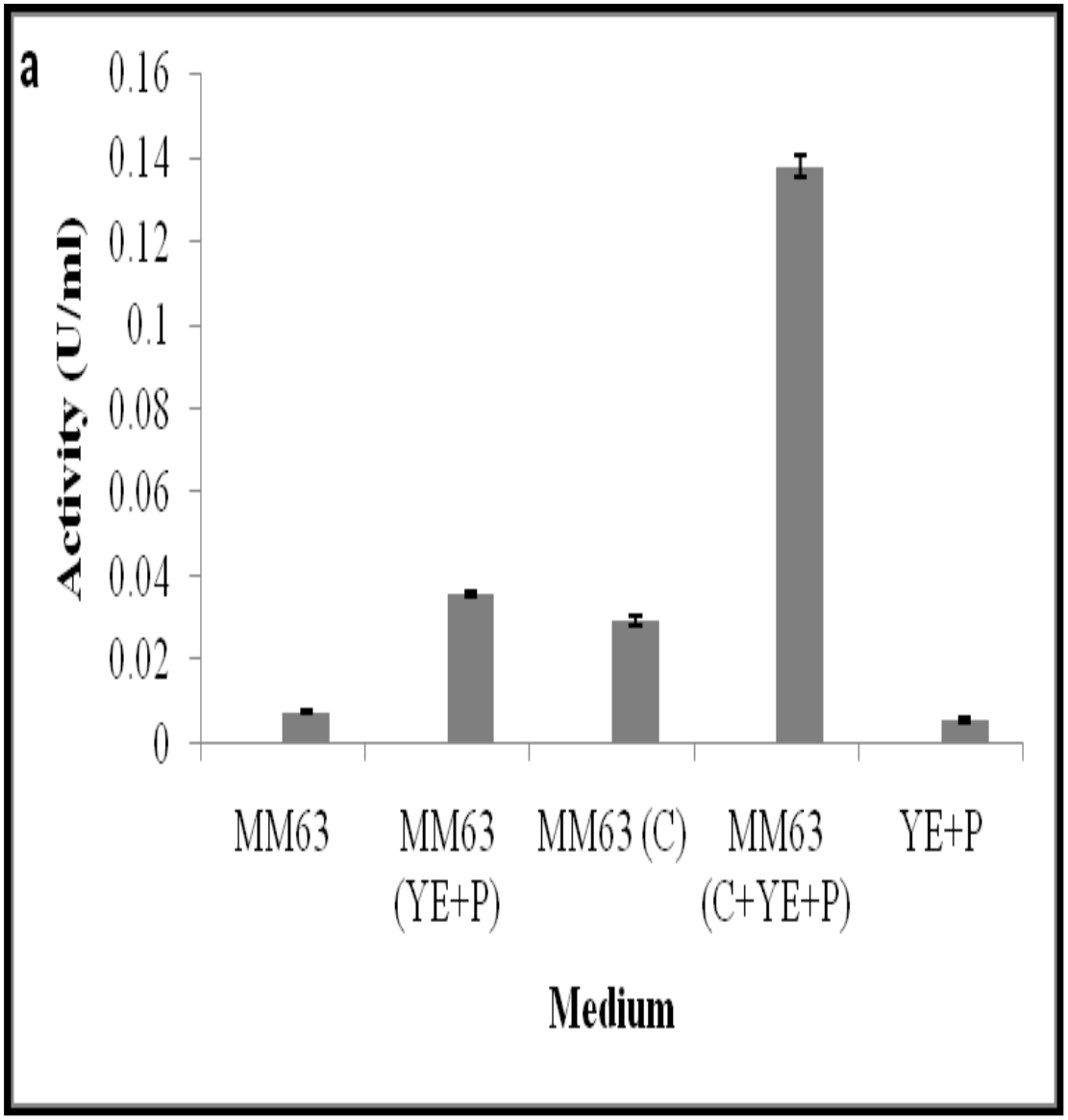


Figure 8.3 (a) Cellulase production of *Halomonas* sp. PS47 in MM63 medium. C-CMC; P-peptone; YE-yeast extract.

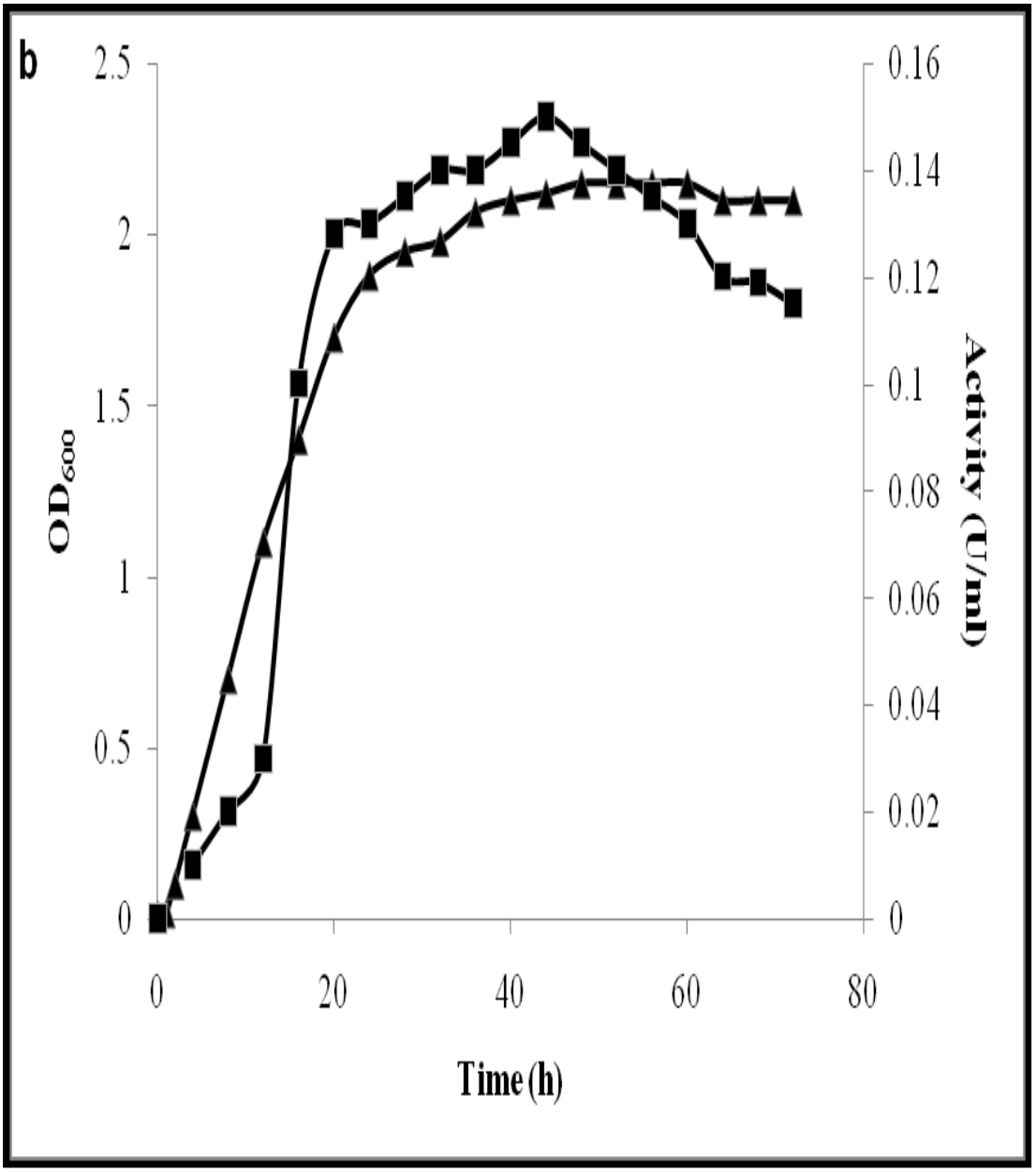


Figure 8.3 (b) Growth curve and cellulase secretion of *Halomonas* sp. PS47 in MM63 medium containing CMC. Samples were withdrawn at 4 h interval for the determination of cell growth by OD_{600} (▲) and cellulase activity (■).

Deka et al. (2011) reported cellulase activity of *Bacillus subtilis* AS3 in the unoptimised CMC medium to be 0.07 U/ml. By optimising the medium further (CMC, peptone and yeast extract) a six fold increase in enzyme activity was obtained (0.43 U/ml). A maximum cellulase activity of 0.043 U/ml has been reported from cell-free culture supernatants of *Geobacillus* sp. isolated from deep goldmine environment (Rastogi et al. 2009). Li et al. (2008) reported maximum cellulase activity (0.26 U/ml) of a *Bacillus* sp. when the culture was grown in LB medium supplemented with 1% CMC. In another study, a cellulase activity of 0.0113 U/ml was observed under optimised conditions from *Geobacillus* sp. (Tai et al. 2004). These studies give a brief idea of the different cellulolytic potentials of microorganisms. In general, higher production of enzyme requires the presence of complex nitrogen sources. Yeast extract and peptone have been reported to have a significant effect on cellulase production. Yeast extract is known to be an effective medium component for the growth of halophilic bacteria (Shivanand and Jayaraman, 2009). A low-cost fermentation medium can be designed for the production of halostable cellulase by using agricultural byproducts. These complex carbohydrate sources can serve as basal and optimized medium for obtaining higher yields of the enzyme.

8.2.4 Cellulase production on different agricultural residues

Among the different agricultural residues used, wheat bran was found to yield the highest amount of cellulase (0.079 U/ml) as shown in Fig. 8.4a. Rice bran and cobs could also yield substantial amounts of cellulase (0.059 and 0.06 U/ml, respectively). Lower levels of cellulase activity were detected when sugarcane bagasse (0.04 U/ml) and groundnut shells (0.049 U/ml) were used as the carbon source. The addition of yeast extract and peptone to the pre-treated wheat bran medium resulted in over two-fold increase in activity to 0.12 U/ml. The enzyme secretion in the wheat bran medium was higher in the stationary phase of growth (Fig. 8.4b). Higher production of enzyme requires the presence of complex nitrogen sources (Singh et al. 2001).

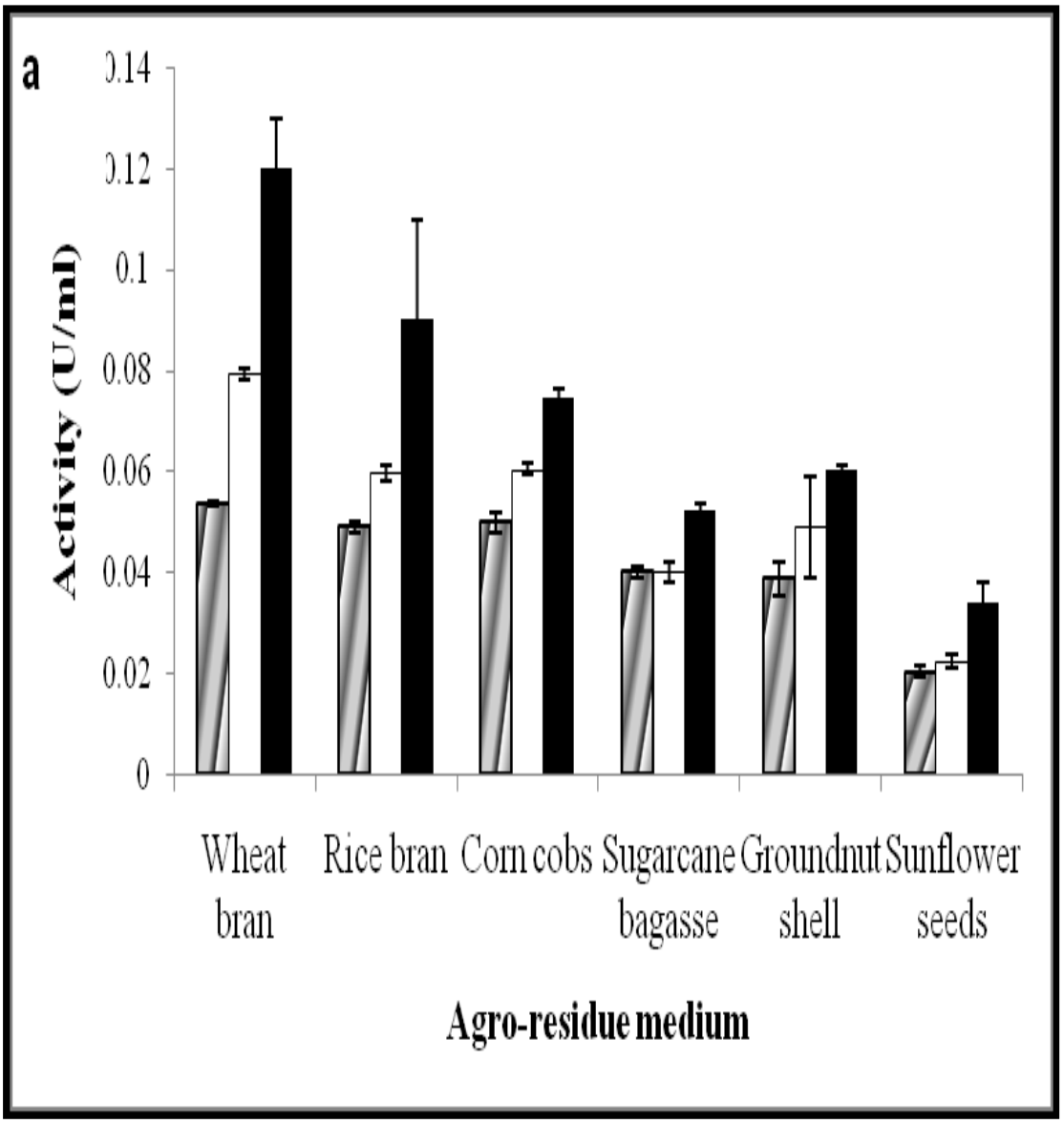


Figure 8.4 (a) Cellulase production of *Halomonas* sp. PS47 in MM63 medium containing (▨) – untreated agro-residue; (□) – treated agro-residue and (■) – treated agro-residue along with yeast extract and peptone.

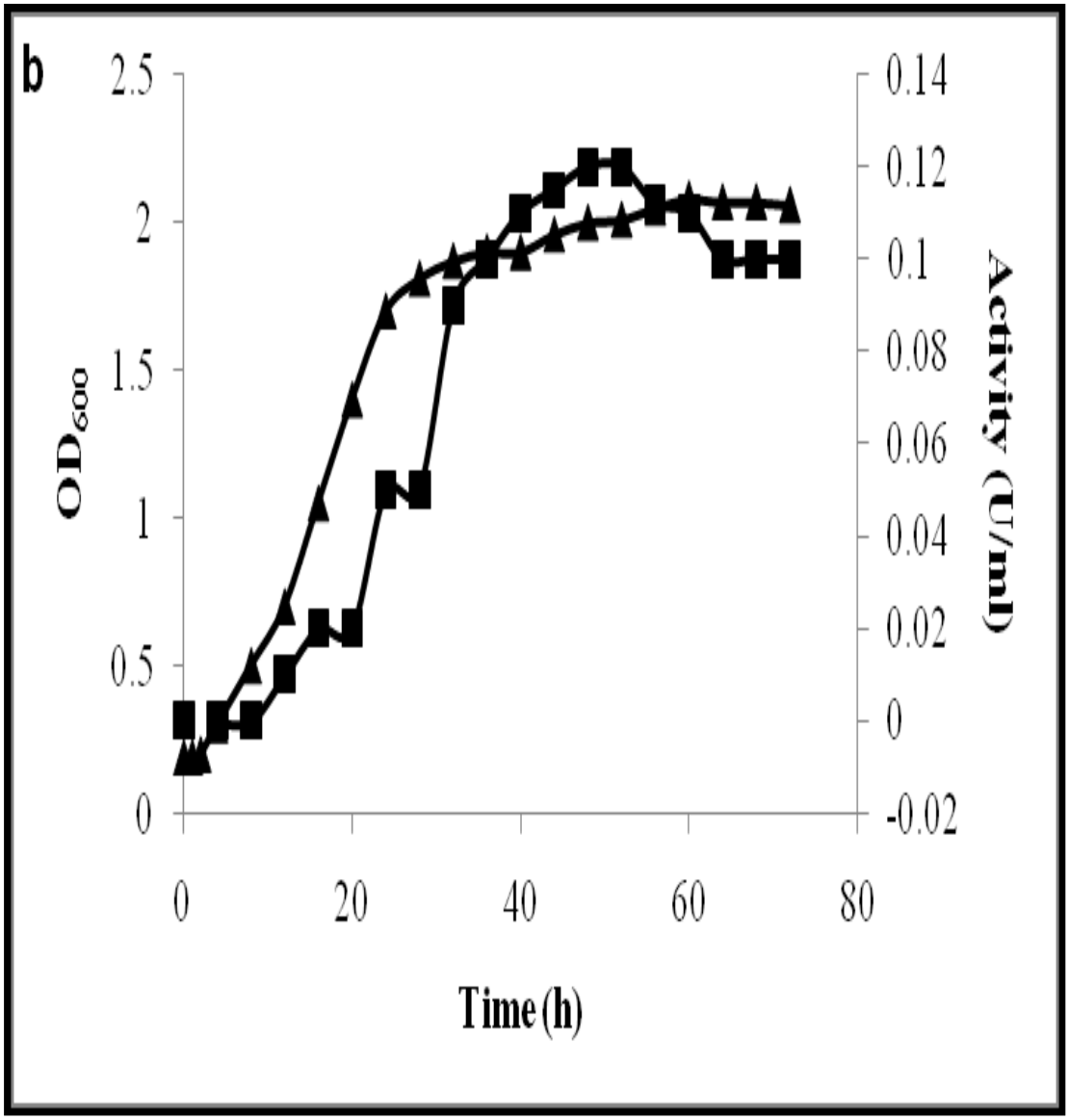


Figure 8.4 (b) Growth curve and cellulase secretion of *Halomonas* sp. PS47 in MM63 medium containing wheat bran. Samples were withdrawn at 4 h interval for the determination of cell growth by OD_{600} (▲) cellulase activity (■).

Utilization of cellulosic wastes as cheap carbon and energy sources can serve as alternative to our rapidly depleting finite sources of fossil fuels. Halophilic bacteria capable of utilizing cheap renewable agricultural wastes could be exploited as an economic alternative to existing production processes.

Utilization of pure carbon sources is impractical for commercial production of enzymes owing to their high costs. The production of cellulase demonstrated in this study can be used for the hydrolysis of lignocellulosic biomass which can in turn be used for the cost-effective production of ethanol.

8.2.5 Growth and cellulase production in media with different salt concentration

Growth (Fig. 8.5a) in media with different concentrations of NaCl followed a similar pattern, where exponential phase was observed from 4 h – 24 h followed by stationary phase. The bacterium was able to grow well up to NaCl concentration of 15% (w/v). Efficient growth was not seen at higher concentrations of NaCl. Growth was optimal at NaCl concentrations in the range of 3% - 9% (w/v) where the bacterium followed similar growth patterns.

Cellulase production (Fig. 8.5b) was also higher in this range of NaCl (3- 9%, w/v), the optimum being at 6% (w/v). Though growth was much stunted at 12% and 15% (w/v) NaCl, the bacterium was able to produce extracellular cellulase. These studies confirm the moderately halophilic nature of the bacterium and its versatility in adaptations to increasing levels of salinity.

8.2.6 Effect of pH and temperature

Halomonas sp. PS47 could grow and produce extracellular cellulase over a wide range of pH (6.0-10.0). Higher activity was seen in the pH range of 6.5 – 8. Maximum cellulase production was obtained at pH 7.5 (Fig. 8.6a). The optimum temperature for cellulase production was 30 °C (Fig. 8.6b). Production was significantly reduced at 45 °C, which was not favourable for growth.

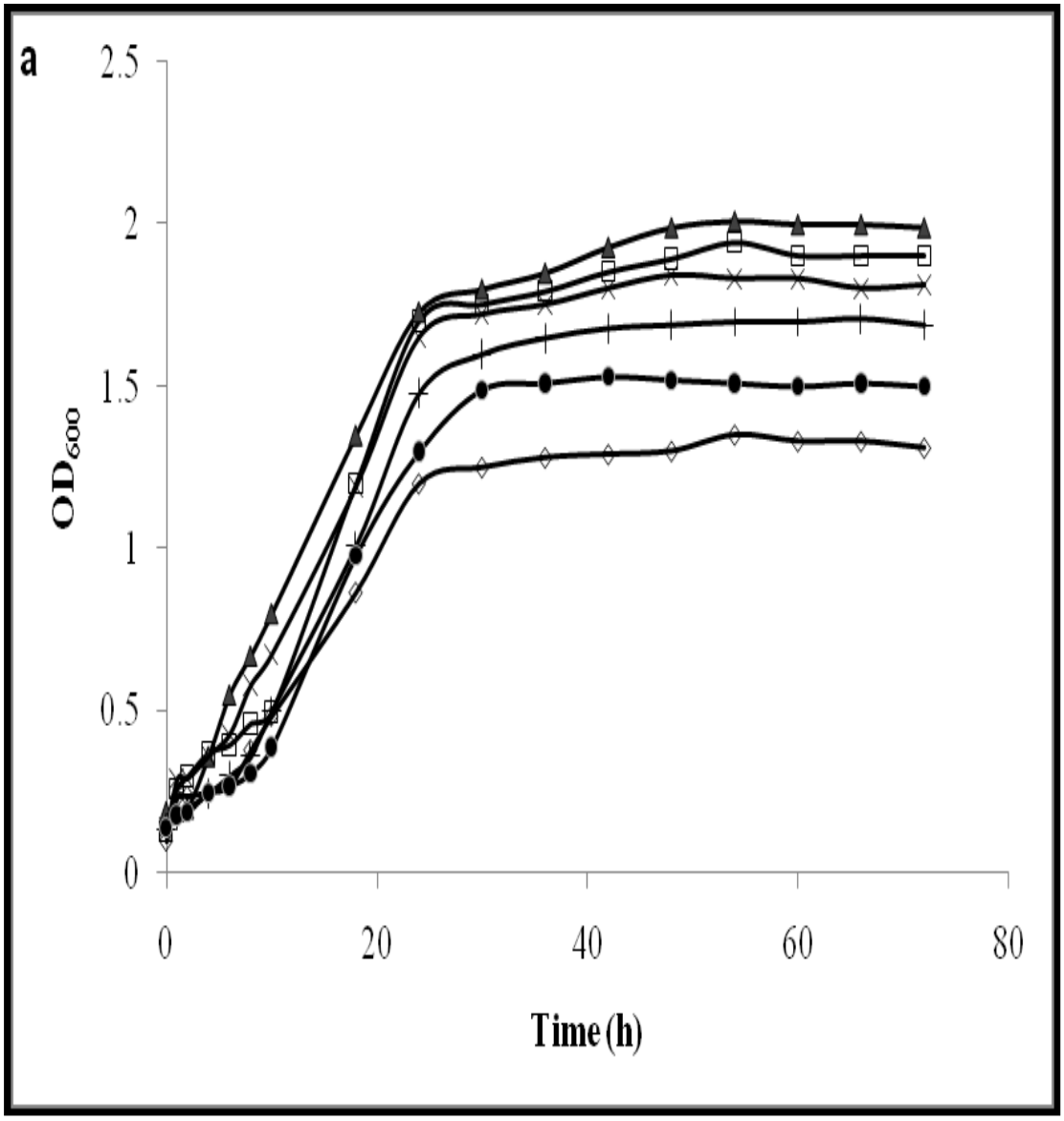


Figure 8.5 (a) Growth curves of *Halomonas* sp. PS47 in media with different salt concentrations, % (w/v) - 0 (◊); 3 M (□); 6 M (▲); 9 (×); 12 (+) and 15 (●). Samples were withdrawn at every 6 h interval for the determination of cell growth (OD_{600}).

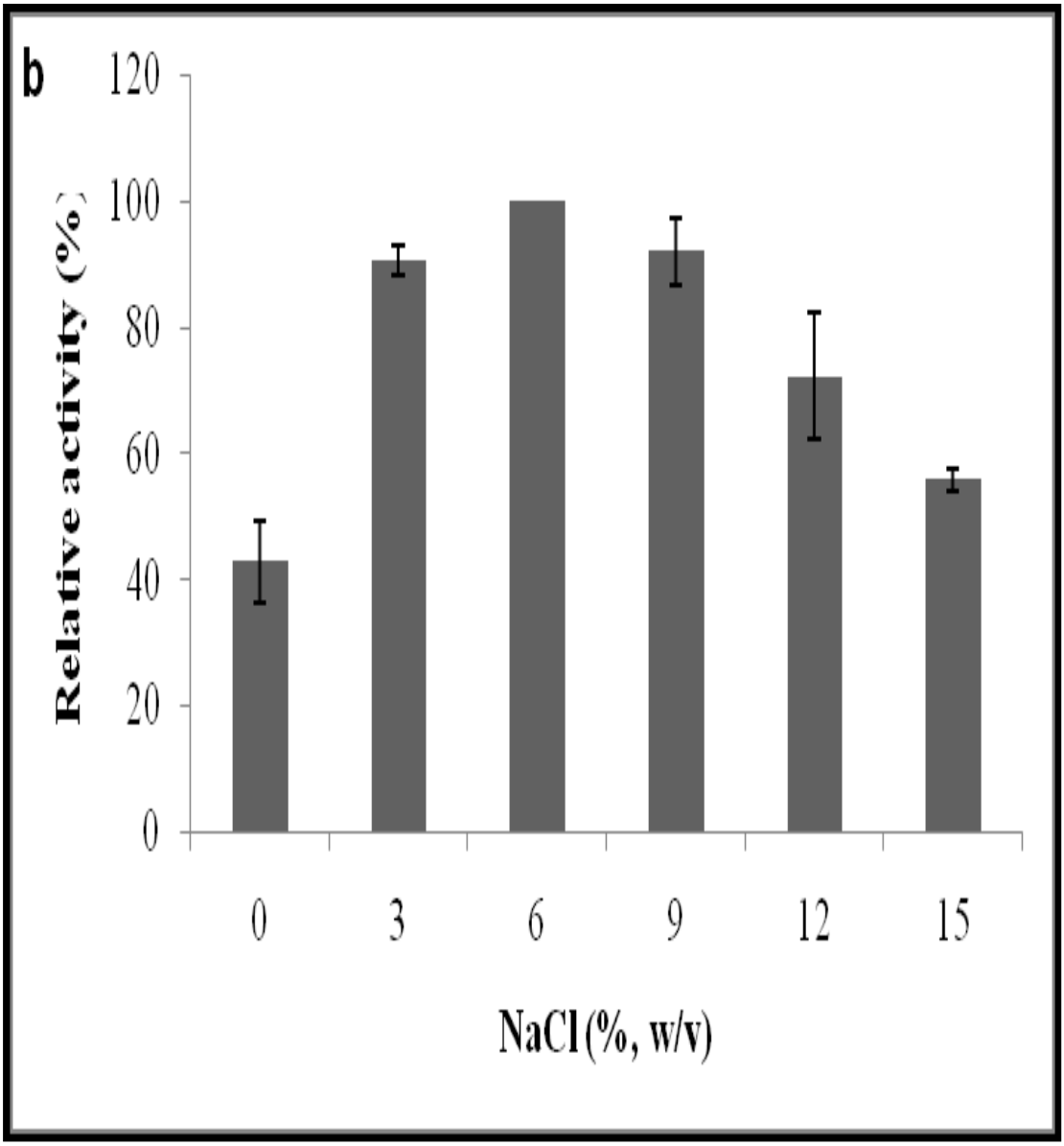


Figure 8.5 (b) Cellulase production of *Halomonas* sp. PS47 in media with different salt concentrations. The highest activity in 6% (w/v) NaCl medium is taken as 100%.

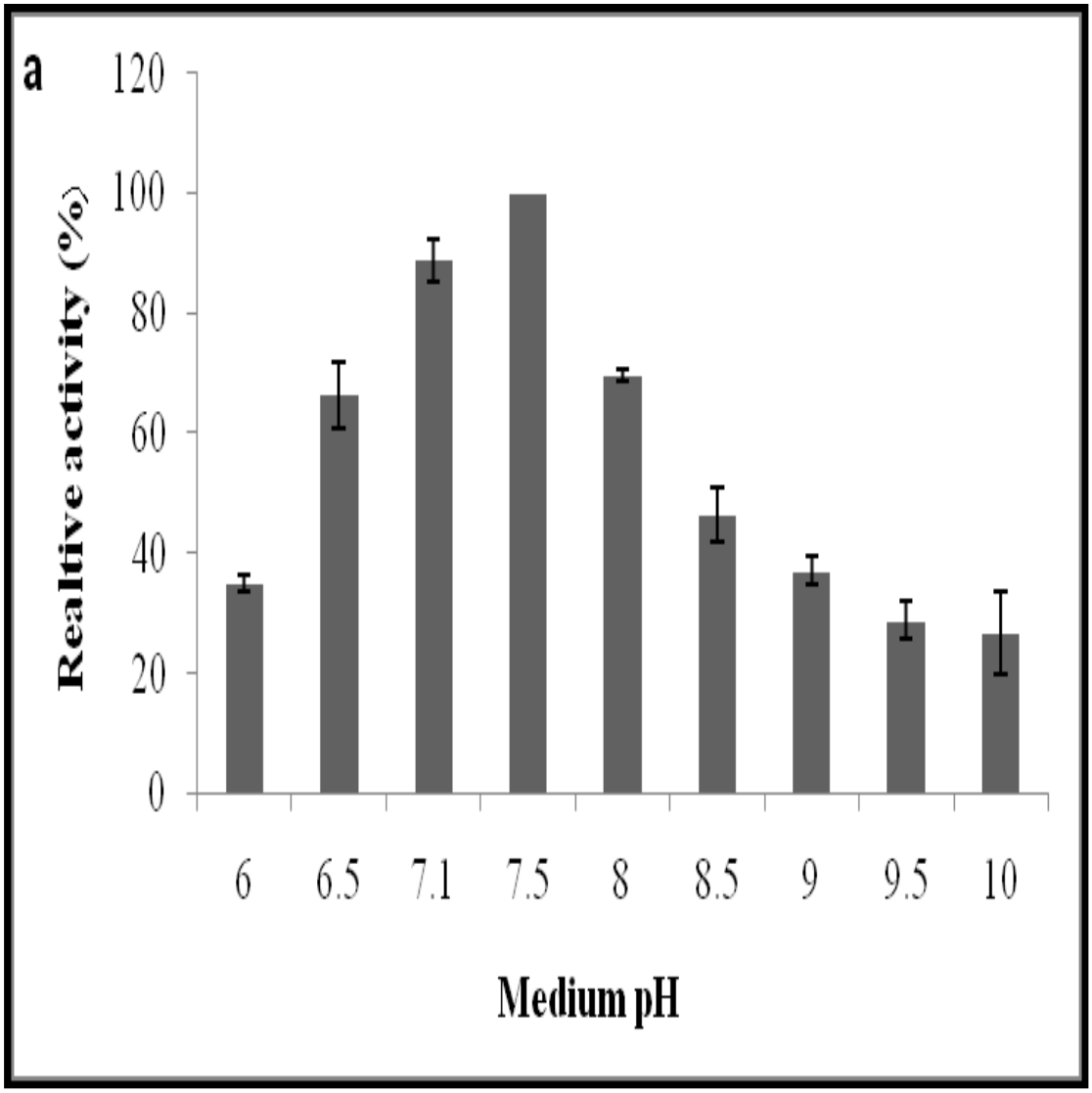


Figure 8.6 (a) Effect of medium pH on cellulase production by *Halomonas* sp. PS47. Samples were taken after incubation of 48 h at 30 °C, for the determination of cellulase activity (U/ml). Highest production at pH 7.5 is taken as 100%.

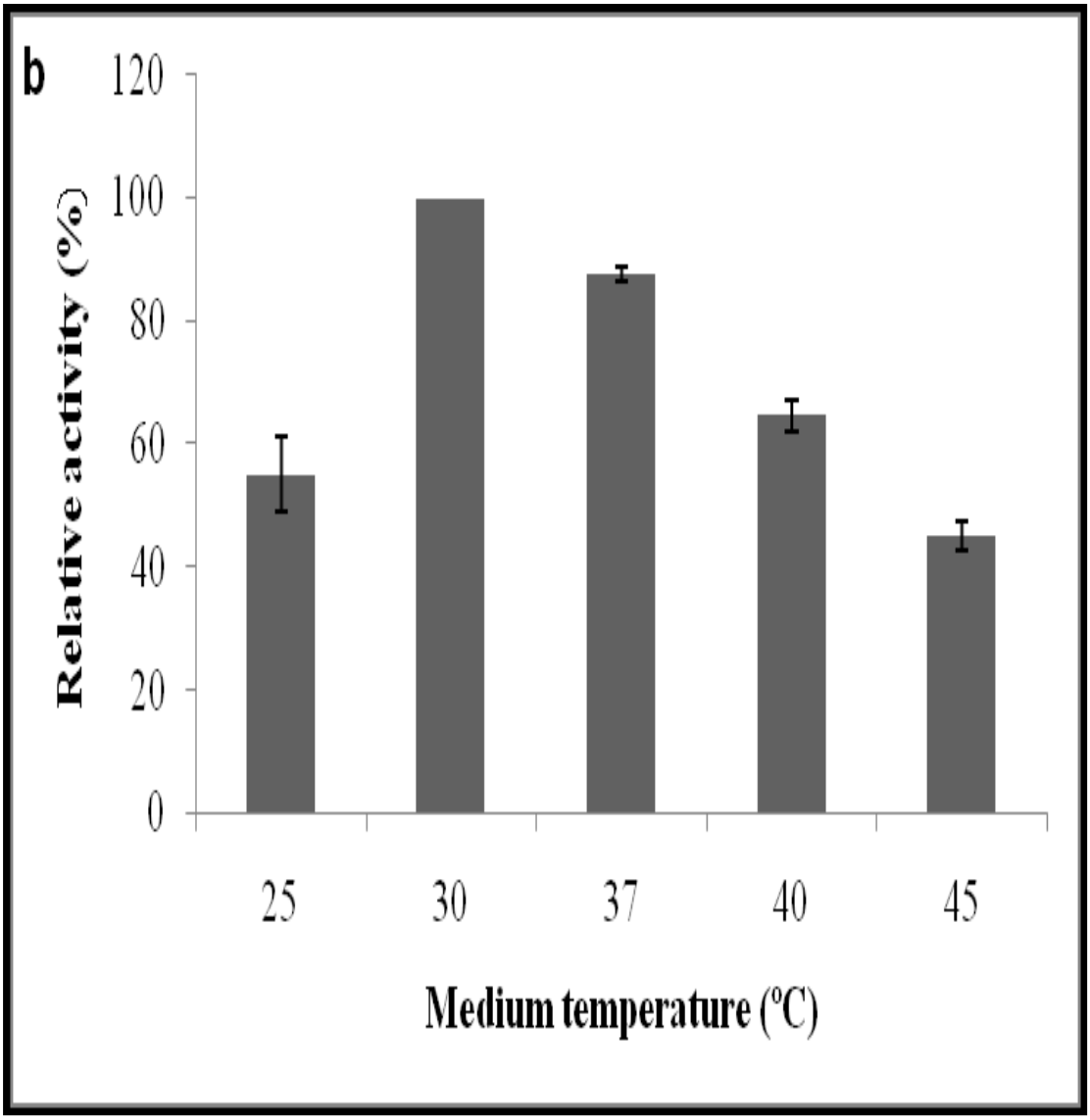


Figure 8.6 (b) Effect of medium temperature on cellulase production by *Halomonas* sp. PS47. Samples were withdrawn after incubation of 48 h at pH 7.5, for the determination of cellulase activity (U/ml). Highest production at 30 °C is taken as 100%.

8.2.7 Properties of the extracellular cellulase

The cellulase was active in the pH range of 7.0 -10.0, with an optimum at pH 7.5 (Table 8.1). The enzyme had maximum cellulolytic activity at 50 °C, but is active over a wide range of temperature (35-65 °C). The enzyme exhibited appreciable activity in the presence of NaCl up to a concentration of 4 M, although the highest activity was seen in the presence of 0 – 1 M NaCl. The activity was reduced to 65% of the original value at 3 - 4 M NaCl. Halostable enzymes constitute an excellent model for structural adaptations. Although halophilic enzymes display identical enzymatic functions as their non-halophilic counterparts, they have different properties, including stability and activity at different range of salinity (Amoozegar et al. 2007). Halostability is an important characteristic that will facilitate the future application of the cellulase in biotechnological processes containing high salinity or osmotic pressures. The ability of the moderately halophilic bacteria to survive and produce enzymes that are active over a very wide range of salinities made them attractive candidates for the isolation of novel enzymes.

Table 8.1 Properties of the extracellular cellulase produced by *Halomonas* sp. PS47

pH opt.	7.1
T opt.	50 °C
NaCl stability	
0 M	0.12±0.00057 U/ml
0.5 M	0.117±0.0006 U/ml
1 M	0.115±0.003 U/ml
1.5 M	0.099±0.0011 U/ml
2 M	0.087±0.0025 U/ml
3 M	0.079±0.001 U/ml
4 M	0.0783±0.003 U/ml

8.3 SUMMARY

Halomonas sp. PS47 (GenBank ID: JQ425853) produced cellulase (0.0076 U/ml) on basal MM63 medium. Higher activity (0.14 U/ml) was seen when glucose in MM63 medium was replaced by CMC and $(NH_4)_2SO_4$ was replaced by a combination of yeast extract and peptone. Strain PS47 produced higher cellulase activities (U/ml) - on wheat bran (0.079), corn cobs (0.06), rice bran (0.059 U/ml), sugarcane bagasse (0.04) and groundnut shells (0.049). Addition of yeast extract and peptone to the pre-treated wheat bran medium resulted in over two-fold increase in activity to 0.12 U/ml. PS47 cellulase exhibited highest activity at pH 7.5 and 50 °C. Enzyme exhibited stability up to 4 M NaCl and higher activity at 0 – 1 M NaCl. The activity was reduced to 65% of the original value at 3 - 4 M NaCl.

The study assumes significance in the ability of the halophilic bacterium to survive in a wide range of salinity and yield optimum levels of extracellular halostable cellulase using salt solution in approximately the same proportion as found in sea water, using biopolymers and agro-residues as medium components. Results show that *Halomonas* sp. PS6 is a potential candidate for halostable cellulase production and that the culture conditions can be further optimised to get higher yields of extracellular cellulase.

Chapter 9

Optimisation of halostable cellulase production from *Halomonas* sp. PS47

9.1 MATERIALS AND METHODS

9.1.1 Screening of significant factors by Plackett-Burman design

Medium components and conditions for cellulase production were screened and significant factors identified by the Plackett-Burman design using statistical software package 'Design-Expert® Version 8.0.4', Stat-Ease, Inc., and (Minneapolis, MN, USA). A total of eleven parameters were considered at two levels: −1 (low level) and +1 (high level) namely A- wheat bran (10-50 g/l), B- yeast extract (2-5 g/l), C- peptone (2-5 g/l), D- NaCl (50-100 g/l), E- K_2HPO_4 (8-13 g/l), F- KOH (1-4 g/l), G- pH (7-8), H- temperature (25-40 °C), J- $MgSO_4 \cdot 7H_2O$ (0.25-1 g/l), K- $(NH_4)_2SO_4$ (2-5 g/l), L- Rice bran (10-50 g/l). Cellulase activity was taken as the response. All experiments were carried out in duplicate.

The design is based on the first-order polynomial model: $Y = \beta_0 + \sum \beta_i X_i$, where Y is the response (enzyme activity), β_0 is the model intercept, β_i is the linear coefficient, and X_i is the level of the independent variable. From the regression analysis the variables, which were significant at or above 95% level (*p-value* < .05), were considered to have greater impact on cellulase activity and were further optimized by central composite design.

9.1.2 Optimization of key determinants and statistical analysis

The next stage in the medium formulation was to determine the optimum levels of the significant variables, viz. A - wheat bran (30-60 g/l), B - yeast extract (2-4 g/l) and C - $MgSO_4.7H_2O$ (0.3-0.5 g/l) and study the interaction between them. For this purpose, central composite design was adopted for improving cellulase activity using the Design Expert software. A total of 20 experiments were conducted. All the

variables were taken at a central coded value, which was considered as zero. The average maximum cellulase yield was taken as the independent variable or response (Y).

The data obtained were subjected to analysis of variance (ANOVA). Once the cellulase activity (U/ml) was determined, a second order polynomial model was fitted to the response data obtained from the design by the multiple regression procedure. This resulted in an empirical model that related the response measured to the independent variables of the experiment. The polynomial equation for a three-variable system is in the following form:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC \text{.....(9.1)}$$

with, Y, predicted response; A, B, C, variables; β_0, intercept; β_1, β_2, β_3, linear coefficients; β_{11}, β_{22}, β_{33}, squared coefficients; β_{12}, β_{13}, β_{23}, interaction coefficients. The statistical significance of the model equation and model terms was evaluated via the Fisher's test. The quality of fit of the second-order polynomial model equation was expressed via the coefficient of determination (R^2) and the adjusted R^2. The three-dimensional response surface curves were then plotted to understand the interaction of the medium components and the optimum concentration of each component required for maximum cellulase production. The combination of different optimized variables, which yielded the maximum response, was determined to verify the validity of the model. An experiment was conducted with initial and optimized media to verify the accuracy of the predicted model.

9.2 RESULTS AND DISCUSSION

9.2.1 Screening of significant factors by Plackett-Burman design

The effects of eleven medium components and conditions were investigated for cellulase production in 12 runs using Plackett-Burman design. The variation in cellulase activity (Table 9.1) reflected the significance of factors on the enzyme activity.

Table 9.1 Plackett-Burman design for eleven variables along with cellulase activity taken as response

Run	A g/l	B g/l	C g/l	D g/l	E g/l	F g/l	G	H ºC	J g/l	K g/l	L g/l	R U/ml
1	50	5	2	50	8	4	7	40	1	2	50	0.079
2	50	2	5	100	13	1	7	25	1	2	50	0.125
3	50	5	2	100	13	4	7	25	0.25	5	10	0.131
4	10	2	2	50	8	1	7	25	0.25	2	10	0.052
5	50	2	5	100	8	4	8	40	0.25	2	10	0.062
6	10	5	2	100	13	1	8	40	1	2	10	0.053
7	10	5	5	50	13	4	8	25	0.25	2	50	0.062
8	10	2	5	50	13	4	7	40	1	5	10	0.032
9	10	2	2	100	8	4	8	25	1	5	50	0.072
10	50	2	2	50	13	1	8	40	0.25	5	50	0.045
11	50	5	5	50	8	1	8	25	1	5	10	0.137
12	10	5	5	100	8	1	7	40	0.25	5	50	0.037

A-Wheat bran, B-Yeast extract, C-Peptone, D-NaCl, E-KH_2PO_4, F-KOH, G-pH, H-Temperature, J-$MgSO_4.7H_2O$, K-$(NH_4)_2SO_4$, L-Rice bran

Among the variables screened, the most effective factors with high level of significance were in the order of wheat bran, yeast extract and $MgSO_4.7H_2O$ as shown in Fig. 9.1. Hence these variables were chosen for further optimization studies with the ranges given in Table 9.2. The model F value of 198.26 implies the model is significant. There is only a 0.5% chance that a model F-value this large could occur due to noise. The value of multiple correlation coefficient, R^2 (0.9989) indicates good correlation between predicted and experimental responses. The predicted R^2 of 0.9597 is in reasonable agreement with the adjusted R^2 of 0.9938. *Adequate precision* measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 39.521 indicates an adequate signal and that the model can be used to navigate the design space.

9.2.2 Optimization of significant variables using central composite design

Higher enzyme activity was observed with increased concentrations of wheat bran. The results of the twenty-trial design for studying the effects of the three independent variables, viz., wheat bran (A), yeast extract (B) and $MgSO_4.7H_2O$ (C) on cellulase production are presented in Table 9.3 along with the predicted and mean observed responses.

Halomonas sp. PS47 synthesizes substantial amount of cellulase to degrade the high amount of cellulose present in wheat bran. Yeast extract contains amino acids like metheonine, asparagine and tryptophan, which are found to be stimulatory for cellulase secretion (Vyas et al. 2005).

Moreover, yeast extract provides a good source of nutrients for the growing cells in a halophilic medium (Shivanand and Jayaraman 2009). The activity of enzyme is influenced by the presence of cofactors. Certain cofactors like metal ions are found to stimulate and in some cases inhibit the activity of enzymes. Mg^{2+} ions act as co-factors for cellulases enhancing the enzyme activity in many cases (Moreno et al. 2009). Hence an attempt is made to optimize the levels of these three components in the medium to get a higher yield of extracellular cellulase from *Halomonas* sp. PS47.

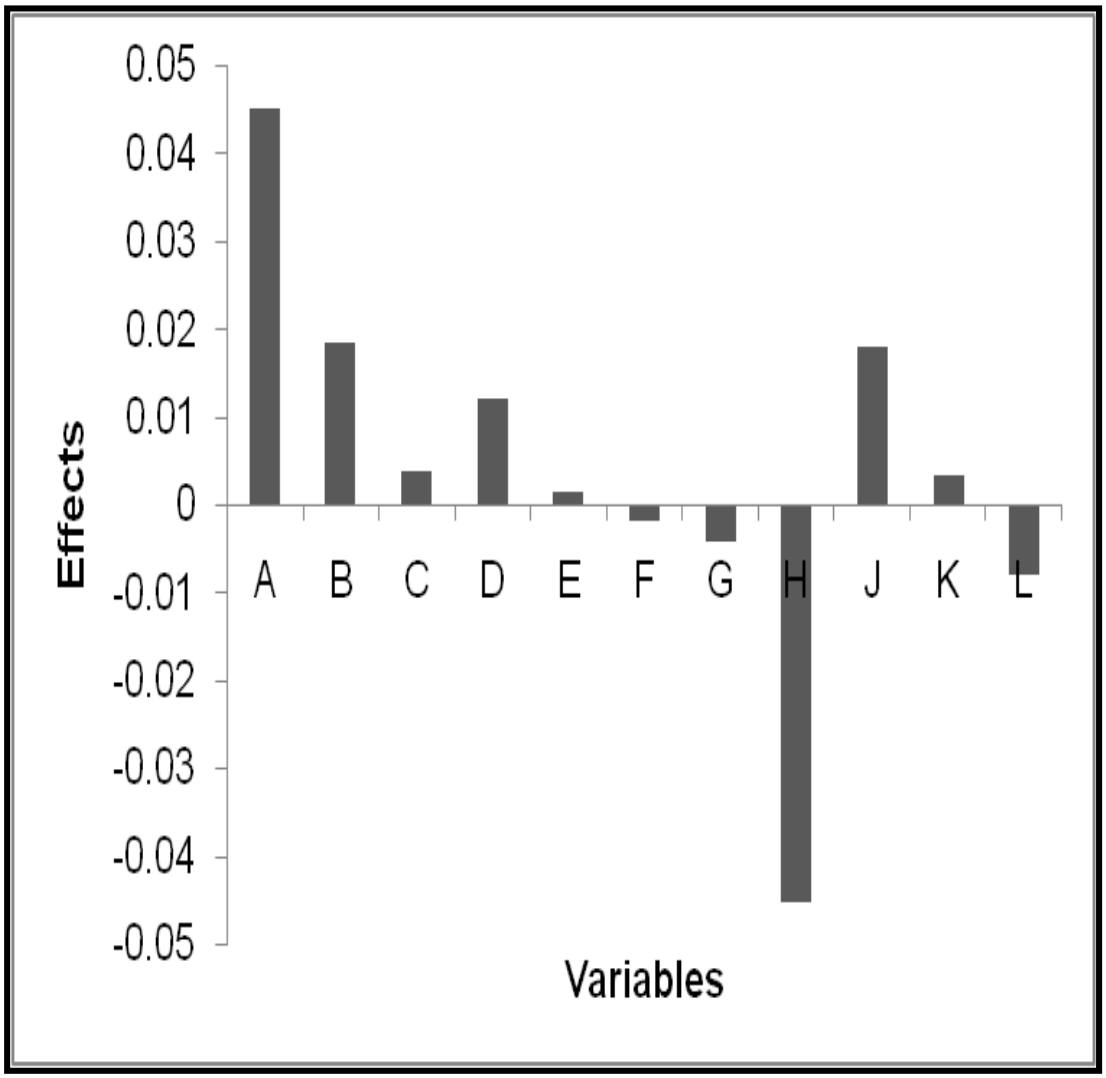


Figure 9.1 Main effects of different variables on cellulase production in Plackett-Burman design. A-Wheat bran, B-Yeast extract, C-Peptone, D-NaCl, E-KH_2PO_4, F-KOH, G-pH, H-Temperature, J-$MgSO_4.7H_2O$, K-$(NH_4)_2SO_4$, L-Rice bran

Table 9.2 Range of variables for central composite design of cellulase production

Variables	Symbol	Range of levels				
		-α	**-1**	**0**	**+1**	**+α**
Wheat bran (g/l)	A	19.77	30	45	60	70.23
Yeast extract (g/l)	B	1.32	2	3	4	4.68
$MgSO_4.7H_2O$ (g/l)	C	0.23	0.3	0.4	0.5	0.57

The adequacy of the model was checked using analysis of variance (ANOVA) which confirms a satisfactory adjustment of the reduced quadratic model to the experimental data. The regression equation indicated the R^2 value of 0.9873 (a value >0.75 indicates fitness of the model), which ensured a satisfactory adjustment of the quadratic model to the experimental data and indicated that 99.75% of the variability in the response could be explained by the model. The 'adjusted R^2' is 0.9749 and the 'predicted R^2' is 0.9626, which are in reasonable agreement. This indicates a high significance of the model as nearer the value of R^2 to 1.0, the more fit the model is deemed to be.

The '*adequate precision* value' of 65.214 indicates an adequate signal. At the same time, a relatively lower value of the coefficient of variation (CV%: 1.47) indicates improved precision and reliability of the conducted experiments. The high *F*-value (412.58) and low *p*-value (<0.0001) indicated that the experimental model is significant. The *p*-value denotes significance of coefficients and also important in understanding the pattern of mutual interactions among variables. The *Lack of Fit* of the model at *p*-value of 0.1477 is not significant suggesting that the obtained experimental data were in good fit.

Table 9.3 Central composite design for three variables along with predicted and observed response

Run	A Wheat bran g/l	B Yeast extract g/l	C $MgSO_4.7H_2O$ g/l	Cellulase Activity U/ml	
				Observed	Predicted
1	45	3	0.57	0.317	0.303
2	60	4	0.3	0.298	0.319
3	45	3	0.4	0.347	0.351
4	30	2	0.5	0.21	0.208
5	45	3	0.4	0.355	0.351
6	60	2	0.5	0.31	0.303
7	30	4	0.3	0.2	0.198
8	45	3	0.4	0.349	0.351
9	45	1.32	0.4	0.292	0.289
10	45	3	0.4	0.35	0.351
11	45	3	0.23	0.297	0.294
12	19.77	3	0.4	0.152	0.149
13	60	4	0.5	0.35	0.343
14	45	4.68	0.4	0.301	0.303
15	30	4	0.5	0.245	0.250
16	60	2	0.3	0.347	0.345
17	45	3	0.4	0.348	0.351
18	30	2	0.3	0.215	0.221
19	70.2	3	0.4	0.329	0.331
20	45	3	0.4	0.346	0.351

The coefficients of the regression equation were calculated using the Design Expert software and the data was fitted to a second order polynomial equation. The cellulase production by *Halomonas* sp. PS46 can be expressed in terms of the following regression equation:

$$Y = + 0.35 + 0.054A + 0.004B + 0.002C - 0.006AB – 0.007AC - 0.016BC – 0.039A^2 –0.019B^2–0.018C^2 \quad .....(9.2)$$

Where Y is cellulase activity (response); A, wheat bran; B, yeast extract and C, $MgSO_4.7H_2O$. The above equation indicates that wheat bran contributed most and also has a significant interaction with yeast extract. The three-dimensional response surface curves were then plotted to understand the interaction effects and optimum levels of the variables. This is done by plotting the response (cellulase activity) on the Z-axis against any two independent variables, while maintaining the other variables at fixed levels (zero, for instance). Fig. 9.2a shows the response surface plot obtained as function of wheat bran *vs.* yeast extract, while MgSO4.7H2O was maintained at zero level. Fig. 9.2b shows the response surface plot obtained as function of yeast extract *vs.* $MgSO_4.7H_2O$, while wheat bran was maintained at zero level. Fig. 9.2c shows the response surface plot obtained as function of wheat bran *vs.* $MgSO_4.7H_2O$, while yeast extract was maintained at zero level. The model predicted a maximum cellulase activity of 0.365 U/ml at wheat bran (50 g/l), yeast extract (3 g/l) and $MgSO_4.7H_2O$ (0.4 g/l).

9.2.3 Experimental validation of the model

To validate the predicted model an experiment was conducted in triplicate using the optimum medium composition. Cellulase activity of 0.35 U/ml was observed at this optimized medium composition against the predicted response of 0.365 U/ml, with a validity of 95.8%. The correlation between predicted and experimental values justifies the validity of the model and the existence of an optimum point. Higher amounts of wheat bran in the medium led to higher enzyme activity. This may be due wheat bran serving as both carbon and nitrogen source.

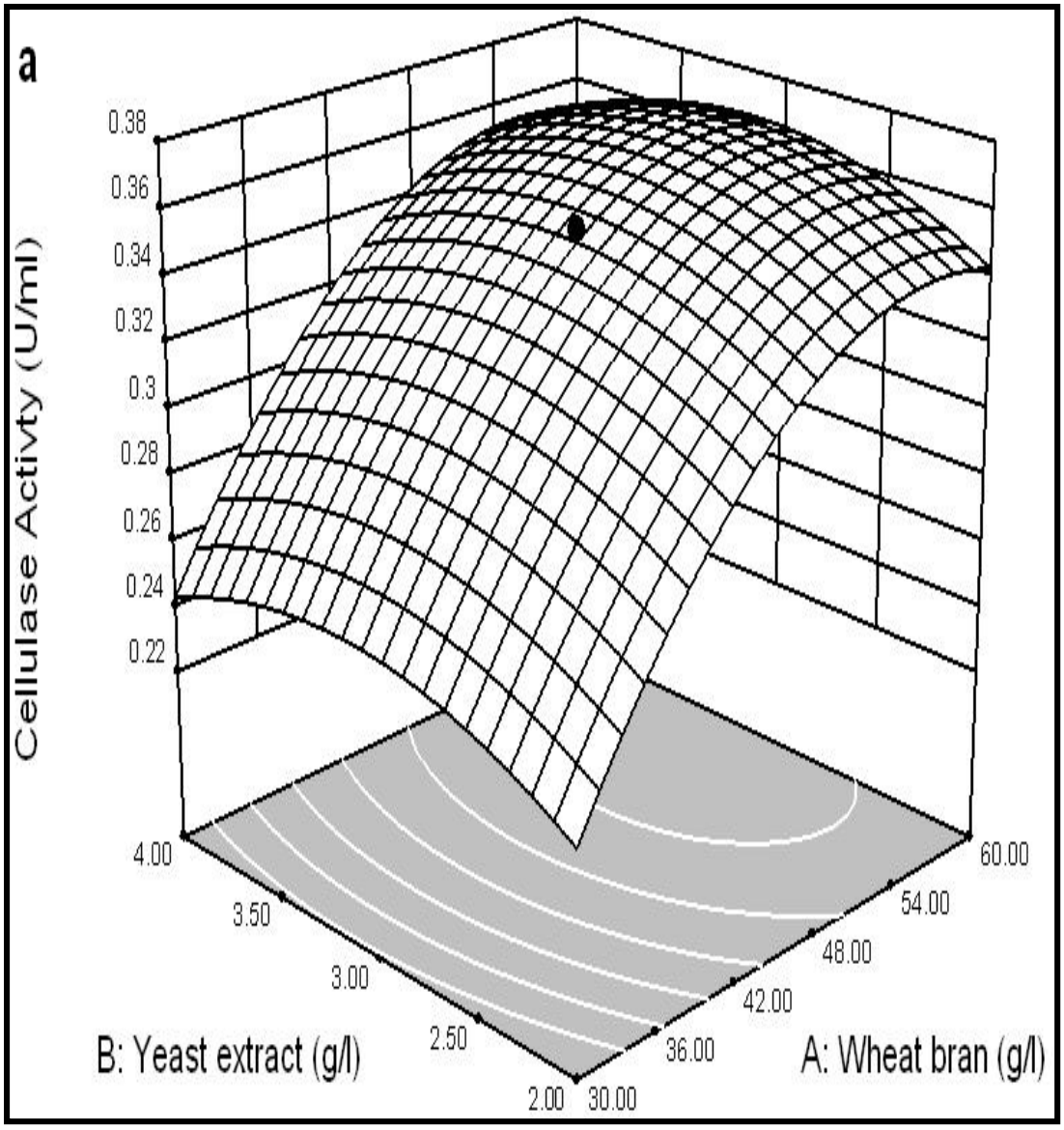
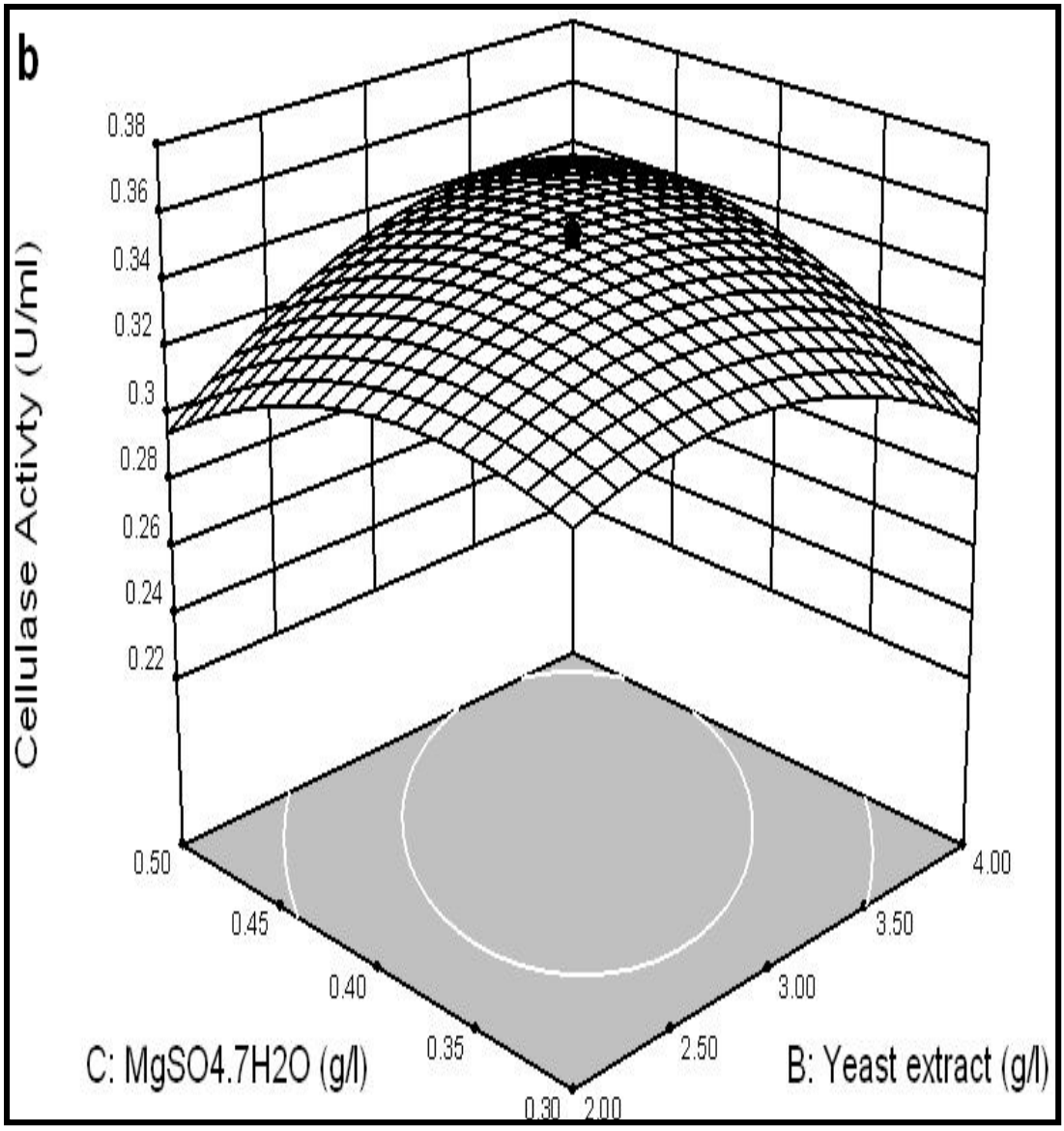
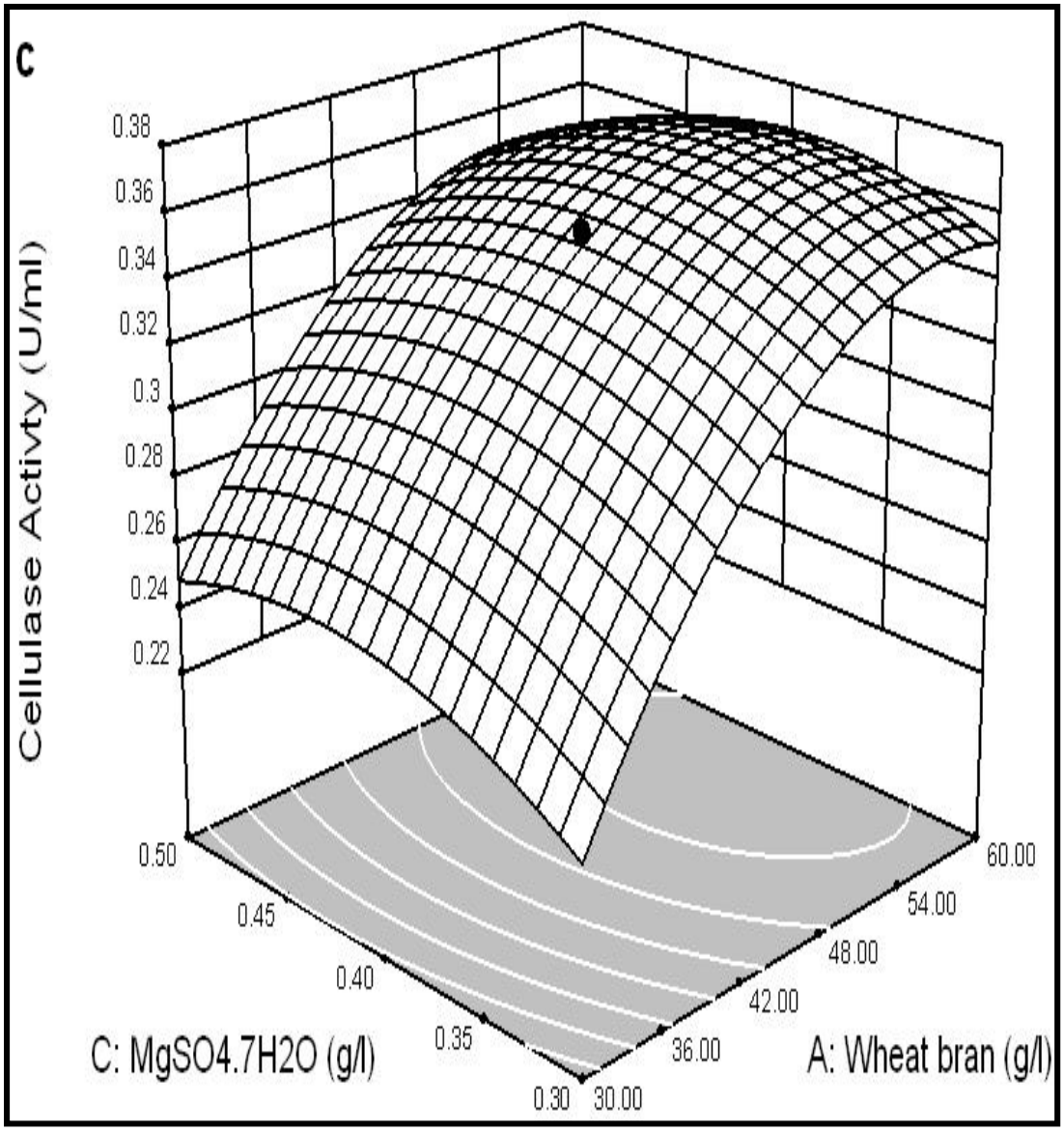


Figure 9.2 Response surface plots for cellulase production by *Halomonas* sp. PS47 (a) wheat bran vs. yeast extract.



(b) yeast extract vs. $MgSO_4.7H_2O$.



(c) wheat bran vs. $MgSO_4.7H_2O$.

9.3 SUMMARY

Using Plackett-Burman design wheat bran, yeast extract and $MgSO_4.7H_2O$ were found to significantly influence cellulase production from *Halomonas* sp. PS47. The concentration of these three medium variables was optimised by response surface methodology. A maximum cellulase activity of 0.365 U/ml at wheat bran 50 g/l, yeast extract 3 g/l and $MgSO_4.7H_2O$ 0.4 g/l was predicted by the model. Cellulase activity of 0.35 U/ml was observed with validity of 95.8% and 3 three-fold increase over the unoptimised medium. The present study establishes an optimized medium with an improvement in cellulase production for *Halomonas* sp. PS47 which is able to utilise different renewable agro-residues for cellulase production. This serves as another example for the application of response surface methodology to biological systems.

Chapter 10

Summary and conclusion

The present investigation on 'The utilization of renewable agricultural resources for ectoine, xylanase and cellulase production from newly isolated halophilic bacteria' was described in the thesis in three parts namely, i. ectoine production ii. xylanase production and iii. cellulase production. Following is the summary of the study:

10.1 SUMMARY

1. Based on Gram staining and morphological studies, several biopolymer degrading bacterial strains were isolated from sea coasts and solar salterns of coastal Karnataka.

2. The isolates were screened for the production of ectoine, xylanase and cellulase and the potential strains were identified by 16S rRNA gene sequence analysis.

3. *Halomonas* sp. PS6 (GenBank ID: KC295600) produced higher amount of ectoine (mg/gdw) - on glucose (71.3) and on a variety of biopolymeric substrates including xylan (45.2), CMC (22.6), inulin (11.8), chitin (8.1) and carob (22.7).

4. Strain PS6 produced substantial amounts of ectoine on treated agro-residues (mg/gdw) - rice bran (60.2), wheat bran (36.2), sugarcane bagasse (34.7), corn cobs (25.5), groundnut shells (18.3) and coir pith (14.1). Higher production of ectoine was seen at increased NaCl concentration (85.4 mg/gdw ectoine at 12% w/v NaCl) and higher temperature (70.5 mg/gdw ectoine at 37 ºC).

5. Substantial amount of ectoine was also detected on un-treated agro-residues. Strain PS6 has an efficient machinery of biopolymer-degrading enzymes.

6. Using response surface methodology studies, a maximum ectoine production of 113.6 mg/gdw at rice bran 50 g/l, NaCl 110 g/l and temperature 37 ºC was predicted. Ectoine production of 112.3 mg/gdw was observed with validity of 98% and two-fold increase over the unoptimised medium.

7. *Brachybacterium* sp. PS3 (GenBank ID: JQ425852) produced xylanase (1.37 U/ml) on basal MM63 medium. Higher activity (4.2 U/ml) was seen when glucose in MM63 medium was replaced by xylan and $(NH_4)_2SO_4$ was replaced by a combination of yeast extract and peptone.

8. Strain PS3 produced higher xylanase activities (U/ml) - on wheat bran (0.9), corn cobs (0.75), rice bran (0.7 U/ml), sugarcane bagasse (0.44), groundnut shells (0.48) and coir pith (0.4) Addition of yeast extract and peptone to the pre-treated wheat bran medium resulted in over two-fold increase in activity to 1.91 U/ml.

9. Using Plackett-Burman design and response surface methodology, a maximum xylanase activity of 8.5 U/ml at pH 9.0, wheat bran 40 g/l, NaCl 90 g/l and corn cobs 30 g/l was predicted. Xylanase activity of 8.23 U/ml was observed with validity of 95.2% and over four-fold increase over the unoptimised medium.

10. PS3 xylanase exhibited highest activity at pH 9.0 and 55 °C. Enzyme exhibited stability up to 4 M NaCl and higher activity at 0 – 1 M NaCl. The activity was reduced to 52% of the original value at 3 M NaCl and to 37% at 4 M NaCl.

11. *Halomonas* sp. PS47 (GenBank ID: JQ425853) produced cellulase (0.0076 U/ml) on basal MM63 medium. Higher activity (0.14 U/ml) was seen when glucose in MM63 medium was replaced by CMC and $(NH_4)_2SO_4$ was replaced by a combination of yeast extract and peptone.

12. Strain PS47 produced higher cellulase activities (U/ml) - on wheat bran (0.079), corn cobs (0.06), rice bran (0.059 U/ml), sugarcane bagasse (0.04)

and groundnut shells (0.049). Addition of yeast extract and peptone to the pre-treated wheat bran medium resulted in over two-fold increase in activity to 0.12 U/ml.

13. Using Plackett-Burman design and response surface methodology, a maximum cellulase activity of 0.365 U/ml at wheat bran 50 g/l, yeast extract 3 g/l and $MgSO_4.7H_2O$ 0.4 g/l. Cellulase activity of 0.35 U/ml was observed with validity of 95.8% and 3 three-fold increase over the unoptimised medium.

14. PS47 cellulase exhibited highest activity at pH 7.5 and 50 °C. Enzyme exhibited stability up to 4 M NaCl and higher activity at 0 – 1 M NaCl. The activity was reduced to 65% of the original value at 3 - 4 M NaCl.

10.2 SIGNIFICANT FINDINGS

1. The ability of the halophilic bacterium to utilize *bio-polymers* in the *synthetic medium* for growth and ectoine production.

2. Production of ectoine using *untreated* agricultural residues. This is significant from industrial viewpoint since pretreatment of agro-wastes and neutralization, two cost-intensive steps, can be avoided.

3. Production of two industrially essential saline hydrolases (xylanases and cellulases), which show optimal activity over a wide range of salinity, temperature and pH.

4. Advantage of using halophilic bacteria is that the salt created after neutralization does not need to be removed. Moreover, for halo-alkaliphilic bacteria the costly step of neutralization may be avoided which is cost effective.

5. The utilization of cheap renewable agro-residues provides economic alternative to existing production processes, thereby adding value to the waste.

6. Utilization of halophilic reserves of coastal Karnataka, India, the region which has been less explored for halophiles and production of high value biomolecules from them.

10.3 CONCLUSION

The present investigation introduces three biopolymer degrading halophilic bacteria, *Halomonas* sp. PS6, *Brachybacterium* sp. PS3 and *Halomonas* sp. PS47 as potential candidates for the production of ectoine, halostable xylanase and halostable cellulase, respectively, utilising agro-residues. The study on compatible solute production using agricultural wastes is less attempted. Moreover, the study on biopolymer degrading enzymes from halophiles sheds light on the broad spectrum of saline hydrolases and their potential applications. Thus, the present study contributes towards the limited information available with regards to alternate production processes available for high value products from halophilic bacteria.

10.4 SCOPE FOR FUTURE WORK

1. Development of innovative fermentation processes for large scale production of compatible solutes and saline hydrolases.
2. Purification studies of ectoine, xylanase and cellulase can be undertaken. Cellulases and xylanases are active de-polymerising enzyme systems that work through concerted action and dynamic synergism. Purification and characterisation of these enzymes is a challenging task.
3. Applications of ectoine, xylanase and cellulase. Ectoine can be used in the preparation of advanced buffer systems - for the stabilization of biomolecules, cosmetic actives and therapeutic agents. Xylanases and cellulases can be used in saline waste water treatment studies, bio-bleaching of kraft pulp and in the production of biofuels from saline biomass.

APPENDIX I

Materials used in the present study

Chemicals	**Company**
yeast extract, peptone, potassium dihydrogen phosphate, di-potassium hydrogen phosphate, potassium hydroxide, ammonium sulphate, magnesium sulphate, ferrous sulphate, sodium chloride, Tris base, methanol, chloroform, sodium hydroxide, potassium sodium tartarate, 3,5-dinitrosalicylic acid, sodium nitrate, manganous suphate	Merck
Ectoine, hydroxyectoine	Fluka
Xylan, carboxymethyl cellulose, cellulose, inulin, xylose	Hi media
Agar, Gram staining kit, iodine, sodium molybdate, malachite green, alpha naphthol, gelatin, Kovac's reagent, oxidase discs, phenol, ethanol,	Nice/Rankem
Bacterial DNA mini kit	Invitek
Acetonitrile, chitin	Sigma
Sodium nitrate, sodium chloride	SRL
Glucose	Thomas baker

APPENDIX II

Composition of MM63 medium (Larsen et al. 1987)

Medium component	pH 7.1 (g/l)	pH 8.5 (g/l)
KH_2PO_4	13.61	_
K_2HPO_4	_	2.2
KOH	4.21	_
Tris	_	12
$(NH_4)_2SO_4$	1.98	1.98
$MgSO_4.7H_2O$	0.25	0.25
$FeSO_4.7H_2O$	0.0011	0.011
NaCl	6% (w/v)	6% (w/v)
Glucose	5	5

APPENDIX III

Harvesting microbial biomass for compatible solute extraction

100 ml of culture broth is taken in centrifuge tubes

Broth is centrifuged at 8500 rpm for 20 min at 20 °C

The biomass pellets are scrapped onto filter paper for drying

Biomass is then transferred to 2 ml eppendorf tubes

Eppendorf tubes are spun down for 2 min to let the biomass pellet settle down

The tubes are sealed with parafilm and tubes frozen at -20 °C

Tubes are kept for lyophilisation overnight

The freeze-dried sample is used for extraction of solutes

APPENDIX IV

Modified protocol of Bligh and Dyer (1959) for extraction of compatible solutes

30 mg crushed, freeze-dried material

taken in 1.5 ml reaction vial

⇩

500 µl of B&D solution is added

(10 methanol:5 chloroform: 4 water)

10 min shaking at RT

⇩

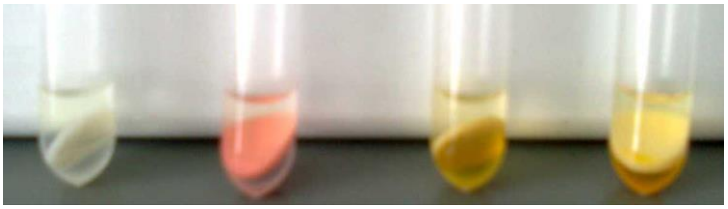
130 µl chloroform and 130 µl water is added

10 min shaking at RT

⇩

Centrifuged at 10,000 rpm for 10 min

- Phase separation occurs



⇩

Upper phase containing the solutes

transferred to a clean vial

⇩

Diluted appropriately with 80% (v/v) acetonitrile

for HPLC analysis

APPENDIX V

16S rRNA gene sequencing of *Halomonas* sp. PS6

- The genomic DNA was isolated using a modified protocol by James (2010).
- The PCR assay was performed with 0.5µl of DNA extract in a total volume of 50µl. The PCR master mixture contained 5µl of 10X PCR reaction buffer (with $MgCl_2$), 5µl of 2 mM dNTPs, 0.5µl each of oligonucleotide primer 16S_8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 16S_U1492R (5'-GGTTACCTTGTTACGACTT-3'), 1µl Taq DNA polymerase and 23.5µl of glass-distilled PCR water.
- Initially denaturation accomplished at 95 °C for 3 min. Thirty cycles of amplification consisted of denaturation at 95°C for 20 s, annealing at 45 °C for 20sec and extension at 72°C for 2 min. A final extension phase at 72°C for 10 min was performed.
- The amplified DNA was sent for sequencing in a DNA analyzer. The rDNA sequence contigs generated were then analysed using online databases viz. NCBI-BLAST to find the closest match of the contig sequence.
- Phylogenetic analysis was performed using the software packages PHYLIP (Felsenstein 1985) and MEGA version 4 (Kumar et al. 2004) after obtaining multiple alignment data available from databases by CLUSTAL_X (Thomson et al. 1997).
- Pairwise evolutionary distances were computed using the correction method (Jukes and Cantor 1969) and clustering was performed using the neighbor-joining method (Saitou and Nei 1987). Sequence was deposited in the NCBI GenBank under the accession number KC295600.

APPENDIX VI

DNS assay for estimation of xylanase/cellulase activities (Miller 1959; Ghose 1987)

1ml of crude enzyme added to mixture containing

1 ml xylan/CMC solution + 1ml buffer

⇩

Incubate in water bath at 50 ºC for 30 min

⇩

Add 3 ml of DNS reagent, mix and place tubes

In boiling water bath for 15 min

⇩

Control contains 1 ml buffer +1 ml xylan/CMC

Placed in water bath for 30 min

Add 3ml DNS followed by 1ml enzyme

Place in boiling water bath for 15 min

⇩

Standard made from 0 to 1 mg/ml xylose/glucose

⇩

OD_{540}

Xylose standard graph

$Y=0.884x$, $R^2=0.9959$

Glucose standard graph

$Y=0.819x$, $R^2=0.9963$

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LIST OF PUBLICATIONS

PUBLICATIONS

1. Pooja Shivanand, Gopal Mugeraya and Anubhav Kumar (2012). "Utilization of renewable agricultural residues for the production of extracellular halostable cellulase from newly isolated *Halomonas* sp. strain PS47." *Annals of Microbiology*. DOI 10.1007/s13213-012-0583-8

2. Pooja Shivanand and Gopal Mugeraya (2011). "Halophilic bacteria and their compatible solutes – osmoregulation and potential applications." *Current Science*, Vol. 100:10; 1516-1521.

CONFERENCE PROCEEDINGS

3. Pooja Shivanand, Gopal Mugeraya and Hitesh Chhabra, 'Optimization of halostable alkaline xylanase production from newly isolated *Brachybacterium* sp. strain PS3 using renewable agro-residues', International Conference on Advances in Chemical Engineering (ACE 2013), IIT Roorkee, India (Feb 22 – 24, 2013).

4. Pooja Shivanand and Gopal Mugeraya, 'Production studies of ectoine, xylanase and cellulase from newly isolated halophilic bacteria using renewable agro-residues', Chemcon-2012, International Conference on Sustainable technologies for energy and environment in process industries, Dr. B. R. Ambedkar NIT, Jalandhar, Punjab, India (Dec 27 – 30, 2012).

5. Pooja Shivanand and Gopal Mugeraya, 'Utilization of renewable agricultural residues for solute and enzyme production from newly isolated halophilic bacteria', 3rd World Congress on Biotechnology, Hyderabad, India (Sept 13 – 15, 2012). Abstract reviewed - J. Biotechnol. Biomater. (2012), 2(6), 247.

6. *Hitesh Chhabra, Pooja Shivanand and Gopal Mugeraya, 'Screening of xylan degrading bacteria isolated from saline environments and their use in

bioleaching of kraft pulp' 2nd World Congress on Biotechnology, Hyderabad, India (May 4-6, 2012).

7. Pooja Shivanand, Gopal Mugeraya and Hitesh Chhabra, 'Screening of halophilic bacteria for ectoine, xylanase, cellulase production from agro-wastes', 2nd International Engineering Symposium (IES 2012), Kumamoto University, Japan (Mar 5-7, 2012), International Journal of Biological Sciences and Engineering, Vol. 3 (2), 80-84.

8. Pooja Shivanand and Gopal Mugeraya, 'Survival with a pinch a salt offers a gold mine for the future', 9th triennial International conference on halophilic microorganims, Beijing, China (Jun 29 – Jul 3, 2010).

9. Pooja Shivanand and Gopal Mugeraya, 'Therapeutic potentials of compatible solutes of halophilic bacteria', International symposium on 'Gene to vial - Concept for biotechnology based health care molecules' at CBST, VIT University (Feb 7 – 10, 2010).

10. Pooja Shivanand and Gopal Mugeraya, 'Halophilic bacteria – diversity, perspectives and potential for biotechnology', National conference on 'Emerging concepts in biotechnology (ECB 2009)' at NIT Calicut (Dec 11 – 12, 2009).

* This work on bio-bleaching of kraft pulp was carried out as a part of the M. Tech thesis of Mr. Hitesh Chhabra. Present-day bleaching of kraft pulp uses large amounts of chlorine-based chemicals. Xylanases are very specific for hydrolysis of hemicelluloses in order to preserve cellulose structure during bio-pulping. PS3 xylanase was used to depolymerise hemicellulose precipitated on the surface of newspaper, notepad paper, lab-waste paper and tissue paper thereby producing smaller lignin molecules easier to remove. Pulp was present in tanks at higher temperature (55-60°C) and alkaline pH. The cellulase-free alkaline PS3 xylanase being halo-tolerant exhibited activity at higher temperature, increasing the brightness of the paper pulps. This is significant from industrial view-point.

The work received 'Best poster award' at the above mentioned congress.

INTERSHIPS DURING Ph.D. STUDIES

1. DAAD (German Academic Exchange Service) - funded Short-term Ph.D. studies on ectoine production at the Institute for Microbiology and Biotechnology, University of Bonn, Germany under the guidance of Prof. Dr. Erwin A. Galinski (Jun 15 – Sep 15, ’11).

2. Summer internship program on ‘Culture and molecular techniques of halophilic microorganisms’, National Institute of Oceanography, Goa, India under the guidance of Dr. Samir Damare (May 17-June 25, ’10).

POOJA S. Breh

ADDRESS | PRESENT
Flat No. 1,
Akshaya apartment,
Annasandrapalya 3rd Cross,
VinayakaNagara,
Vimanapura Post,
H.A.L.
Bangalore - 560017
Karnataka, India

ADDRESS | PERMANENT
Janani,
Nellikeri, College Road,
Behind New Busstand,
Kumta – 581 343
Karnataka, India
Phone: +91-9886618224
E-mail: pooja.shivanand@gmail.com
poojasbreh@gmail.com

WORK EXPERIENCE

- **Current: Research Scientist (R&D), Richcore Lifesciences Pvt. Ltd.** Bangalore, Karnataka (Aug 1, '13 onwards).
- Past: Served as TRA (Teaching-cum-Research Assistant), VIT University, Vellore (Dec 18, '06 to Jun 17, '09).
- Past: Served as Lecturer in Bioprocess, Dept. of Biotechnology, K.I.T.'s College of Engineering, Kolhapur (Jul 6, '06 to Aug 10, '06).

EDUCATION

Degree	**Ph. D.**
Institution	National Institute of Technology Karnataka, Surathkal, India
Department	Chemical Engineering
Research Work	'Utilization of renewable agricultural resources for ectoine, xylanase and cellulase production from newly isolated halophilic bacteria.'
Date of Joining	July 2009
Completion	27th September 2013

Degree	**M. S. (by Research)**
Institution	Vellore Institute of Technology University, Vellore, India
Department	Biotechnology, Chemical and Biomedical Engineering
Research Work	'Production and properties of extracellular protease fromhalotolerant bacterium isolated from saltern'
Year	2006-2009

Degree	**Bachelor of Engineering**
Institution	B.V.B. College of Engineering & Technology, Hubli, India
University	Visveswaraya Technological University, Belgaum, India
Department	Biotechnology, Chemical and Biomedical Engineering
Year	2002-2006
Marks / Division	75% / Distinction

PUC (Class XII) Board	**Pre-University Board, Karnataka**
Institution	Dr. A.V. Baliga College of Arts & Science, Kumta, India
Year	2002
Marks / Division	85% / Distinction

SSLC (Class X) Board	**Karnataka Secondary Education Examination Board**
Institution	Nirmala Convent School, Kumta, India
Year	2000
Marks / Division	88% / Distinction

PAPER PUBLICATIONS

- Pooja Shivanand, GopalMugeraya and Anubhav Kumar (2012). Utilization of renewable agricultural residues for the production of extracellular halostablecellulase from newly isolated *Halomonas* sp. strain PS47, ***Annals of Microbiology***, DOI 10.1007/s13213-0120583-8.

- Pooja Shivanand and GopalMugeraya (2011). Halophilic bacteria and their compatible solutes – osmoregulation and potential applications. ***Current Science***, Vol. 100:10; 15161521.

- Pooja Shivanand and GurunathanJayaraman (2011). Isolation and characterisation of a metal ion-dependent alkaline protease from a halotolerant*Bacillus aquimaris* VITP4, ***Indian Journal of Biochemistry and Biophysics***, Vol. 48; 95-100.

- Pooja Shivanand and GurunathanJayaraman (2009). Production of extracellular protease from halotolerant bacterium, *Bacillus aquimaris* strain VITP4 isolated from Kumta coast, ***Process Biochemistry***, Vol. 44:10; 1088-1094.

AWARDS AND HONOURS

- **A1 level certification (95%) in German language** from Goethe Institute (Max Mueller Bhavan) Bangalore.

- National award for ***Best Technical Paper*** – "Learning challenges and business models for biotechnology – an Indian perspective", presented at the 39th annual convention and national conference of ISTE held in NITK Surathkal (Dec 18 – 20, '09).

- **DAAD Research Fellowship** for short-term Ph.D. studies at Institute for Microbiology and Biotechnology, University of Bonn, Germany (Jun 15 – Sep 15, '11).

- **Special prize for B.E. project** 'Lipase production using economical Jatrophacurcas meal', in the State-level project competition, "Srishti-2006", GMIT Davangere, India (May 18-21, '06).

- **DAAD funded participation** in the inaugural event of German House for Research & Innovation (DWIH New Delhi) on Oct 27, '12.

EXTRACURRICULAR

- Organizing secretary of Journal Club, Department of Chemical Engineering, NITK Surathkal (2009-2010).

- Academic secretary of biotechnology association NEOBT, Department of Biotechnology, BVBCET, Hubli (2003-2005)

- School Pupil Leader of Nirmala Convent School, Kumta (1999-2000).

CONFERENCE PROCEEDINGS

- Pooja Shivanand, GopalMugeraya and Hitesh Chhabra, 'Optimization of halostable alkaline xylanase production from newly isolated *Brachybacterium* sp. strain PS3 using renewable agro-residues', **International conference on Advances in Chemical Engineering (ACE-2013), IIT Roorkee**, Uttaranchal, India (Feb 22 – 24, 2013)

- Pooja Shivanand and GopalMugeraya, 'Production studies of ectoine, xylanase and cellulase from newly isolated halophilic bacteria using renewable agro-residues', **Chemcon-2012, Dr. B. R. Ambedkar NIT, Jalandhar**, Punjab, India (Dec 27 – 30, 2012).
- Pooja Shivanand and GopalMugeraya, 'Utilization of renewable agricultural residues for solute and enzyme production from newly isolated halophilic bacteria', **3rd World Congress on Biotechnology, Hyderabad**, India (Sept 13 – 15, 2012). Abstract reviewed - J. Biotechnol. Biomater. (2012), 2(6), 247.

- Pooja Shivanand, GopalMugeraya and Hitesh Chhabra, 'Isolation of halophilic bacteria for ectoine, xylanase, cellulase production from agro-wastes', **2nd International Engineering Symposium (IES 2012), Kumamoto University, Japan** (Mar 5-7, 2012), International Journal of Biological Sciences and Engineering, Vol. 3 (2), 80-84.

- Pooja Shivanand and GopalMugeraya, 'Survival with a pinch a salt offers a gold mine for the future', **Halophiles-2009, Beijing, China** (Jun 29 – Jul 3, 2010).

- Pooja Shivanand and GopalMugeraya, 'Therapeutic potentials of compatible solutes of halophilic bacteria', International symposium on 'Gene to vial - Concept for biotechnology based health care molecules' at **CBST, VIT University** (Feb 7 – 10, 2010).

- Pooja Shivanand and GopalMugeraya, 'Halophilic bacteria – diversity, perspectives and potential for biotechnology', National conference on 'Emerging concepts in biotechnology (ECB 2009)' at **NIT Calicut** (Dec 11 – 12, 2009).

- Hitesh Chhabra, Pooja Shivanand and GopalMugeraya, 'Screening of xylan degrading bacteria isolated from saline environments and their use in bioleaching of kraft pulp' **2nd World Congress on Biotechnology, Hyderabad, India** (May 4-6, 2012) – Best Poster Award.

PROFESSIONAL TRAINING

- Short-term Ph.D. studies at Institute for Microbiology and Biotechnology, University of Bonn, Germany (Jun 15 – Sep 15, '11).

- Summer internship program on 'Culture and molecular techniques of halophilic microorganisms', National Institute of Oceanography, Goa, India (May 17-June 25, '10).

- Workshop on 'Technology transfer and management for biotech industry and academia' by Dr. Ashley Stevens organized by BIRAC, India, in Bangalore (Feb 1112, '13).

- Workshop on 'Research methodology', VIT University, Vellore, India (Nov 12-13, '08).

- TEQIP-N sponsored Faculty-development program on 'Applications of molecular techniques in bioprocess engineering', GCT, Coimbatore, India (Oct 21-25, '07).

- Indian Academy of Science -sponsored National Lecture Workshop on 'Trends in Medical Biotechnology', VIT University, Vellore, India (Dec 27-29, '07).

- Short-term Course on 'Advanced Instrumentation', NITK, Surathkal (Jun 22-27, '06).