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PRODUCTION OF ALKALINE PROTEASE BY FREE AND IMMOBILISED CELLS OF *VIBRIO* sp. UNDER DIFFERENT FERMENTATION SYSTEMS AND ITS APPLICATION ON DEPROTEINISATION OF PRAWN SHELL WASTE FOR CHITIN RECOVERY

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By

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*To my **parents**,*
the origin of every virtue I posses

*my **teachers**,*
the wisdom that shaped my destiny

*my **sister**,*
the fortune I grew up with

*my **friends**,*
the youth that inspired me to be the best I can be

And

*my **husband**,*
the promise to live a lifetime together

DECLARATION

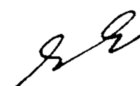
I hereby do declare that the thesis entitled **"PRODUCTION OF ALKALINE PROTEASE BY FREE AND IMMOBILISED CELLS OF *VIBRIO* sp. UNDER DIFFERENT FERMENTATION SYSTEMS AND ITS APPLICATION ON DEPROTEINISATION OF PRAWN SHELL WASTE FOR CHITIN RECOVERY"** *is a genuine record of research work done by me under the supervision of Dr. A.V. Saramma, Reader in Microbiology, School of Marine Sciences, Cochin University of science and technology, Kochi-682 016, and that no part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title of any university or institution.*

Kochi- 682 016
December 2005

Maya Paul T.

CERTIFICATE

This is to certify that the thesis entitled **"PRODUCTION OF ALKALINE PROTEASE BY FREE AND IMMOBILISED CELLS OF *VIBRIO* sp. UNDER DIFFERENT FERMENTATION SYSTEMS AND ITS APPLICATION ON DEPROTEINISATION OF PRAWN SHELL WASTE FOR CHITIN RECOVERY"** *is an authentic record of research work carried out by Ms. Maya Paul T. under my supervision and guidance in the department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology in partial fulfilment of the requirements for the degree of Doctor of Philosophy and no part thereof has been presented before for the award of any degree, diploma or associateship in any university.*



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CONTENTS

Chapter 1 **Introduction** **1- 9**

Chapter 2 **Production of alkaline protease under solid state fermentation** **10-50**

2.1 Materials and methods 15

2.1.1 Experimental organism 15

2.1.2 Inoculum preparation 17

2.1.3 Alkaline protease production under SSF 17

2.1.4 Enzyme extraction 18

2.1.5 Alkaline protease assay 18

2.1.6 Optimisation of fermentation process under SSF 19

2.1.7 Optimisation of extraction parameters for protease recovery 21

2.2 Statistical analysis 22

2.3 Results 22

2.4 Discussion 33

Chapter 3 **Production of alkaline protease under submerged fermentation** **51- 70**

3.1 Materials and methods 54

3.1.1 Inoculum preparation 54

3.1.2 Alkaline protease production under SmF 54

3.1.3 Enzyme extraction 54

3.1.4 Alkaline protease assay 54

3.1.5 Optimisation of fermentation process under SmF 54

3.2 Statistical analysis 57

3.3 Results and Discussion 57

Chapter 4 **Production of alkaline protease under biphasic fermentation** **71- 79**

4.1 Materials and methods 72
4.1.1 Inoculum preparation 72
4.1.2 Alkaline protease production under biphasic fermentation 72
4.1.3 Enzyme extraction 73
4.1.4 Alkaline protease assay 73
4.1.5 Optimisation of fermentation process under SmF 73
4.2 Statistical analysis 74
4.3 Results 74
4.4 Discussion 77

Chapter 5 Comparative techno-economic implications of solid state, submerged and biphasic fermentations 80- 97

5.1 Results and discussion 83
5.1.1 Comparative analysis of solid state, submerged and biphasic fermentations 83
5.1.2 Comparitive analysis of nutritional preferences of *Vibrio* sp. V26 under solid state and submerged fermentations 89
5.1.3 Comparison of optimum temperature required by *Vibrio* sp. V26 for maximum protease production under solid state and submerged fermentations 95

Chapter 6 Production of alkaline protease using immobilised cell technology 98-123

6.1 Materials and methods 103
6.1.1 Calcium alginate entrapment of cells 103
6.1.2 Production of alkaline protease by Immobilised cells in calcium alginate beads 104
6.1.3 Recovery of calcium alginate beads and enzyme extraction 104
6.1.4 Determination of cell leakage 105

6.1.5 Alkaline protease assay 105

6.1.6 Effect of Immobilisation conditions on alkaline protease production and cell leakage 105

6.1.7 Alkaline protease production and cell density of free and Immobilised cells 106

6.1.8 Estimation of operational stability of beads 106

6.1.9 Estimation of shelf life of beads 106

6.2 Statistical analysis 107

6.3 Results 107

6.4 Discussion 117

Chapter 7 Application of alkaline protease on deproteinisation of prawn shell waste for chitin recovery 124-136

7.1 Materials and methods 128

7.1.1 Pre-treatment of prawn shell waste 128

7.1.2 Demineralisation of prawn shell waste 128

7.1.3. Preparation of crude enzyme 129

7.1.4 Deproteinisation of prawn shell waste 129

7.1.5 Protein estimation 129

7.1.6 Measurement of percentage of deproteinisation 129

7.1.7 Optimisation of process parameters for deproteinisation of prawn shell waste 130

7.2 Statistical analysis 131

7.3 Results 131

7.4 Discussion 134

Chapter 8 Summary 137-141

References 142-172

Appendix

General introduction

Enzymes have been used by man throughout the ages, either in the form of vegetable or animal tissues rich in enzymes or in the form of microorganisms. The properties of these proteins that lend themselves to extensive use include their specificity and efficiency of action at low concentrations and under mild conditions of pH and temperature, their lack of toxicity and the easy termination of their action by mild treatments. Enzymes are ubiquitous in all living systems of which a myriad of microbes from many different exotic environments turn out to be the most explored and exploited.

Enzymes are well-known biocatalysts that perform a multitude of chemical reactions and are commercially exploited in various industries such as detergent, food, pharmaceuticals, diagnostics and fine chemicals. The technological application of enzymes under demanding industrial conditions makes the currently known arsenal of enzymes inadequate. Thus, the search

for new microbial sources is a continual exercise. The microorganisms from diverse and exotic environments are considered as important source of enzymes and their specific properties are expected to result in novel process applications (Govardhan and Margolin, 1995; Robertson *et al.*, 1996).

The estimated value of the worldwide sales of industrial enzymes is $1 billion (Godfrey and West, 1996). Of the industrial enzymes, 75% are hydrolytic. Proteases represent one of the three largest groups of industrial enzymes which constitute more than 65% of the total industrial enzyme market (Rao *et al.*, 1998). There is renewed interest in the study of proteolytic enzymes, mainly due to the recognition that these enzymes not only play an important role in the cellular metabolic processes but also have gained considerable attention in the industrial community. They find application in different sectors *viz.* detergent formulations, food and feed, pharmaceuticals, peptide synthesis, pulp and paper, leather, meat tenderisation, waste treatment, diagnostics, therapeutics, cosmetics, photographic industry, animal cell culture, degumming of silk, contact lens cleaning etc. (Kumar and Takagi, 1999; Gupta *et al.*, 2002a)

Proteases form a complex group of biocatalysts collectively known as peptidyl-peptide hydrolases and are responsible for hydrolysis of peptide bonds in a protein molecule. They show varying substrate specificities and are classified into acidic, neutral and alkaline proteases depending on the pH optima. Of the various types of proteases, alkaline proteases which are active from neutral to alkaline pH, have a wide market all over the world. There is a large scope for developing versatile proteases towards various industrial applications.

Alkaline Proteases

Alkaline proteases (or Subtilisins, E.C.3.4.21.14) are a physiologically and commercially important group of enzymes which play a specific role in the

hydrolysis of proteins. They are defined as those proteases, which are active in a neutral to alkaline pH range (Gupta *et al.*, 2002a). They are either serine proteases or metalloproteases, and the alkaline serine proteases are the most important group of enzymes exploited commercially. In Japan, alkaline protease sales were estimated as 15000 million yen in 1994 (equivalent to $ 116 million) (Horikoshi, 1996). An upward trend in the use of alkaline proteases is expected by the turn of the decade. Amongst the various sources, microorganisms account for a two-third share of commercial protease production in the world.

Microbial sources of alkaline proteases

A large number of microbes are known to produce alkaline proteases of serine type (Kumar and Takagi, 1999), although very few are recognised as commercial producers.

Of all the alkalophilic microorganisms that have been screened for use in various industrial applications, members of the genus *Bacillus* were found to be predominant and a prolific source of alkaline proteases (Kumar and Takagi, 1999). Some of the Gram–negative bacteria producing alkaline proteases were identified as *Pseudomonas aeruginosa* (Morihara, 1963); *Pseudomonas maltophila* (Kobayashi *et al.*, 1985); *Vibrio alginolyticus* (Deane *et al.*, 1987); *Xanthomonas maltophila* (Debette, 1991); *Pseudomonas* sp. strain B45 (Chakraborty and Srinivasan, 1993) and *Vibrio metschnikovii* strain RH530 (Kwon *et al.*, 1994).

Alkaline proteases are also produced by some rare microorganisms. *Kurthia spiroforme*, a spiral shaped Gram–positive bacterium possessing a distant relationship to genus *Bacillus*, was reported to produce alkaline proteases (Steele *et al.*, 1992).

Halophiles that were described to produce alkaline proteases included *Halobacterium* sp. (Ahan *et al.*, 1990); *Halomonas* sp.ES–10 (Kim *et al.*, 1991, 1992) and *Halobacterium halobium* ATCC 43214 (Ryu *et al.*, 1994). The alkalopsychrotrophic and alkalopsychrophilic bacteria represent a new potential source of alkaline proteases (Margesin and Schinner, 1994). These organisms are characterised by their adaptation to both cold temperatures and alkaline conditions. An alkalopsychrotrophic *Bacillus* sp. capable of producing alkaline proteases of high activity at low temperatures was isolated by Margesin *et al.* (1992).

Among actinomycetes, strains of Streptomyces and Nocardia are the potential ones (Dash *et al.*, 1989). In fungi, aspergilli is the most exploited group and the strains of *Neurospora*, *Pencillium*, *Ophiostoma*, *Tritirachium album*, *Basidiobolus*, *Myxococcus* and *Rhizopus* also produce alkaline proteases (Gupta *et al.*, 2005). Among yeast, *Yarrowia* and *Candida* sp. have been studied in detail as potential alkaline protease producers. Besides, thermophilic and thermostable alkaline proteases with optimal activity from 70-100^{0}C have also been obtained from thermophiles and extremophiles, *viz.*, *Aquifex*, *Desulphurococcus*, *Pyrococcus*, *Staphylothermus* and *Thermococcus*.

Looking into the vast diversity of microbial population, there is always a chance of finding microorganisms producing novel enzymes with better properties that are suitable for commercial applications.

Production of alkaline proteases

Protease production is an inherent property of all organisms and these enzymes are generally constitutive, however, at times they are partially inducible (Gupta *et al.*, 2002b). Under most culture conditions, microorganisms produce extracellular proteases during post-exponential and

stationary phases. The culture conditions that promote protease production were found to be significantly different from the culture conditions promoting cell growth (Moon and Parulekar, 1991).

The unravelling nature of fermentation was a milestone in the history of enzymes. Different methods of fermentations have been used to regulate the protease synthesis by combinations of the strategies such as fed-batch, continuous and chemostat cultures (Christiansen and Nielsen 2002a,b; Hameed *et al.*, 1999). Through these strategies, high yields of alkaline protease in the fermentation medium can be obtained over a longer period of incubation during prolonged stationary phase (Beg *et al.*, 2002).

The overall cost of enzyme production and downstream processing is the major obstacle against the successful application of any technology in the enzyme industry. Researchers and process engineers have used several methods to increase the yields of alkaline proteases to meet their industrial requirements.

The cost-effective approach towards enhanced production at profitable levels has led to the fermentation designs like solid state, submerged and biphasic systems using cheap agro-industrial residues. Moreover, the use of agro-industrial and other solid waste substrates for fermentation helps to provide a substitute in conserving the already dwindling global energy resources.

Solid state and submerged Fermentation

Solid state fermentation (SSF) involves the growth of microbes on moist solid materials in the absence or near absence of free water (Mitchell, 1992). The moisture content could vary between 40 and 80 per cent (Cannel and Moo-Young, 1980a). This limited availability of water makes SSF quite different from submerged state fermentation (SmF). The major difference

between SSF and SmF is that in the former, the substrate is a moist solid, which is insoluble in water but not suspended in liquid (primarily water), whereas, in the latter, the substrate is a solid dissolved or submerged in liquid. The solid substrates act as a source of carbon, nitrogen and minerals as well as growth factors, and they have a capacity to absorb water, which meets the vital requirement for water by the microorganism. SSF stimulates the fermentation reactions that occur in nature, which include wood rotting, composting and food spoilage by moulds. SSF processes can be conducted under controlled conditions, which are useful for producing valuable products such as enzymes or secondary metabolites (Hesseltine, 1977a; Bailey and Ollis, 1977; Ulmer *et al.*, 1981; Pandey *et al.,* 2000a,b). Solid substrate fermentation may be used advantageously for enzyme production, especially in those agrobiotechnological applications where the crude fermented product may be used directly as enzyme source. Such applications are enzyme assisted ensiling, bioprocessing crops and crop residues, fibre processing, enzyme enriched feed supplements, biopulping and directed composting for soil enrichment, enhancing biopesticide action, post harvest residue decomposition, waste recycling and soil remediation.

Biphasic fermentation

Biphasic growth system consists of a layer of solid nutrient medium over layered with a small volume of nutrient broth. It is a well established fact that the biomass yield can be increased upto 30 fold in a biphasic growth system (Tyrell *et al.*, 1958). In this method, there is a slow release of nutrients from the lower solid phase of the medium, which support the growth of the organism when the medium is exhausted just as in batch culture (Kaur *et al.*, 2001).

Immobilisation of enzymes

An alternative method for producing a desired product is by the use of immobilisation technique. In this technique, microbial enzymes and/or microbial cells are adsorbed or bonded on a solid support. Such bounded and thus immobilised enzymes, act as catalysts when biochemical (nutrient) medium is passed across these catalysts. The method involves control of optimal temperature, pH and oxygen concentration to achieve maximum conversion. This type of technique is very useful when the desired product is formed by single step transformation. Methods have been used for improving protease production by different microorganisms employing cell immobilisation of *B. licheniformis* (Lee and Chang, 1990), *B.firmus* 44 (Landau *et al.*, 1995; 1997), *B. thuringiensis* (Hotha and Banik, 1997) etc.

The immobilised enzyme technique makes the industrial process far more economical. It avoids the wasteful expense of continuously growing microorganisms. However, in the process if cells are immobilised then it is required to maintain their viability. If only enzymes are immobilised then it is required to maintain their activities. Special care is taken so that the enzymes or cells are not washed off while removing the fermented liquor.

The application of properly designed approaches with multi-factor models allows process and biochemical engineers to develop scale-up strategies for increasing enzyme production. Owing to the multitude of applications in a variety of industrial sectors, there had always been an increasing demand for novel producers and resources of alkaline proteases as well as for innovative methods of production on a commercial scale. This existing scenario stirred the inspiration which materialised the present study.

In a previous study conducted in our laboratory (Venugopal, 2004), a bacterial strain *Vibrio* sp. V26 isolated from a mangrove sediment sample was proved to have great potential as an alkaline protease producer. The protease was partially purified and the characteristics were studied. The molecular weight of the enzyme was calculated to be 22.4 kDa. It was proved to be active as well as stable in the pH range 7 to 11, with an optimal activity at pH 9. The enzyme was heat stable upto 60^{0}C having an optimum temperature of activity at 75^{0}C. Co^{2+}, Mg^{2+}, Na^+ and K^+ ions act as enhancers whereas Hg^{2+} and Cu^{2+} are inhibitors of the enzyme. Based on the inhibitor studies the enzyme was found to belong to the metalloprotease group of alkaline proteases. The present study was aimed at exploring and exploiting the protease production potential of *Vibrio* sp. V26 in a cost effective manner.

The objectives of the investigation can be briefed as follows :

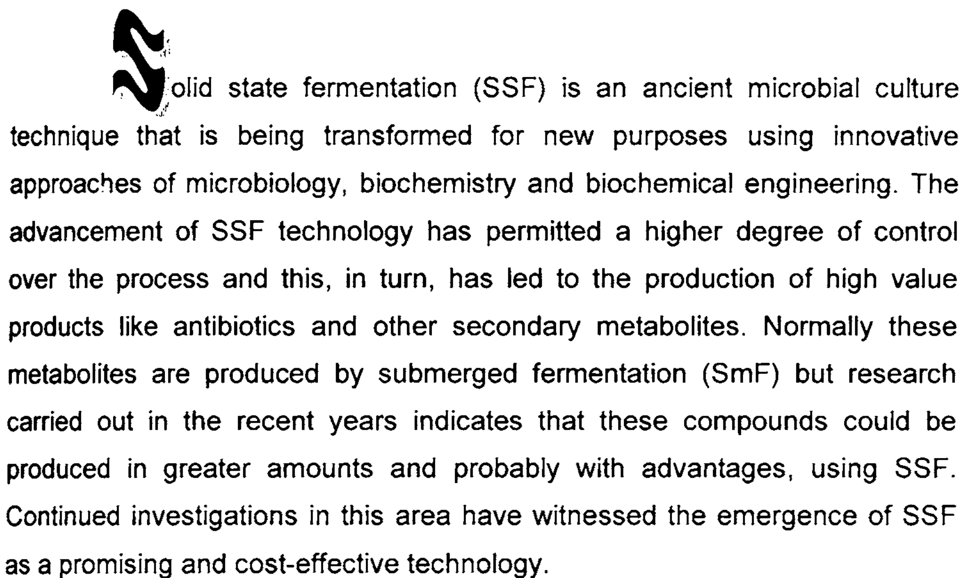
- Optimisation of process parameters for alkaline protease production under solid state fermentation (SSF)
- Optimisation of process parameters for alkaline protease production under submerged fermentation (SmF)
- Optimisation of process parameters for alkaline protease production under biphasic fermentation system
- Comparative analysis of the solid state, submerged and biphasic fermentation systems for the production of alkaline protease
- Production of alkaline protease by immobilised growing cells

- Application of alkaline protease in deproteinisation of crustacean waste for chitin recovery

The thesis is presented in eight chapters. The first chapter gives a brief introduction to the subject. The second chapter constitutes the detailed account of the optimisation studies of alkaline protease production under solid state fermentation. Chapter three deals with similar experiments conducted under submerged fermentation. Biphasic fermentation studies are illustrated in chapter four. A comparison of the different fermentation systems have been presented in the fifth chapter. Chapter six describes the attempts to immobilise the producer cells for alkaline protease production and to optimise conditions of immobilisation for maximum enzyme production. The seventh chapter explores the possible utilisation of alkaline protease for deproteinisation of prawn shell waste for the recovery of chitin. The major findings of the thesis are summarised in chapter eight. This chapter is followed by appendix and the list of literature consulted.

2

Production of alkaline protease under solid state fermentation



Solid state fermentation (SSF) is an ancient microbial culture technique that is being transformed for new purposes using innovative approaches of microbiology, biochemistry and biochemical engineering. The advancement of SSF technology has permitted a higher degree of control over the process and this, in turn, has led to the production of high value products like antibiotics and other secondary metabolites. Normally these metabolites are produced by submerged fermentation (SmF) but research carried out in the recent years indicates that these compounds could be produced in greater amounts and probably with advantages, using SSF. Continued investigations in this area have witnessed the emergence of SSF as a promising and cost-effective technology.

Solid state (or substrate) fermentation (SSF) is characterised by a fermentation process on a solid support, which has a low moisture content (lower limit ~ 12%) and occurs in a non – septic and natural state (Nigam and Singh, 1994). It can be defined as "growth of microorganisms on moist solid

materials in the absence of free-flowing water (Cannel and Moo-Young, 1980a; Moo-Young *et al.*, 1983). The two terms, solid state fermentation and solid substrate fermentation have often been ambiguously used. As has been pointed out by Pandey *et al.* (1999a), it would be logical to distinguish between these two terms. Solid substrate fermentation could be defined as those processes in which the substrate itself acts as carbon/energy source, occurring in the absence or near-absence of free flowing water, whereas, solid state fermentation may be defined as any fermentation process occurring in the absence or near-absence of free flowing water, employing a natural substrate as above or an inert substrate used as solid support.

Solid state fermentations have been used from time immemorial. There has been a renewed interest in the methodology by many groups of workers in recent years, culminating in evaluation studies and there are quite a large number of reviews on the subject dealing with food fermentations, enzyme production, general methodology and the advantages and limitations of the technique (Windish and Mhatre, 1965; Vezina *et al.*, 1968; Knapp and Howell, 1980). Hesseltine (1977a,b) reviewed and discussed SSF for the production of secondary metabolites and fermentation of animal wastes. The history and developments in SSF have been reviewed by several authors. Cannel and Moo-Young (1980a,b) reviewed the development of SSF as it was initiated and improved in the oriental food industry and composting. Aidoo *et al.* (1982) made an attempt to trace the history of the growth of microorganisms on solid substrates, a process that led to the development of the term 'solid state fermentation'. Knapp and Howell (1985) reviewed the literature on SSF focusing the microorganisms and microbial enzymes involved. The advantages and disadvantages of SSF have been discussed by Hesseltine (1972), Moo-Young *et al.* (1983) and Lonsane *et al.* (1985).

Solid state fermentation (SSF) has been usually exploited for the production of value-added products, biofuels, enzymes, organic acids, aroma compounds, pharmaceutical products and also for bioremediation of hazardous compounds, biological detoxification of agro-industrial residues, nutritional enrichment, biopulping etc. This technology gained renewed attention from industry as a more attractive alternative to liquid fermentation for many products. SSF was exploited for the production of food (Bhumiratna *et al.*, 1980; Hesseltine, 1983), feeds (GumbinaSaid, 1996; Nigam and Singh, 1996; Sandhu and Joshi, 1997), fuel (Hinman *et al.*, 1992; Ingram *et al.*,1999; Lapadatesecu and Bonnarme, 1999) and also for dye degradation (Nigam *et al.,* 2000; Robinson *et al.*, 2000). In recent years, new applications of SSF in the environmental pollution control have been developed including bioremediation and biodegradation of hazardous compounds and the detoxification of agro-industrial residues (Pandey *et al.*, 2000a).

SSF produces a high product concentration and has a relatively low energy requirement (Yang and Yuan, 1990; Mudgett *et al.,* 1992). Because of the lack of free water, smaller fermenters are required for SSF, and therefore, less effort is needed for downstream processing. Wild type strains of bacteria and fungi tend to perform better in SSF conditions than do genetically modified microorganisms, reducing energy and cost requirements even further (Barrios-Gonzalez *et al.*, 1993). Cultures reported for the production of protease by SSF are limited to the genus *Bacillus* and some fungi (Tunga *et al.,* 1998, 1999 and 2003). Microorganisms utilise various substrates as the source of nutrient for growth and metabolic activities. In SSF, the microorganism secretes the necessary enzymes for degradation of the available substrate molecules in order to meet their nutritional requirements. SSF processes are usually simpler and can use wastes or agro-industrial substrates, such as defatted soybean cake, gram bran, wheat bran, rice bran,

banana waste, etc. for enzyme production (Krishna and Chandrashekaran, 1996; Germano *et al.*, 2003; Kashyap *et al.*, 2003). Recently SSF has generated much interest, because of lower manufacturing costs by utilising unprocessed or moderately processed raw materials.

SSF has a bright future in both developed and developing countries due to its numerous advantages, such as superior productivity, simpler technique, low capital investment, reduced energy requirement, low wastewater output, improved product recovery, elimination of foam problems and dispensation with rigorous control of a few parameters. However, limitations are encountered, such as heat build-up, bacterial contamination, problems in scale up, growth estimation and difficulty in control of the moisture level of the substrate (Lonsane *et al.*, 1982, Hesseltine, 1972, 1977a and Ralph, 1976). An important engineering problem is heat removal from the solid medium, but in general terms, the problem centres on the lack of engineering experience for the design and scale-up of solid-state fermenters and the non-existence of standard fermenters for these processes. However, there have been noticeable advances in this field as reviewed by several authors (Mudgett, 1986; Mitchell *et al.*, 1992; Prior *et al.*, 1992).

In general, SSF is a well-adapted and cost-effective process for the production of a wide spectrum of bioproducts. The use of SSF for the production of commercially valuable metabolites is at present under-utilised, with a strong preference towards conventional and familiar liquid fermentations. This lack of adoption by industry seems strange, since research in this area shows that SSF produces higher yields in a shorter time. If SSF parameters are controlled properly and the purity of the product is defined, then SSF may be a more competitive process than is commonly thought of and it may be a viable option for the industrial production of value-added products.

SSF holds tremendous potential for the production of enzymes. It can be of special interest in those processes where the crude fermented product may be used directly as enzyme source (Tengerdy, 1996). Enzyme production has been an area in which several bacterial strains have been used successfully (Babu and Satyanarayana, 1996; Pandey *et al.*, 1999b). In addition to the well established applications in food and fermentation industries, microbial enzymes have attained a significant role in biotransformations involving organic solvent media, mainly for bioactive compounds. Several reports are available on the production of enzymes of industrial importance, under SSF (Pandey, 1992; Pandey and Radhakrishnan, 1992, 1993; Pandey, 1995; Benjamin and Pandey, 1998; Pandey and Soccol, 1998; Soccol and Krieger, 1998; Pandey *et al.*, 1999a,b,c,d; Pandey *et al.*, 2000a,b). These include proteases, cellulase, ligninases, xylanase, pectinase, amylase, glucoamylase etc. Attempts were also made to study SSF processes for the production of inulinases, phytases, tannase, phenolic acid esterase, microbial rennet, aryl-alcohol oxidase, oligo-saccharide oxidase, tannin acyl hydrolase etc. Most of the results, however, are from laboratory, or semi-pilot-scale experiments. In 1999, Alltech established a large-scale enzyme production facility, which was claimed to be the first enzyme technology based on SSF (Anon, 1999).

The present study is focussed on the optimisation of process parameters for maximising the production of alkaline protease under SSF conditions, a project with an attractive economic profile. Direct estimation of growth in SSF is not possible as it is impossible to separate residual solid substrate from the fermented biomass. Therefore, the present investigation is confined to the factors affecting protease production rather than elucidating the relation between growth parameters and enzyme production.

2.1 Materials and methods

2.1.1 Experimental organism

The experimental organism was selected from the collection of protease-producing bacterial strains maintained in the Microbiology laboratory of the Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology. Out of the 180 isolates, a strain of *Vibrio* sp. V26 isolated from the mangrove sediments of Puduvyppu, Cochin was chosen, based on its suitability for SSF with excellent potential as an alkaline protease producer. The morphological and biochemical characteristics of selected strain are given in Table 2.1.

Morphological and biochemical characteristics of *Vibrio* sp. V26 (Venugopal, 2004)

Gram reaction	-
Motility	+
Oxidase	+
O/F	+/+
Growth on TCBS agar	+ yellow colony, become green afterwards
Arginine dihydrolase	-
Lysine decarboxylase	+
Ornithine decarboxylase	+
Indole	+
Citrate	+
MR	+
VP	+
Amylase	-
Phosphatase	+
Mannitol utilisation	+
Arabinose utilisation	-
Gas from glucose	-
Growth in 0% NaCl	+
Growth in 6% NaCl	+
Growth at 0^0C	-
NO_3 reduction	+
ONPG	+
Resistance to O/129 (150 μg)	-
Catalase	+
Phenylalanine deaminase	-
Identified as	*Vibrio* sp.*

*could not be assigned to any known sp. based on these tests

2.1.1.1 Plasmid curing

Plasmid curing was carried out using the meyhod of Silhavy *et al.* (1984). The bacterial culture was inoculated into nutrient broth containing sublethal concentrations of Acridine orange (0.05mg/ml and 0.1mg/ml) and SDS (1% and 3%). The cells were allowed to grow in the media overnight at 37^{0}C under vigorous shaking. On the second day, 10% of the culture was transferred to the fresh medium and was incubated again. This was continued till the fourth day. Everyday a loopful of the culture was streaked on to nutrient agar slants which on growth after 24 h were stored at 4^{0}C until subsequent analysis. On obtaining the fourth day culture, all the samples were spot inoculated on gelatin agar plates in order to confirm and compare the gelatinase production. A plasmid uncured sample was also spotted as the positive control.

2.1.1.2 Gelatinase production

Frazier's gelatin agar medium (modified) of Harrigane and McCane (1972) of the following composition was used.

Peptone	5 g
Beef extract	3 g
NaCl	5 g
Gelatin	10 g
Agar	20 g
Water	1000 ml
pH	7.2

Gelatin agar plates were spot inoculated with the test culture and incubated at 28^{0}C for 24 h. After incubation, the plates were overlaid with 15% $HgCl_2$ in 20% con. HCl. Clear transparent zones around the colony indicated gelatinase production.

2.1.2 Inoculum preparation

Pre-inoculum was prepared by transferring a loopful of 24 hr old slant culture into 250ml Erlenmeyer flask containing 50ml of sterile pre-inoculum medium. The culture medium was incubated at 37^{0}C on a rotary shaker at 100 rpm for 24 h. Flasks containing 100ml fresh medium were inoculated with 20% (v/v) of this pre-inoculum and incubated at 37^{0}C at 100rpm to serve as the inoculum for SSF.

Pre-inoculum medium

Nutrient broth supplemented with 0.2% gelatin was used

Peptone	5 g
Beef extract	3 g
NaCl	15 g
Gelatin	2 g
Water	1000 ml
pH	7.2

2.1.3 Alkaline protease production under SSF

The SSF experiments were carried out in 250ml Erlenmeyer flasks using 5g of the substrate with 70% (w/v) moistening medium (0.2M carbonate/bicarbonate buffer, pH 9.2). Care was taken to maintain the final moisture content by taking into account the volume of the inoculum and the moisture content present in the bran. Cotton-plugged flasks were autoclaved at 121^{0}C under 15lb pressure for 15min, cooled to room temperature and uniformly inoculated with 5%(v/w) inoculum (6x10^{8}cells/ml) of 24 hr culture and incubated at 37^{0}C for 120 h and samples were drawn for enzyme assay. Plate 1(A) shows the solid state fermentation set up in a 250 ml conical flask.

A. SSF set up in a conical flask

B. Enzyme extraction

SSF set up and enzyme extraction

Plate 1

2.1.4 Enzyme extraction

After the required period of incubation, the fermented mass was soaked in 4 volumes of the extractant, 0.2M carbonate/bicarbonate buffer, pH 9.2 (based on the initial dry weight of the substrate). The slurry was mixed at 37^{0}C on a rotary shaker at 100 rpm for 2 h. The slurry was then filtered and squeezed through muslin cloth (Plate 1(B)). The extracts were clarified by centrifugation at 10,000 g at 4^{0}C for 15 min and the clear supernatant was used as crude enzyme for assays.

2.1.5 Alkaline protease assay

Protease production was measured in terms of protease activity exhibited by the culture supernatant in the enzyme assay. Protease activity was assayed by a modification of the casein digestion method of Kunitz (1947) as standardised by Venugopal (2004).

Protease activity was assayed in a reaction mixture containing 0.5 ml of suitably diluted enzyme extract and 3ml of 0.6% casein (Hammersten) dissolved in 0.2M carbonate/bicarbonate buffer (pH 9.2) and incubated at 75^{0}C for 30 min. The reaction was terminated by addition of 3ml of pre-chilled trichloroacetic acid (5%) and allowed to stand for 15 min for the complete precipitation of residual protein. The reaction mixture was then filtered through Whatman no.1 filter paper and the filtrate was run in a Hitachi 2000-20 UV-Visible spectrophotometer at 280nm to measure the absorbance corresponding to the tyrosine liberated during hydrolysis, as inferred from the standard calibration curve prepared with pure tyrosine. Unit proteolytic activity was defined as the amount of enzyme that liberated 1 µg tyrosine per ml per minute under the specific assay conditions and was reported in terms of enzyme units per gram initial dry weight of the substrate (PU/g).

2.1.6 Optimisation of fermentation process parameters under SSF

The protocol adopted for optimisation of various process parameters influencing protease production was to evaluate the effect of an individual parameter and to incorporate it at the standardised level before optimising the next parameter. The parameters optimised were:

2.1.6.1 Effect of different substrates on protease production

A variety of substrates such as wheat bran, rice bran, rawa, maize bran, barley, ragi powder, bread powder, cassava powder, black gram bran and soy bean bran were procured from the local market and used as the solid substrate to study their effect on the production of protease. The best substrate in terms of enzyme yield was employed in subsequent experiments.

2.1.6.2 Effect of incubation period on protease production

Different incubation periods 24, 48, 72, 96 and 120 h were employed to study their effect on protease production. The optimum incubation period obtained was fixed for the conduct of further experiments.

2.1.6.3 Effect of initial moisture content on protease production

With a view to investigate the influence of initial moisture content (before autoclaving) of the substrate, the fermentation was carried out under various initial moisture contents 30, 40, 50, 60 and 70 (%, w/v), adjusted with adequate volume of buffer. Moisture content most suitable for maximum protease production was adopted for the rest of the study.
Percent moisture content of the solid substrate was calculated as follows,

Percent of moisture content = (wt. of the substrate – dry wt.) x 100/dry wt.

The dry wt. was recorded after drying the substrate at 80^0C until a constant weight is obtained.

2.1.6.4 Effect of inoculum size on protease production

Various inoculum levels of 5,10,15,20 and 25 (%, v/w) were tried to study their impact on enzyme production. The optimum inoculum level achieved by this step was used for subsequent experiments.

2.1.6.5 Effect of temperature on protease production

Fermentation was carried out at temperatures of 20^{0}C, 25^{0}C, 30^{0}C, 37^{0}C and 45^{0}C to optimise the temperature for maximum protease production under SSF. The experiments followed were conducted at the optimal temperature.

2.1.6.6 Effect of amount of substrate on protease production

The influence of the amount of substrate on protease production was studied by changing the ratio of weight of substrate to flask volume. Different amounts 5g, 10g, 15g and 20g of substrate were used for fermentation. The minimum quantity of substrate yielding maximum protease output was selected for further study.

2.1.6.7 Effect of particle size of substrate on protease production

The substrate wheat bran was sieved through various mesh size to obtain fine, medium, coarse and large particles of < 425 µm, 425-600 µm, 600-1000 µm and 1000-1425 µm size respectively and the optimum particle size for enzyme production was determined.

2.1.6.8 Effect of NaCl concentration on protease production

The effect of sodium chloride was evaluated by incorporating it at various concentrations of 0,0.5, 1.0, 1.5 and 2.0 (%, w/w) in the fermentation medium. The optimum NaCl concentration obtained was fixed for all the subsequent experiments.

2.1.6.9 Effect of supplementary carbon sources on protease production

The requirement of additional nutrient supply was studied, by adding different supplementary carbon sources (1%w/w) like glucose, sucrose, maltose, lactose, glycerol, starch, mannose and molasses to the fermentation medium. The ideal carbon source thus obtained was included in the medium formulation to continue the optimisation studies of the remaining parameters.

2.1.6.10 Effect of supplementary nitrogen sources on protease production

Whether the addition of supplementary organic nitrogen sources could enhance the production of protease was determined by supplying beef extract, casein, gelatin, peptone, tryptone, yeast extract and corn steep liquor in 1%w/w concentration to the medium. The possibility of an increase in the amount of protease produced due to the addition of any inorganic nitrogen source was also studied, incorporating $NaNO_3$, NH_4Cl, $(NH_4)_2SO_4$, NH_4NO_3 and urea at a level of 1%w/w in the medium.

2.1.7 Optimisation of extraction parameters for protease recovery

2.1.7.1 Effect of different extractants on protease extraction

In order to determine the most suitable extractant for the recovery of the protease produced by fermentation, different extractants such as carbonate/bicarbonate buffer, distilled water and physiological saline were used.

2.1.7.2 Effect of volume of extractants on protease extraction

The optimal volume of the extractant that has to be used for the maximum extraction of the enzyme was estimated by using different volumes 20ml, 30ml, 40ml, 50ml and 60ml of the extractant for extraction.

2.1.7.3 Effect of soaking time of extractants on protease extraction

Keeping the volume of the extractant at optimum level, incubation time for soaking was optimised for maximum enzyme recovery from the fermented bran. The soaking time was varied from 30 min to 270 min at 30 min intervals.

2.1.7.4 Effect of repeated extractions on protease extraction

Repeated extractions were carried out more as a confirmatory test in order to determine whether most of the protease could be recovered in one extraction with the optimum volume of the ideal extractant.

All the experiments were carried out in triplicates in order to corroborate the validity of the results obtained.

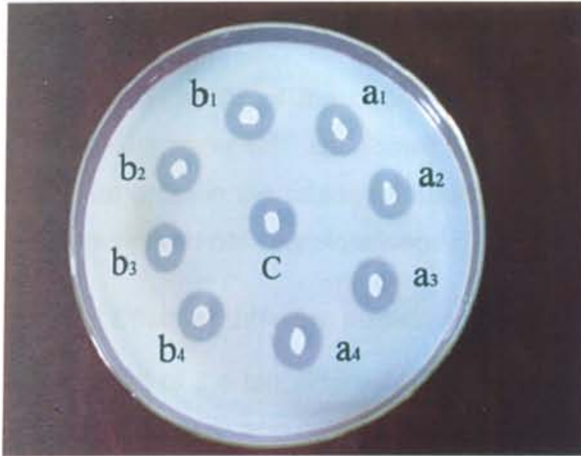
2.2 Statistical analysis

Data generated from the above experiments were analysed by One-way Analysis of Variance (ANOVA). Mean of the results was compared using SPSS 10.0 for windows at a significance level of $p<0.05$. Values with the same superscripts did not vary significantly.

2.3 Results

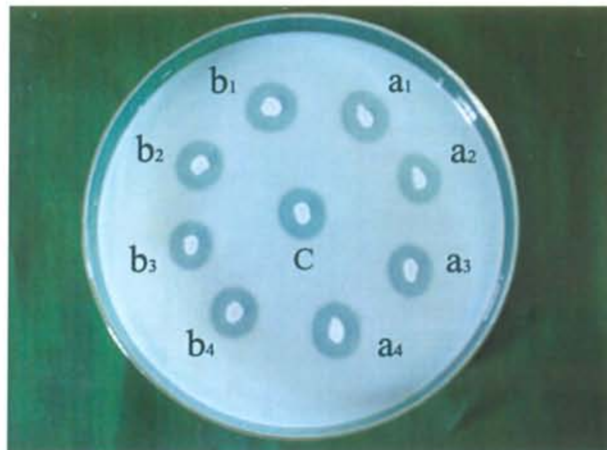
2.3.1 Plasmid curing

Even after treatments with sublethal concentrations of both the curing agents (acridine orange and SDS), and repeated subculturing, the culture samples gave clear transparent zones of gelatine hydrolysis around the colony as good as the positive control (Plate 2). It can be inferred that they still retained the property of enzyme production and the genes responsible for the protease production is located on chromosome and the property is not plasmid mediated.



C- positive control
a- 2.5 mg/ml acridine orange
b- 5.0 mg/ml acridine orange

Integers denote consecutive sub-culture samples



C- positive control
a- 0.5 g/ml S D S
b- 1.5 g/ml S D S

Integers denote consecutive sub-culture samples

Demonstration of gelatinase production by the plasmid cured *Vibrio* sp. using different curing agents

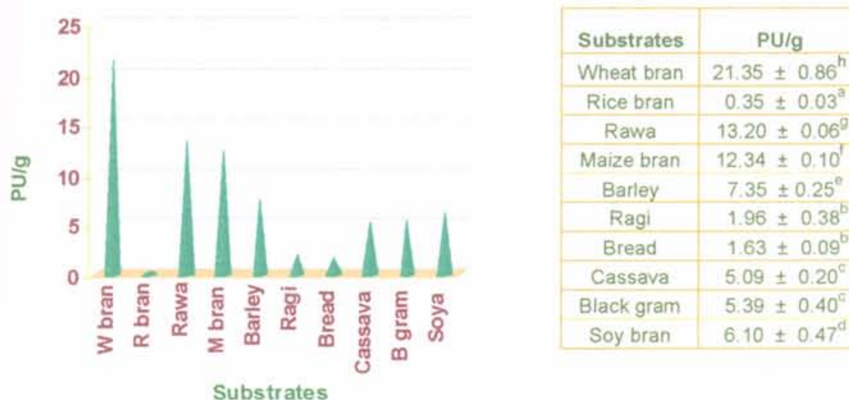
Plate 2

2.3.2 Optimisation of fermentation process parameters under SSF

The results of the optimisation studies of the fermentation process parameters under SSF are shown as graphs and tables. The ANOVA results are also given in the tables as superscript alphabets. Values with the same superscripts do not vary significantly.

2.3.2.1 Effect of different substrates on protease production

Out of the ten different substrates used for the study, only three *viz.* wheat bran, rawa and maize bran were utilised by *Vibrio* sp. for growth and protease production to a convincing level. Among the three, wheat bran was proved to be superior with a high titre of protease activity as evident from Fig.2.1. Statistical analysis also showed that the values obtained for wheat bran was significantly higher than all other substrates. The order of substrate suitability was wheat bran > rawa > maize bran > barley > soy bean bran > black gram bran and cassava > bread powder and ragi > rice bran. In subsequent experiments, therefore, wheat bran was used as the substrate for the production of protease by SSF.

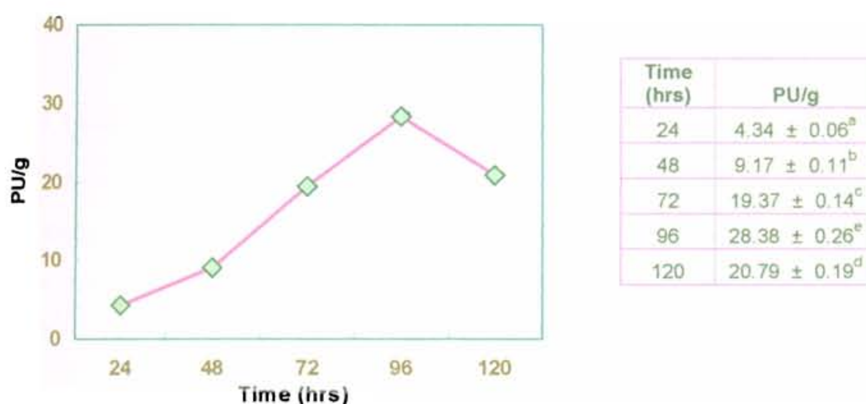


Substrates	PU/g
Wheat bran	21.35 ± 0.86^{h}
Rice bran	0.35 ± 0.03^{a}
Rawa	13.20 ± 0.06^{g}
Maize bran	12.34 ± 0.10^{f}
Barley	7.35 ± 0.25^{e}
Ragi	1.96 ± 0.38^{b}
Bread	1.63 ± 0.09^{b}
Cassava	5.09 ± 0.20^{c}
Black gram	5.39 ± 0.40^{c}
Soy bran	6.10 ± 0.47^{d}

Fig.2.1 Effect of different substrates on protease production: SSF

2.3.2.2 Effect of incubation period on protease production

Maximum enzyme production was obtained after 96 h of incubation. The protease production showed a progressive increase from 24 h reaching a maximum after 96 h and further incubation lowered the production considerably. Fig. 2.2 describes the protease production at different periods of incubation. ANOVA showed that incubation period has a considerable effect on protease production as the values differed significantly from each other.

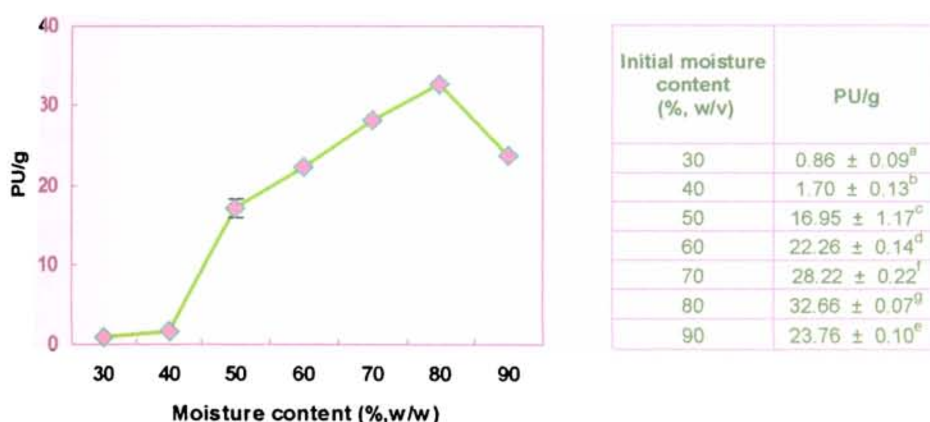


Time (hrs)	PU/g
24	4.34 ± 0.06[a]
48	9.17 ± 0.11[b]
72	19.37 ± 0.14[c]
96	28.38 ± 0.26[e]
120	20.79 ± 0.19[d]

Fig. 2.2 Effect of incubation period on protease production: SSF

2.3.2.3 Effect of initial moisture content on protease production

Significantly higher enzyme titre was attained when the moisture level was 80% in comparison with lower levels of moisture content in the medium and an even higher moisture content brought down the enzyme production (Fig. 2.3).

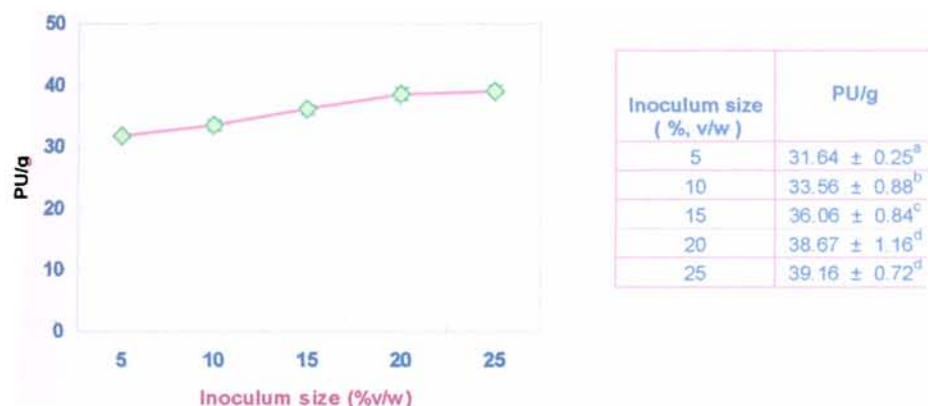


Initial moisture content (%, w/v)	PU/g
30	0.86 ± 0.09^{a}
40	1.70 ± 0.13^{b}
50	16.95 ± 1.17^{c}
60	22.26 ± 0.14^{d}
70	28.22 ± 0.22^{f}
80	32.66 ± 0.07^{g}
90	23.76 ± 0.10^{e}

Fig. 2.3 Effect of initial moisture content on protease production: SSF

2.3.2.4 Effect of inoculum size on protease production

Protease production increased with increasing concentrations of inoculum. The maximum enzyme yield was obtained at an inoculum concentration of 20% (v/w) and further increase in inoculum did not produce any significant effect in the enzyme output as depicted in Fig. 2.4, supported by the ANOVA results.

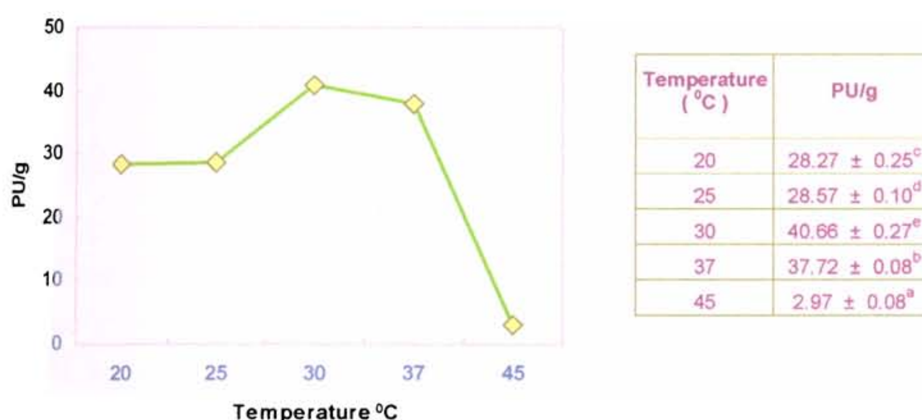


Inoculum size (%, v/w)	PU/g
5	31.64 ± 0.25^{a}
10	33.56 ± 0.88^{b}
15	36.06 ± 0.84^{c}
20	38.67 ± 1.16^{d}
25	39.16 ± 0.72^{d}

Fig.2.4 Effect of inoculum size on protease production: SSF

2.3.2.5 Effect of temperature on protease production

All temperatures selected for the study supported growth and enzyme production by *Vibrio* sp. except 45^{0}C. The optimal temperature for maximum protease production was 30^{0}C. Enzyme production declined sharply when the temperature of incubation was higher (Fig. 2.5). The enzyme units obtained at each temperature varied significantly from one another proving the direct influence of temperature on protease production.

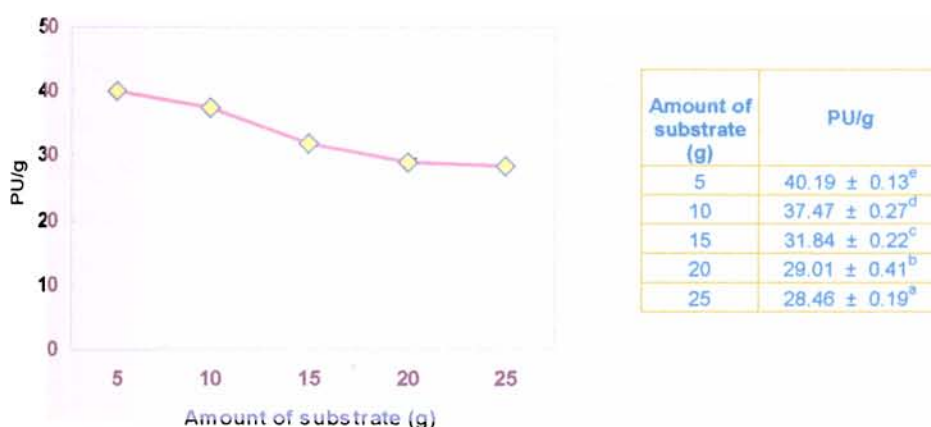


Temperature (°C)	PU/g
20	28.27 ± 0.25^{c}
25	28.57 ± 0.10^{d}
30	40.66 ± 0.27^{e}
37	37.72 ± 0.08^{b}
45	2.97 ± 0.08^{a}

Fig.2.5 Effect of temperature on protease production: SSF

2.3.2.6 Effect of amount of substrate on protease production

Amount of substrate or the ratio of the weight of substrate to volume of flask has a significant effect on enzyme production. The highest enzyme production was observed when 5g substrate (weight of substrate to volume of flask ratio, 1:50) was taken in a 250ml flask, for fermentation. Increasing the amount of substrate in the flask decreased the enzyme yield (Fig. 2.6).

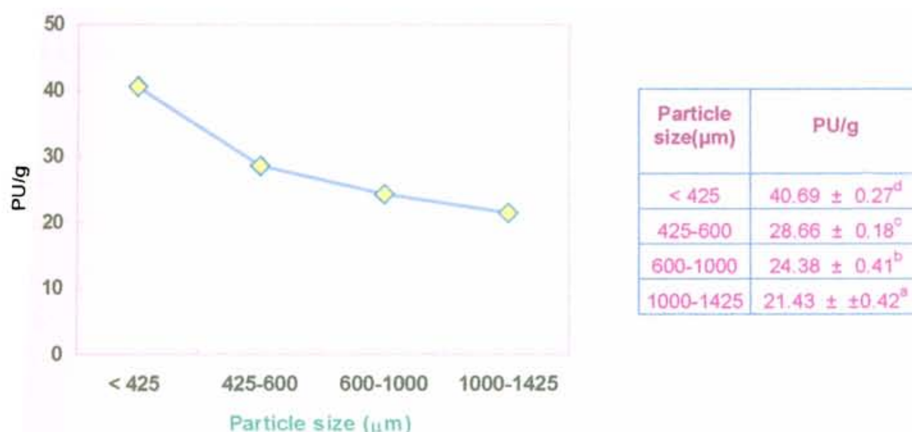


Amount of substrate (g)	PU/g
5	40.19 ± 0.13^{e}
10	37.47 ± 0.27^{d}
15	31.84 ± 0.22^{c}
20	29.01 ± 0.41^{b}
25	28.46 ± 0.19^{a}

Fig.2.6 Effect of amount of substrate on protease production: SSF

2.3.2.7 Effect of particle size of substrate on protease production

Fig. 2.7 shows the results of fermentation with different particle sizes of the substrate wheat bran. Maximum protease production was obtained with substrate particles of average size < 425 µm. Larger particles did not support enzyme production.

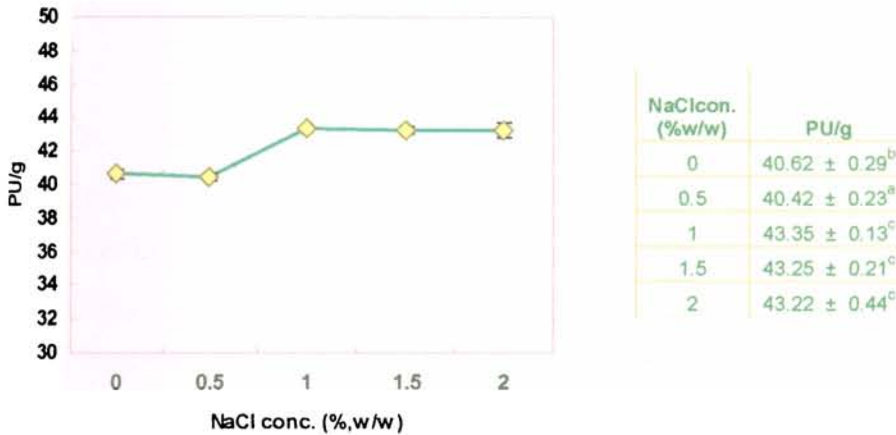


Particle size(µm)	PU/g
< 425	40.69 ± 0.27^{d}
425-600	28.66 ± 0.18^{c}
600-1000	24.38 ± 0.41^{b}
1000-1425	21.43 ± ±0.42^{a}

Fig.2.7 Effect of particle size of substrate on protease production: SSF

2.3.2.8 Effect of NaCl concentration on protease production

The NaCl requirement of *Vibrio* sp. for maximal yield of protease production under SSF conditions is shown in Fig.2.8. It was observed that 1 % (w/w) NaCl is optimum for maximum production and any further increase could not improve the yield.

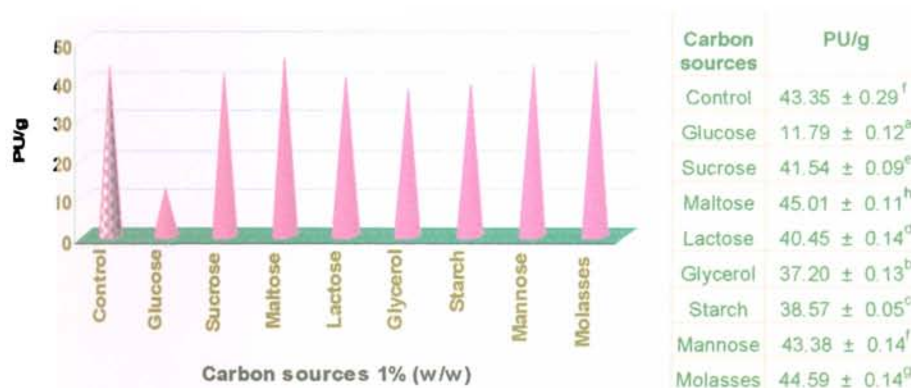


NaClcon. (%w/w)	PU/g
0	40.62 ± 0.29[b]
0.5	40.42 ± 0.23[a]
1	43.35 ± 0.13[c]
1.5	43.25 ± 0.21[c]
2	43.22 ± 0.44[c]

Fig.2.8 Effect of NaCl concentration on protease production: SSF

2.3.2.9 Effect of supplementary carbon sources on protease production

Of all the carbon sources tested, maltose was found to cause a slight increase in protease production. The yield was found to be much impeded in the presence of glucose (Fig. 2.9). ANOVA results showed that maltose was giving significantly higher production followed by molasses, mannose and sucrose.

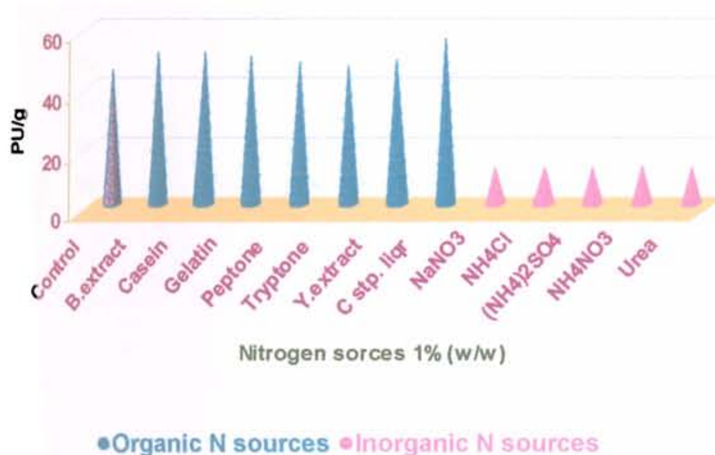


Carbon sources	PU/g
Control	43.35 ± 0.29^{f}
Glucose	11.79 ± 0.12^{a}
Sucrose	41.54 ± 0.09^{e}
Maltose	45.01 ± 0.11^{h}
Lactose	40.45 ± 0.14^{d}
Glycerol	37.20 ± 0.13^{b}
Starch	38.57 ± 0.05^{c}
Mannose	43.38 ± 0.14^{f}
Molasses	44.59 ± 0.14^{g}

Fig.2.9 Effect of supplementary carbon sources on protease production: SSF

2.3.2.10 Effect of supplementary nitrogen sources on protease production

Unlike inorganic nitrogen sources, the supplement of organic nitrogen sources favoured protease production. Addition of corn steep liquor increased protease production to the maximum followed by beef extract and casein. The addition of inorganic nitrogen sources had an adverse effect on protease production as demonstrated by the reduced enzyme titres (Fig.2.10).



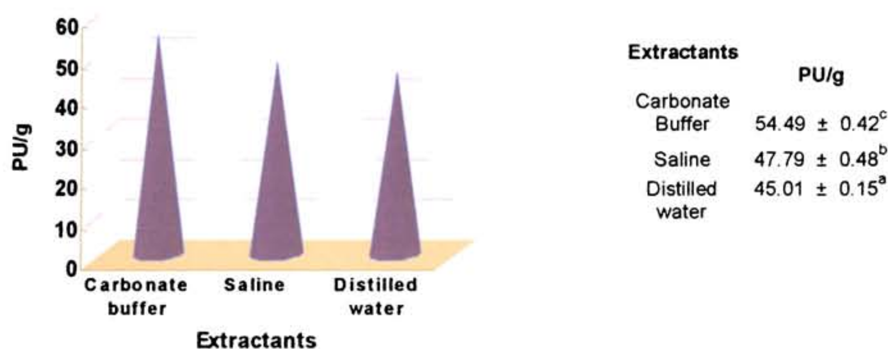
Nitrogen sources	PU/g
Control	45.01 ± 0.11^{b}
Beef extract	50.41 ± 0.06^{f}
Casein	50.38 ± 0.11^{f}
Gelatin	49.07 ± 0.19^{e}
Peptone	47.52 ± 0.27^{c}
Tryptone	45.34 ± 0.15^{b}
Yeast extract	47.87 ± 0.54^{d}
Corn steep liquor	54.98 ± 0.19^{g}
$NaNO_3$	18.42 ± 0.28^{a}
NH_4Cl	18.28 ± 0.40^{a}
$(NH_4)_2SO_4$	18.34 ± 0.20^{a}
NH_4NO_3	18..53 ± 0.64^{a}
Urea	18.29 ± 0.25^{a}

Fig.2.10 Effect of supplementary nitrogen sources 1% (w/w) on protease production: SSF

2.3.3 Optimisation of extraction parameters for protease recovery

2.3.3.1 Effect of different extractants on protease extraction

Different extractants were used for enzyme extraction and carbonate/bicarbonate buffer was found to be the most suitable followed by saline and distilled water (Fig. 2.11). ANOVA results also supported the same.

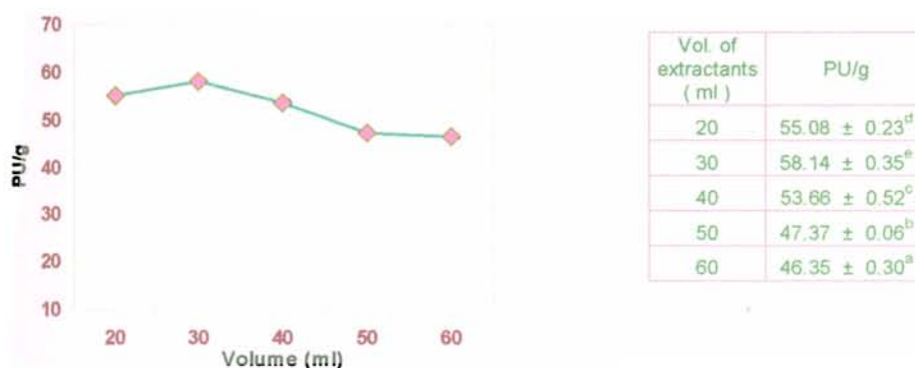


Extractants	PU/g
Carbonate Buffer	54.49 ± 0.42^{c}
Saline	47.79 ± 0.48^{b}
Distilled water	45.01 ± 0.15^{a}

Fig.2.11 Effect of different extractants on protease extraction: SSF

2.3.3.2 Effect of volume of extractants on protease extraction

Fig. 2.12 shows the effect of volume of carbonate/bicarbonate buffer used as extractant for enzyme recovery. The protease recovery increased when the volume of extractant was increased to 30ml and decreased thereafter as is evident in the statistical analysis.

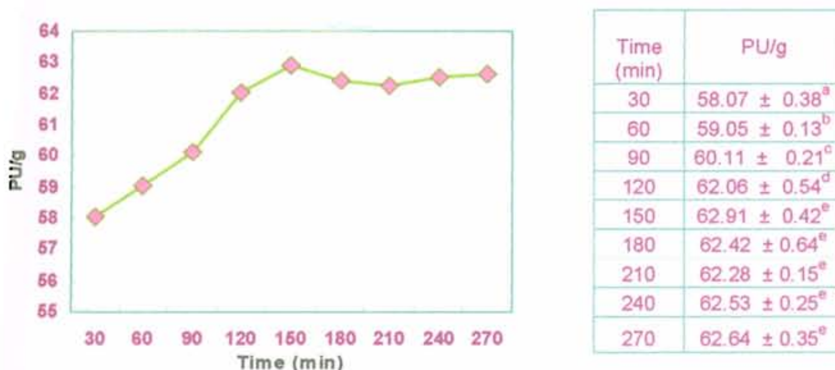


Vol. of extractants (ml)	PU/g
20	55.08 ± 0.23^{d}
30	58.14 ± 0.35^{e}
40	53.66 ± 0.52^{c}
50	47.37 ± 0.06^{b}
60	46.35 ± 0.30^{a}

Fig.2.12 Effect of volume of extractants on protease extraction: SSF

2.3.3.3 Effect of soaking time of extractants on protease extraction

It was found that 150 min soaking time was optimum and beyond that it did not have any additional effect on protease extraction (Fig. 2.13).

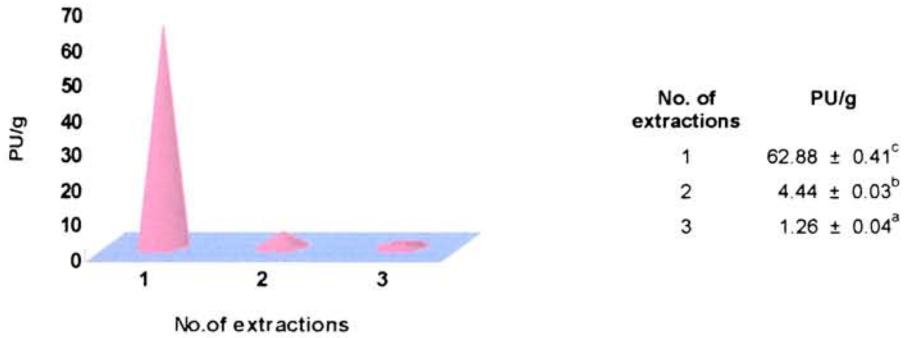


Time (min)	PU/g
30	58.07 ± 0.38^{a}
60	59.05 ± 0.13^{b}
90	60.11 ± 0.21^{c}
120	62.06 ± 0.54^{d}
150	62.91 ± 0.42^{e}
180	62.42 ± 0.64^{e}
210	62.28 ± 0.15^{e}
240	62.53 ± 0.25^{e}
270	62.64 ± 0.35^{e}

Fig.2.13 Effect of soaking time of extractants on protease extraction: SSF

2.3.3.4 Effect of repeated extractions on protease extraction

It was observed that out of the three washes the first two were sufficient for maximum leaching of the enzyme. Evidently, the first wash was more effective giving 92% of the available yield (Fig. 2.14 a and b)



No. of extractions	PU/g
1	62.88 ± 0.41[c]
2	4.44 ± 0.03[b]
3	1.26 ± 0.04[a]

Fig.2.14 a Effect of repeated extractions on protease extraction: SSF

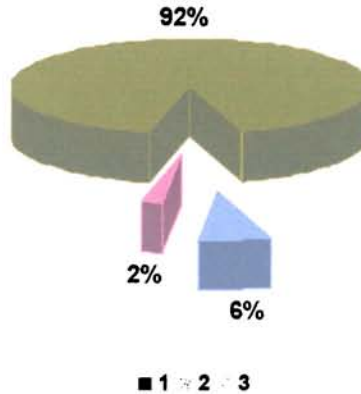


Fig.2.14.b. Percentage recovery of enzyme after fermentation: SSF

2.4 Discussion

In solid state fermentation, the moist water-insoluble substrate is fermented by microorganisms in the absence of any free flowing water. Although it has been used from ancient times to obtain fermented foods and cheese and in composting, the refinement and automation of the technique as well as its industrial exploitation have been confined mainly to the countries of the Orient (Yamada, 1977).

There are several important factors, which affect SSF processes. Among these, selection of a suitable strain and substrate and selection of process parameters (physical, chemical and biochemical) are crucial.

2.4.1 Selection of microorganism

Based upon the type of microorganisms involved, SSF processes can be categorised into two main groups, *viz.* natural (indigenous) SSF and pure culture SSF (individual or mixed). Pure cultures are generally used in industrial SSF processes to improve the control of substrate utilisation and end-product formation. In nature, SSF is often carried out by mixed cultures in which several microorganisms show development and symbiotic cooperation. Selection of a suitable microorganism is one of the most important criteria in SSF. The vast majority of wild type microorganisms are incapable of producing commercially acceptable yields of the product. While efforts largely continued to exploit filamentous fungi and yeast for the production of various products, attempts have also been made to explore the possibilities of using bacterial strains in SSF systems (Babu and Satyanarayana, 1996; Balakrishnan and Pandey, 1996; Pandey and Soccol, 1998; Soccol and Kreiger, 1998; Pandey *et al* .,1999a,b; Pandey *et al*., 2000a,b).

In the present study, a potent strain of *Vibrio* sp. V26 proven to produce appreciable amounts of protease under liquid batch fermentation

(Venugopal, 2004) has been selected for successful exploitation under SSF. In contrast to the usually high water requirements of vibrios, this particular strain of interest gave substantial enzyme yields under SSF. Hence the optimisation of process parameters for maximising the production was carried out.

Other instances of *Vibrio* protease producers include *Vibrio alginolyticus* (Deane *et al.*, 1987), *Vibrio metschnikovii* strain RH530 (Kwon *et al.*, 1994) and *Vibrio fluvialis* (Venugopal, 2004). There is hardly any reference available regarding protease producing vibrios suitable for SSF. Nagendra and Chandrasekaran (1996) produced L- glutaminase using a marine isolate of *Vibrio costicola* under solid state fermentation using different substrates.

Plasmid curing

The present isolate was detected as a highly potent protease producer based on its ability to produce gelatinase enzyme indicated by the remarkable clearance zone in a gelatinase agar medium. The desired property is likely to be lost at any stage of the investigation due to repeated subculturing and stressful experimental conditions. So plasmid curing was carried out to verify whether the property of interest is plasmid mediated or not. It was found that the cells were able to produce gelatinase even after treatments with both the curing agents (acridine orange and SDS) at different concentrations. The cells were subcultured four times and they still retained the property of enzyme production. Thus it was inferred that the genes responsible for protease production is located on chromosome. This validates the genetic stability of the organism for the production of protease enzyme.

2.4.2 Optimisation of fermentation process parameters under SSF

In commercial practice, the optimisation of medium composition is done to maintain a balance between the various medium components, thus minimising the amount of unutilised components at the end of fermentation. No defined medium has been established for the optimum production of alkaline proteases from different microbial sources. Each organism or strain has its own special conditions and requirements for maximum enzyme production.

Often SSF processes involve an organism which grows quite rapidly under the low water conditions, and, if an active inoculum is added to a substrate, the organism is able to outcompete the contaminating organisms. This means that strict aseptic operation of the bioreactor may not be essential in SSF, although, of course, operation should be carried out in as clean a manner as possible. Secondary metabolites are generally reported to be produced in higher concentrations in solid culture, often in shorter times and without the need of aseptic conditions (Hesseltine, 1977a; Barrios-Gonzalez *et al.*, 1988). There are a number of products for which SSF has good potential but the process organisms grow generally relatively slowly, resulting in the outgrowth of contaminants (Pasttrana *et al.*, 1995; Dirand *et al.*, 1997; Machado *et al.*, 1999). In such cases, it would be essential to use a bioreactor, which can be operated under aseptic conditions. However, the present study was conducted under strict aseptic conditions to avoid the possible complications of contamination if any.

Although pH is one of the critical factors, the monitoring and control of pH during fermentation is not usually attempted in SSF. The measurement and control of this variable in SSF is very difficult. Nevertheless, the substrates employed in SSF usually have buffering effect due to their complex chemical composition. In these cases, the control of pH is not very

essential. When this variable must be controlled, buffering solutions are added as liquid phase, but this strategy can be inadequate when the process is scaled up. Often, ammonium salts have been used in SSF, in combination with urea or nitrite salts due to the respective effects of acidification and alkalisation of the former and the latter (Torrado *et al.*, 1998 and Raimbault, 1998). In the present study, carbonate/bicarbonate buffer of pH 9.2 was used in appropriate volumes, as the liquid phase, in all the experiments.

Results obtained for the optimisation of process parameters for SSF production of alkaline protease demonstrated clearly the impact of the process parameters on the gross yield of enzyme as well as their independent nature in influencing the organism's ability to synthesise the enzyme.

2.4.2.1 Effect of different substrates on protease production

A number of solid substrates natural as well as synthetic can be used in SSF processes. The major organic materials available in nature are polymeric in structure, e.g. polysaccharides, proteins and lignins. In general, all these can be used by microorganisms as substrate (carbon source). Solid substrates used in SSF are insoluble in water; in practice, water is absorbed on to substrate particles which can be used by microorganisms for growth and metabolic activity. Bacteria and yeast grow on the surface of the substrate while fungal mycelia penetrate into the particles of the substrate.

The selection of a substrate for SSF process depends upon several factors mainly related to cost and availability and thus may involve screening of several agro-industrial residues (Pandey *et al.*, 2000a). In the SSF process, the solid substrate not only supplies the nutrients to the microbial culture growing in it, but also serves as an anchorage for the cells. The substrate that

provides all the needed nutrients to the microorganisms growing in it should be the ideal substrate.

Research on the selection of a suitable substrate mainly centered around tropical agro-industrial crops and residues. These include crops such as cassava, soybean, sugar beet, sweet potato, potato (Bisping *et al.*, 1993; Emelyanove,1996) and crop residues such as bran and straw of wheat and rice, hull of soy, corn and rice, bagasse of sugarcane and cassava (Chiu and Chan, 1992; Nampoothiri and Pandey, 1996; Makkar and Cameotra, 1997,1999), residues of the coffee processing industry such as coffee pulp, coffee husk (Seitz, 1994; Christen *et al.*, 1994; Besson *et al.*, 1997; Bromarski *et al.*,1998; Meza *et al.*, 1998; Larroche *et al.*, 1999; Medeiros *et al.*, 1999; Soares *et al.*, 2000), residues of fruit-processing industries such as pomace of apple and grape, wastes of pine-apple and cariot processing, banana waste (Amin, 1992; Nagadi and Correia, 1992; Saucedo-Castaneda *et al.*, 1992; Roukas, 1994; Joshi *et al.*, 1995; Henk and Linden, 1996; Joshi and Sandhu, 1996; Kiransree *et al.*, 1999) and others such as saw-dust, corn cobs, carob pods, tea waste, chicory roots etc. (Han,1998). Some reviews are available on the biotechnological potential of several agro-industrial residues for value-addition in SSF (Pandey and Soccol, 1998; Soccol and Kreiger 1998; Pandey *et al.*, 2000a,b).

A suitable solid substrate for the fermentation process is a critical factor and thus involves the screening of a number of agro-industrial materials for microbial growth and product formation. In the present study, wheat bran, a cheap agro-residue was found to be the best substrate compared to the ten other substrates tested. Similarly, Uyar and Baysal (2004) also found wheat bran as a better substrate for protease production than lentil husk. Hesseltine (1972) reported that wheat is a good substrate for SSF because the wheat kernels have much less tendency to adhere to one other. Wheat bran is a well

recognised, widely used and most popular substrate in SSF enzyme production (Rivera-Munoz *et al.*, 1991).Wheat bran was also found to be the best substrate for α- amylase production and it has also been used for the production of alkaline protease by *Bacillus* sp. and P–2 *Pseudomonas* sp. (Babu and Satyanarayana, 1996; Kaur et *al.*, 2001).

The biochemical composition of wheat bran is 16% water content, 15% protein, 22% starch, 3% fat and 44% rest ie.,cellulose and minerals (Cui *et al.*, 1998). The uptake of nutrients from the wheat bran by the cultures, due to their ability to penetrate deeply into the particles, has been well established in SSF processes (Lonsane *et al.*, 1985). Some of the vital nutrients necessary for optimum growth and product formation may also be present in wheat bran at an optimum level. Hence, the supplement of wheat bran with other solid and/or water soluble nutrients has been found to enhance product formation in SSF processes (Kumar and Lonsane, 1987). Water-holding capacity of wheat bran was more than that of other brans, due to high initial starch content of wheat bran.

2.4.2.2 Effect of incubation period on protease production

The incubation time needed for maximum enzyme production is governed by the characteristics of the culture and is based on growth rate and phase of enzyme production. The time employed may vary from 48h to 8-9 days depending upon the organism involved (Aikat and Bhattacharya, 2000; Puri *et al.*, 2002).

In the present study, using wheat bran as the substrate, *Vibrio* sp. V26 showed maximum protease production was obtained after 96 h of incubation. This may be the time when the easily degradable part of the substrate in wheat bran was used. The lower enzyme production at the earlier stages of fermentation may be attributed to the lag phase of growth of the

microorganism when introduced into the fresh medium. The decline in enzyme activity observed after 96 h might be due to denaturation and/or decomposition of protease as a result of interactions with other compounds in the fermented medium.

Results similar to that of the present investigation were obtained during glutaminase (Nagendra and Chandrasekaran, 1997), and α-amylase (Ramesh and Lonsane, 1987) production in SSF; so also by other investigators (Qadeer *et al.*, 1980; Beckord *et al.*, 1945). A sharp drop of chitinolytic activity after a maximal peak in *B.bassiana* was reported in liquid culture (Smith and Grula, 1983). The variation in the incubation time required for maximal enzyme production with the type of inoculum could be attributed to differences in the physiological status of the inoculum which consequently influence the rate of growth and product formation during SSF.

Enzyme production usually takes place during the late logarithmic phase or early stationary phase (Keil-Dlouha *et al.*, 1976; Reid *et al.*, 1980; Makino *et al.*, 1983; Philip, 1987).

2.4.2.3 Effect of initial moisture content on protease production

The moisture level of the substrate is one of the key factors influencing the outcome of using a SSF and is governed by the nature of the substrate, the type of end product and the requirement of the microorganism.The role of the water content of the substrate was widely described and reviewed by different authors (Cannel and Moo-Young, 1980a; Oriol, 1987; Oniol *et al.*, 1988; Ramesh and Lonsane, 1990; Acuna-arguelles *et al.*, 1994; Xavier and Lonsane, 1994; Bellon-Maurel *et al.*, 2003; Gervais and Molin, 2003). Moisture content is a critical factor on SSF processes because of its influence on growth and biosynthesis and secretion of different metabolites (Krishna and Chandrasekaran, 1996; Ellaiah, 2002). A lower

moisture content causes reduction in solubility of nutrients of the substrate, low degree of swelling and high water tension. On the other hand, higher moisture levels can cause a reduction in enzyme yield due to steric hindrance of the growth of the producer strain by reduction in porosity of the solid matrix, thus interfering oxygen transfer (Lonsane *et al.*, 1985).

As the optimal value of moisture content depends on both the microorganism and the solid matrix used, for economic production, the microorganism should be grown in optimal moisture levels either for maximising the growth or metabolite production depending on the application. In the present study, a comparatively higher moisture content of 80% favoured maximum protease yield. Moisture content above 80% resulted in the separation of bran from the moistening medium and reduced enzyme production. Therefore 80% moisture content was considered as the optimum and was chosen for further experiments. Increase in moisture level is believed to reduce the porosity of the wheat bran, thus limiting oxygen transfer (Feniksova *et al.*, 1960 and Lonsane *et al.*, 1985).

The optimal moisture level was reported to be 74% with wheat bran for protease production by *Pseudomonas* sp. (Chakraborty and Srinivasan, 1993). Earlier reports indicated the requirement of 55, 63 and 140% initial moisture content for maximum protease production by *Aspergillus flavus* IMI 327634 (Malathi and Chakraborty, 1991), *Rhizopus oryzae* (Tunga *et al.*, 1998) and *Pencillium* LPB-9 (Germano *et al.*, 2003) respectively. Sinha *et al.* (2003) obtained maximum protease production with 70% moisture in the medium using a *Bacillus* sp. A similar result was reported by Uyar and Baysal (2004) in a bacterial strain *Bacillus* sp. under solid state fermentation.

High substrate moisture results in decreased substrate porosity and may facilitate bacterial contamination. At the same time, low moisture levels lead to poor accessibility to nutrients. Gonzalez *et al.* (1990) discussed the

stochiometric relation and yield parameters of growth of fungi in SSF and illustrated the possibility that water may be a limiting substrate. Hang and Woodams (1990) studied the effect of substrate moisture on citric acid production and found that it critically affected mould growth and activity. Kumar and Lonsane (1990) stressed the need for optimal physical factors, including moisture content, for gibberllic acid production in SSF. Ramesh and Lonsane (1990) also reported the critical importance of moisture content of the substrate in α-amylase production in a SSF system.

The water requirements of microorganism must be better defined in terms of water activity (a_w) rather than water content of the solid substrate (Raimbault, 1998). Water activity is defined as the relationship between the vapour pressure of water in a system and the vapour pressure of the pure water. From a microbiological point of view a_w indicates the available or accessible water for the growth of the microorganism. The water activity affects the biomass development, metabolic reactions and the mass transfer processes (Bellon-Maurel *et al.*, 2003; Gervais and Molin, 2003).The adequate value of a_w depends on both the product and the requirements of the microorganism.

2.4.2.4 Effect of inoculum size on protease production

The effect of inoculum size on SSF is well known (Hesseltine *et al.*, 1976 and Mitchell, 1992). In the case of the present isolate of *Vibrio* sp., an increase in protease production was recorded with increasing size of inoculum up to 20% v/w. The optimum inoculum concentration required for maximum protease activity was 20% v/w of the wheat bran and further increase in the size of inoculum did not lead to increased protease activity.

Sen (1995) reported a 10% inoculum level for the production of alkaline protease by *Bacillus licheniformis* S40. Satyanarayana (1994)

observed that high inoculum levels were inhibitory in nature. Inoculum size controls the initial lag phase (Nystorm and Kormuta, 1975). A smaller inoculum extends the lag phase. The larger inoculum quantity favoured more substrate degradation and biomass formation, perhaps because of the larger quantity of culture.

2.4.2.5 Effect of temperature on protease production

The incubation temperature has a profound effect on the enzyme yield and the duration of the enzyme synthesis phase (Ramesh and Lonsane, 1987). Optimum temperature for enzyme production is largely characteristic of the organism irrespective of the type of solid support involved in SSF (Chandrashekaran *et al.*, 1991; Nagendra and Chandrasekaran, 1996). In the present study the best suitable temperature for maximum production was found to be 30^{0}C. Higher temperatures did not favour growth of the organism though the protease under study was highly thermostable, which shows the mesophelic nature of the organism.

The increase in temperature in SSF is a consequence of the metabolic activity when the heat removal is not enough. This directly affects spore germination, growth and product formation. The temperature level reached is a function of the type of microorganism, the porosity, the particle diameter and the depth of the substrate (Raghavarao *et al.*, 2003; Gervais and Molin, 2003 and Raimbault, 1998).

Heat transfer in SSF is very low because of the limited heat transfer capacity of the solid substrates used. The overall heat transfers depends on the rates of the intra- and inter- particle heat transfer and the rate at which heat is transferred from the particle surface to the gas phase (Raimbault, 1998). In an industrial context, monitoring and controlling this variable is critical for scaling up (Bellon-Maurel *et al.*, 2003). Conventionally aeration is

the main method used (Raimbault, 1998 and Raghavarao *et al.*, 2003) to control the temperature of the substrate. Because high aeration rates can reduce the water activity of the substrate by evaporation, water saturated air is usually used (Raghavarao *et al.*, 2003). The agitation of the fermentation mass can also help to control the temperature.

2.4.2.6 Effect of amount of substrate on protease production

The level of substrates influences the porosity and aeration of the substrate. In the present study, the highest enzyme production was observed when the ratio of substrate weight to volume of flask was 1:50. This is in agreement with the results obtained by other investigators (Satyanarayana, 1994 and Ellaiah *et al.*, 2003). The thickness of the substrate layer employed governs the extent of aeration and oxygen transfer rates of the fermentation system.

2.4.2.7 Effect of particle size of substrate on protease production

The particle size and therefore the specific area of the substrate are of importance in SSF (Muniswaran and Charryalu, 1994) and usually smaller particles stimulate greater growth (Hunang *et al.*, 1985). The utilisation of solid substrates by the microorganisms is affected by several physical and chemical factors. Among the physical factors, accessibility of substrate to microbes, film effects and mass effects are important (Knapp and Howell, 1985). The physical morphology especially porosity and particle size of the substrate, governs the accessible surface area of the organism. Among the chemical factors, the chemical nature of the substrate (degree of polymerisation and crystallinity) is an important criterion. Generally, smaller substrate particles would provide larger surface area for microbial attack and thus should be considered as a desirable factor. However, too small substrate particles may result in substrate agglomeration in most of the cases, which

may interfere with microbial respiration/ aeration and at the same time, larger particles provide better respiration/ aeration efficiency.

In the present study, maximum protease production was obtained with substrate particles of average size < 425 µm. With smaller particle, the surface area for growth was greater but the inter-particle porosity was less. The larger the size, the greater the porosity, but the saturated surface area was less. These two opposing factors probably interacted to give the value corresponding to optimum growth and product formation (Muniswaran and Charryalu, 1994). Huang *et al.* (1984) observed that greater growth of fungal cultures was stimulated by smaller particle size substrate. Particles of 30 mesh size were found to be the optimum particle size. Pandey (1991) found that enzyme productivity was higher with a substrate that contained particles of mixed sizes varying from smaller than 180 µm to bigger than 1.4mm.

2.4.2.8 Effect of NaCl concentration on protease production

The NaCl requirement reflects the growth and productivity of the individual organisms in their natural habitat. The present strain under study is a mangrove isolate and requires 1% NaCl in the medium for optimum protease production and tolerates higher concentrations as well without drop in the enzyme yield.

The mangrove from where the present strain was isolated is Puduvyppu, which is one of the biggest mangrove areas on the Kerala coast. The land is regularly inundated by a semidiurnal tidal rhythm of Cochin bar mouth. The salinity–nutrient changes in the Cochin estuary is evidently influenced by the influx of freshwater and intrusion of sea water (Anirudhan *et al.*, 1987). Thus the isolate selected for study is subjected to high salinity variations in their natural habitat, which may account for its tolerance to different levels of salinity.

2.4.2.9 Effect of supplementary carbon sources on protease production

Generally, high carbohydrate concentrations were found to repress enzyme production. Therefore, carbohydrate was added continuously or in aliquots throughout the fermentation to supplement the exhausted component and keep the volume limited and thereby reducing the power requirements (Aunstrup, 1980). Taking this into consideration the carbon supplement in the present study was limited to 1% w/w of the substrate.

Extracellular enzymes are usually susceptible to catabolite repression by rapidly metabolised carbon sources. Earlier studies have indicated a reduction in protease production due to catabolite repression by glucose (Hanlon *et al.*, 1982; Frankena *et al.*, 1986; Kole *et al.*, 1988; Secades and Guijarro, 1999). Similar result was obtained in the present study, where protease production was very low when glucose was added to the medium. However 1.5 fold increase in production of protease by a *Pseudomonas maltophila* strain was reported by using glucose medium over peptone medium (Kobayashi *et al.*, 1985).

In the present study maximum protease yield was obtained when maltose was supplemented in the medium. Increased yields of alkaline proteases were reported by several workers who used different sugars such as lactose (Malathi and Chakraborthy, 1991), maltose (Tsuchiya *et al.*, 1991) and sucrose (Phadatare *et al.*, 1993) as sources of carbon. In the present case the use of molasses, a byproduct in the beverage industry was found to be a good source of carbon for protease production. It gave good enzyme yield comparable with the other carbon sources. Therefore, molasses could be suggested as cheap raw material for the production of the alkaline proteases.

2.4.2.10 Effect of supplementary nitrogen sources on protease production

Alkaline protease comprises 15.6% nitrogen and its production is dependent on the availability of both carbon and nitrogen in the medium (Kole *et al.*, 1988). Although complex nitrogen sources are usually used for alkaline protease production, the requirement for a specific nitrogen supplement differs from organism to organism.

An external nitrogen source is essential for increased utilisation of soluble carbohydrates and reduction in fermentation time (Sandhya and Lonsane, 1994). In the present study inorganic nitrogen sources inhibited protease production whereas organic nitrogen sources enhanced enzyme production. Corn steep liquor was found to be a more suitable supplement though beef extract and casein also gave satisfactory results.

Corn steep liquor was found to be a cheap and suitable source of nitrogen by other workers also (Fujiwara and Yamamoto, 1987; Malathi and Chakraborty, 1991; Sen and Satyanarayana, 1993). Apart from serving as a nitrogen source, corn steep liquor provided several micronutrients, vitamins and growth promoting factors. However, their use is limited by its seasonal and inter-batch variability.

Similar to the findings of the present study, low levels of alkaline protease production were reported with the use of inorganic nitrogen sources in the production medium (Chandrasekaran and Dhar, 1983; Sen and Satyanarayana 1993; Chaphalkar and Dey, 1994). Enzyme synthesis was found to be repressed by rapidly metabolisable nitrogen sources like amino acids, or ammonium ion concentrations in the medium (Cruegar and Cruegar, 1984; Giesecke *et al.*, 1991). The presence of ammonium significantly reduced protease production in *A.salmonicida* (Liu and Hsieh, 1969), *Vibrio*

strain SA1 (Wiersma *et al.,* 1978), *A.hydrophila* (O'Reilly and Day, 1983), and the fish pathogen *Yersinia ruckerii* (Secades and Guijarro, 1999). Ammonium specific repression is likely to be the explanation. The production of alkaline protease is suppressed by rapidly metabolisable nitrogen sources such as amino acids or ammonium in the medium (Hanlon *et al.*, 1982; Kole *et al.*, 1988). The replacement of soy bean flour with ammonium sulphate in a fed batch process proved cost effective and resulted in the elimination of unpleasant odours as well (Mao *et al.*, 1992).

2.4.3 Optimisation of extraction parameters for protease recovery

SSF produces highly concentrated products which are diffused through the solid media and therefore extraction is required. Extraction in solid state fermentation is a process of dissolution and leaching in a heterogeneous liquid-solid system. Depending upon its application on process economics and to reduce further downstream processing costs, various techniques have been developed (Castillo *et al.*, 1999; Bjusrstorm, 1985; Caltron *et al.*, 1986). Negligible information is available in the literature on the extraction of the enzymes from the fermented bran obtained by SSF.

2.4.3.1 Effect of different extractants on protease extraction

Solid state fermentation is fermentation in the absence of free liquid, and recovery of the fermentation product requires its extraction from the solid fermented medium. As the initial moisture levels in the medium was very low, squeezing of the solid medium itself hardly yields any extract and even if there is any, the volume might not be sufficient for complete extraction. The medium must therefore be soaked for some time in an adequate amount of a suitable extractant for the total recovery of the product.

The extraction efficiency is critical to the recovery of the enzyme from the fermented biomass; hence selection of a suitable solvent is necessary. A

commonly used extractant is distilled or deionised water (Silman, 1980; Yano *et al.*, 1991 and Ghildyal *et al.*, 1993). Other extractants have also been used, such as NaCl solution (Yang and Chiu, 1987; Battaglino *et al.*, 1991), buffers (Rivera-Munoz *et al.*, 1991), aqueous mixture of ethanol and glycerol (Tunga *et al.*, 1999), Tween 80 (Agarwal *et al.*, 2004) etc. In the present study three different extractants *viz.* distilled water, saline and carbonate/bicarbonate buffer were used and the best extractant was found to be the buffer.

Adsorption of the enzyme to mouldy bran has been attributed to ionic bond, hydrogen bond and van der Waal forces (Fernandez-Lahore *et al.*, 1998). Sodium chloride (1%) has been shown to be the most suitable protease extractant for *Mucor bacilliformis* (Fernandez-Lahore *et al.*, 1998) and *Rhizopus oligosporous* (Ikasari and Mitchell, 1996). Alternatively, an aqueous mixture of ethanol (10%) and glycerol (3%) has been efficiently used for extraction of *R.oryzae* protease (Tunga *et al.*, 1999).

2.4.3.2 Effect of volume of extractants on protease extraction

In SSF system free flowing liquid is very much limited. Thus adequate amount of solvent is required to leach out the enzyme present. In the present study, it was found that 30ml solvent in 5g of fermented bran was optimum. The amount of recovered enzyme was lower in experiments with lower volume of extractant. This might be due to insufficient volume of solvent to penetrate the solid fermented mass. Higher volumes of extractant decreased the efficiency of recovery which may be due to the increased dilution. Volume of extract naturally increases with volume of the extractant. Use of excessively large volumes of extractant for greater extraction would yield enzyme solutions too dilute to be profitably utilised. Similar result has been obtained by Aikat and Bhattacharya (2000).

2.4.3.3 Effect of soaking time of extractants on protease extraction

The fermented biomass has to be soaked in a suitable extractant for maximum recovery of the product. The extractant should be allowed enough time to penetrate through the fermented bran, which would facilitate the washing out of maximum enzyme during extraction. The minimum time for total penetration is the optimum incubation time for soaking.

In the present study 150 minutes was the optimum and beyond that no additional effect on enzyme extraction was observed. The lower enzyme yield for shorter periods of incubation must be due to poor penetration of the solvent. Increasing the duration of soaking time will improve the yield to the maximum and further incubation is quite unnecessary. It is inferred that the influence of soaking will diminish with time. In a similar study carried out by Aikat and Bhattacharya (2000) complete extraction of protease from the fermented substrate was achieved in 10 h and longer soaking periods did not result in any significant gain in enzyme recovery.

2.4.3.4 Effect of repeated extractions on protease extraction

In order to make sure that the entire available enzyme produced during fermentation is recovered during extraction, the extraction process is repeated a second time to see if there is too much of residual product in the fermented mass. It was found that 92% of the total available enzyme was recovered in the first wash itself. The necessity of a second wash is overruled considering the significantly lower amounts of yield. Unless excessively large volumes of extractant are used, repeated extractions would result in progressively lower amounts of enzyme extracted and many repeated extractions would be required for complete extraction (Aikat and Bhattacharya, 2000).

Optimisation of the process parameters of SSF is an important aspect of improving performance. The main limitations of SSF are the unique geometric conditions for growth which result in poor substrate accessibility and difficulty in controlling fermentation conditions. The present investigation indicates that improvements in SSF may be made by optimising the process parameters. Considering the difficulties in handling the large volume bioreactors related to the production of bulk chemicals and products in SSF, it could be more practical to use SSF for cost effective projects. The observations in the present study are of economic importance as wheat bran is an inexpensive and abundantly available raw material. Therefore, the use of SSF should be considered by industry, especially when large quantities of secondary metabolites are required in short fermentation periods, with minimal expenditure on media and downstream processing.

3

Production of alkaline protease under submerged fermentation

The use of microorganisms for the production of commercially important metabolites has increased rapidly over the past half century and the production of enzymes in submerged fermentation (SmF) has long been established. A significant number of highly economical SmF processes are known to exist not only for enzyme production but also for the production of several other metabolites and are being successfully exploited by industry in most countries of the world. Extensive investigations have accomplished great developments in the technology overriding the earlier shortcomings due *to unoptimised preculture and other growth conditions*.

Submerged fermentation involves the growth of microbes on solid substrates dissolved or submerged in liquid. The limited availability of water makes SSF quite different from SmF. Compared to solid state fermentation, very little information is available on submerged state fermentation. The

advantages of SSF for protein enrichment and bioconversion of substrates over submerged fermentation are very clear and significant. This has led to SmF being utilised exclusively for enzyme production especially by fungal cultures. Most investigations on enzyme production methods are concerned with producing fungal or bacterial biomass in submerged cultures. These culture methods permit better control of environmental factors such as temperature and pH.

Implementation of submerged fermentation for the production of various secondary metabolites was described by several authors. Bartholomew *et al.* (1950) described the mass transfer of oxygen in submerged fermentation of *Streptomyces griseus*. The nature and quantity of by-products formed during conversion of sugar beets to ethanol by *Zymomonas mobilis* in conventional submerged fermentation were investigated by Amin and Allah (1992). Morphology and citric acid production of *Aspergillus niger* PM1 in submerged fermentation has been worked out by Papagianni (1995). Papagianni *et al.* (2001) have made a successful attempt to produce phytase under submerged fermentation using *Aspergillus niger*. Ghildyal *et al.* (1993) compared the economics of submerged and solid state fermentation for the production of amyloglycosidase. Senecal *et al.* (1992) reported that SmF was favoured over SSF in biotransformation of corn stover when used as a carbon source. Crestini *et al.* (1996) described a method for the production and isolation of chitosan by liquid and solid state fermentation from *Lentinus edodes*.

A general opinion about the choice of fermentation method for the production of any microbial product would normally be SmF, unless there appears a particular reason why SSF should be chosen. It is a familiar technique, scale–up from laboratory level to industry is much simplified, with parameters being more easily monitored and controlled. However, there are

problems associated with secondary metabolite production in liquid fermentation such as shear forces, increasing viscosity due to metabolic secretion and reduction in metabolic stability. However, SmF is intrinsically less problematic – heat transfer is better, and homogeneity is maintained. Infact, SmF has many features which would make it the preferred method in cases where SmF and SSF had similar economic performances.

One of the differences between solid-state and submerged cultures is that in the former, the moisture content of the substrate is low, resulting in a limitation of growth and metabolism of the microorganism. The concept of water availability in a substrate, thus, becomes very important. This difference is one of the crucial factors that govern the processes that occur during fermentation. Moisture content being related to many factors can greatly influence the path of enrichment, leading to products that differ both quantitatively and qualitatively.

Several reports are available on enzyme production by SmF. The strain *A.oryzae* NRRL 2160 was reported to produce high levels of extracellular protease (Dworshack, 1952). Klapper *et al.* (1973) reported the production and purification of a serine protease from this strain in submerged culture. Proteases from *Aspergillus oryzae* have been produced in submerged cultures (Nakadai and Nasuno 1988; Fukushima *et al.*, 1989). Comparatively higher lipid enrichment of prawn shell waste in SmF by various protease producing bacterial strains was described by Amar (2001).

The requirement of water for growth and metabolic activities of microorganisms and the consequent potential of the water activity of the medium in controlling biotechnological processes are well established (Hahn-Hagerdal, 1986).Thus it is expected that the increase in moisture content of the medium would lead to increased productivity of the system. It was only recently, that it became apparent that the biochemical and physiological

responses of many organisms in solid state culture differ greatly from those in SmF, leading to variations in productivity. It is also well known that the strains isolated and developed for the use in SSF processes are poor producers of enzyme in the SmF system and vice versa. So it is imperative to study the preferences of the selected strain to obtain optimal performance.

3.1 Materials and methods

3.1.1 Inoculum preparation

Inoculum was prepared as described in section 2.1.2

3.1.2 Alkaline protease production under SmF

The SmF experiments were carried out in 250 ml Erlenmeyer flasks using 1.25g of the substrate submerged in 25ml of 0.2M carbonate/bicarbonate buffer (pH 9.2). The medium was autoclaved at 121^{0}C, 15lb pressure for 15min, cooled to room temperature and inoculated with 80% (v/w) inoculum (5x10^8 cells/ml). The cultures were then incubated at 37^{0}C and 100rpm for 120 h and samples were withdrawn for enzyme assay. Plate 3 shows submerged fermentation set up in a 250 ml conical flask.

3.1.3 Enzyme extraction

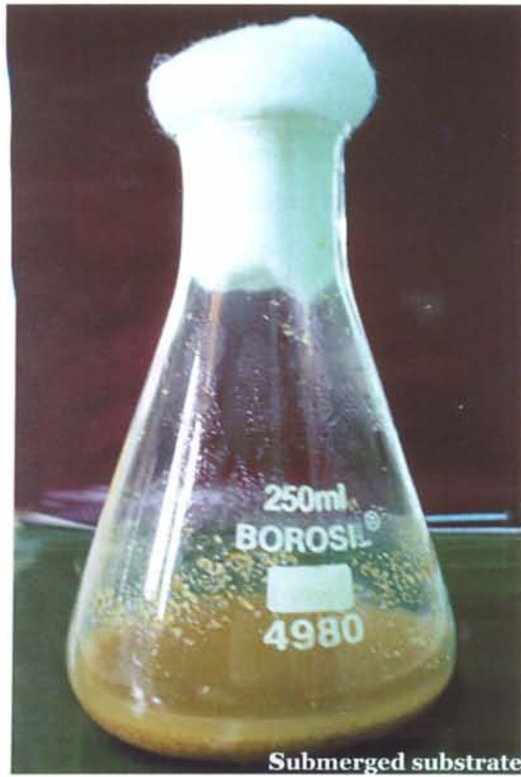
After fermentation the whole contents were filtered and squeezed through a muslin cloth. The clear extract obtained after centrifugation was assayed for proteolytic activity.

3.1.4 Alkaline protease assay

Alkaline protease was assayed as described in section 2.1.5

3.1.5 Optimisation of fermentation process parameters under SmF

The strategy followed was to optimise each parameter, independent of the others and subsequently optimal conditions were employed in all the



SmF set up in a conical flask

Plate 3

experiments. The various process parameters optimised for maximal protease production were as follows:

3.1.5.1 Effect of different substrates on protease production

All the substrates experimented for the production of protease under SSF (2.2.6.1) were tried in SmF as well. The ideal substrate obtained was used for subsequent experiments.

3.1.5.2 Effect of incubation period on protease production

The culture medium was incubated for varying periods of time ,24, 48, 72, 96 and 120h to find the optimum time required for maximum enzyme production.

3.1.5.3 Effect of inoculum size on protease production

The influence of inoculum size on protease production was studied by altering the inoculum levels as 20, 40, 60, 80 and 100 (%,v/w) in the medium.

3.1.5.4 Effect of temperature on protease production

Fermentation was carried out at various temperatures such as 20^{0}C, 25^{0}C, 30^{0}C, 37^{0}C and 45^{0}C to evaluate their influence on protease production. The experiments that followed were conducted at the temperature optimised at this step.

3.1.5.5 Effect of amount of substrate on protease production

Different quantities of substrate (%,w/v) 5,10,15 and 20 were submerged in the fermentation medium to investigate the influence of the amount of substrate on the magnitude of enzyme production. The minimum quantity of substrate yielding maximum enzyme was selected for further experiments.

3.1.5.6 Effect of particle size of substrate on protease production

The substrate was sieved through various mesh size to obtain fine, medium, coarse and large particles of < 425μm, 425-600 μm, 600-1000 μm and 1000-1425 μm size respectively to see whether the particle size has any influence on enzyme production.

3.1.5.7 Effect of NaCl concentration on protease production

The effect of sodium chloride was evaluated by incorporating it at various concentrations of 0, 0.05, 0.1, 0.2 and 0.4 (%,w/w) in the fermentation medium.

3.1.5.8 Effect of supplementary carbon sources on protease production

The requirement of additional nutrient supply was studied, adding different supplementary carbon sources (10%w/w) like glucose, sucrose, maltose, lactose, glycerol, starch, mannose and molasses to the fermentation medium. The ideal carbon source thus obtained was included in the medium formulation to continue the optimisation studies of the remaining parameters.

3.1.5.9 Effect of supplementary nitrogen sources on protease production

Whether the addition of supplementary nitrogen sources could enhance the production of protease was studied by supplying organic sources like beef extract, casein, gelatin, peptone, tryptone, yeast extract and corn steep liquor; and inorganic nitrogen sources like $NaNO_3$, NH_4Cl, $(NH_4)_2SO_4$, NH_4NO_3 and urea at a level of 10% w/w in the medium.

3.1.5.10 Effect of shaking speed on protease production

The effect of aeration and mixing of substrate on protease production under SmF was determined by incubating the culture medium at different rpm (0, 50,100, 150 and 200) in a rotary shaker.

All the experiments were carried out in triplicates in order to corroborate the validity of the results obtained.

3.2 Statistical analysis

Data generated from the above experiments were analysed by One-way Analysis of Variance (ANOVA). Mean of the results was compared using SPSS 10.0 for windows at a significance level of $p<0.05$.

3.3 Results and Discussion

The results of the optimisation studies of the fermentation process parameters under SmF are shown as graphs and tables. The ANOVA results are also given in the corresponding tables as superscript alphabets. Values with the same superscripts do not vary significantly.

3.3.1 Optimisation of fermentation process parameters under SmF

Extracellular protease production in microorganisms is strongly influenced by media components such as, variation in C/N ratio, presence of some easily metabolisable sugars, such as glucose and metal ions. Protease synthesis is also affected by the presence of rapidly metabolisable nitrogen sources in the medium. Besides, several physical factors such as aeration, inoculum density, pH, temperature and period of incubation also affect the amount of protease produced (Hameed *et al.*, 1999; Puri *et al.*, 2002). In the present study also, culture conditions have been found to influence the quantity of protease produced by the selected strain.

3.3.1.1 Effect of different substrates on protease production under SmF

Studies on the effect of different substrates on protease production have shown that different substrates performed quite differently from that of SSF, except for wheat bran, which proved to be the best substrate in SmF as well. Rice bran and ragi which yielded very low quantities of enzyme in SSF

produced good results in SmF while rawa and maize bran which gave good results in SSF were poor producers in SmF. The order of substrate suitability as per the ANOVA results was wheat bran > ragi > rice bran > black gram bran > soyabean bran, rawa, maize bran and bread powder > cassava > barley (Fig.3.1). Wheat bran being the best source for enzyme production was used in the subsequent experiments.

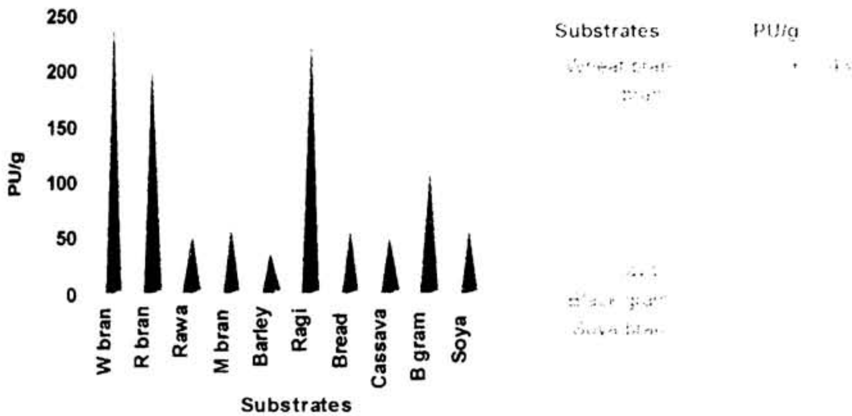


Fig.3.1 Effect of different substrates on protease production: SmF

The present study makes it evident that the choice of substrate is equally important as the producer organism for successful fermentation. Decreased moisture content might have impaired the accessibility of rice and ragi to the microorganism in SSF. With free flowing water in the medium, these substrates yielded higher amounts of enzyme whereas rawa and maize bran favoured low levels of water in the medium as demonstrated by better performance under SSF conditions. Wheat bran is suitable for both SSF and SmF with better productivity under SmF which itself indicates that the microorganism under study prefers higher water content in the medium.

Although the moist solids are constantly degraded in nature by microorganisms, very little information is available on the factors affecting the utilisation of solid substrates. In general, growth is attributed to the action of enzymes in breaking down the solids to the components that are capable of permeating into the microbial cells. The mechanisms of uptake of non-permeating substrates such as chitin (Stolp and Starr, 1965), lignins (Ogelsby *et al.*, 1967), keratin (Martin and So, 1969), collagen (Seifter and Harper, 1970), sulphur (Beebe and Umbreit, 1971), proteins (Costerton *et al.*, 1974), metal sulphides (Tuovinen and Kelly, 1974), hydrocarbons (Wodzinski and Coyle, 1974; Velankar *et al.*, 1975), agar (Day and Yaphe, 1975), granular starch (Dunlap *et al.*, 1976) and cellulose (Nesse *et al.*, 1977; Berg and Patterson, 1977) were investigated under conditions of submerged culture.

Partial hydrolysis of the pre-treated solids during fermentation occurs outside the cells due to extracellular or cell wall bound enzymes, although detachment of the cell wall bound enzymes or the release of intracellular enzymes due to cell autolysis may also play an important role (Pollock, 1962; Knapp and Howell, 1980). Uptake of moist solids by microorganisms will be influenced by various physical and chemical factors such as the shape of solid particles, porosity, particle size, fibrousness, surface-to-mass ratio, crystallinity, amorphism, stickiness, diffusivity within the solids, mass transfer to and from the solids, degree of polymerisation, hydrophobic or hydrophilic nature, surface electrochemical properties and chemical properties decisive in allowing adsorption of microorganisms on the substrate surfaces (Lonsane *et al.*, 1985). The microbial modification of the substrate resulting in partial hydrolysis is also governed by a variety of factors such as production of enzymes by the cells, uptake of permeable products, diffusion of enzyme into mass of solids, accessibility of solids to cells, heterogeneity of solid surfaces,

organism proximity, requirement of the presence of more readily degradable carbon sources in the system and overall enzyme kinetics (Erickson, 1978).

3.3.1.2 Effect of incubation period on protease production under SmF

Enzyme assay results showed that maximum protease production could be attained at a shorter time in SmF than SSF. A period of 48 h of incubation was enough for maximum yield in SmF compared to 96 h in SSF (Fig.3.2). ANOVA results also substantiated the same results.

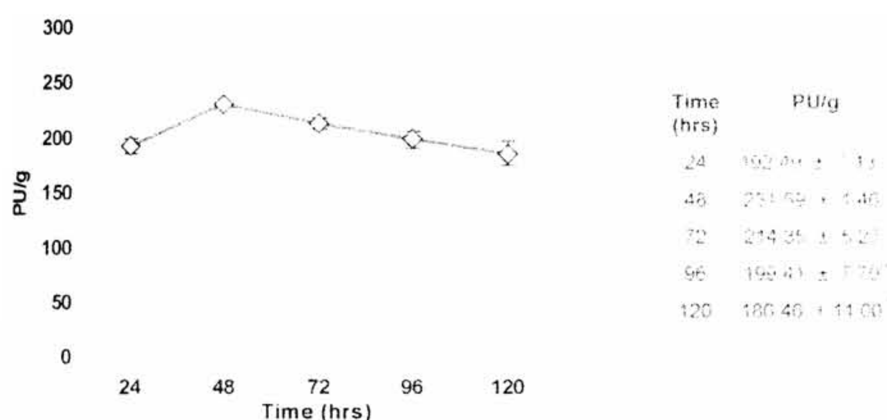


Fig.3.2 Effect of incubation period on protease production: SmF

An optimal incubation period for maximum protease production is characteristic of the microorganism involved and is largely based on growth characteristics. Bacteria when grown in batch cultures exhibit a characteristic growth curve involving phases of lag , log , stationary and decline , and the phase at which protease production begins in the cell is determined by measuring enzyme production at different time intervals, after inoculation. In the present study, enzyme production begins at the logarithmic phase of growth, reaching the maximal level at the stationary phase of growth. Maximum yield was obtained at 48 h which must apparently be the stationary

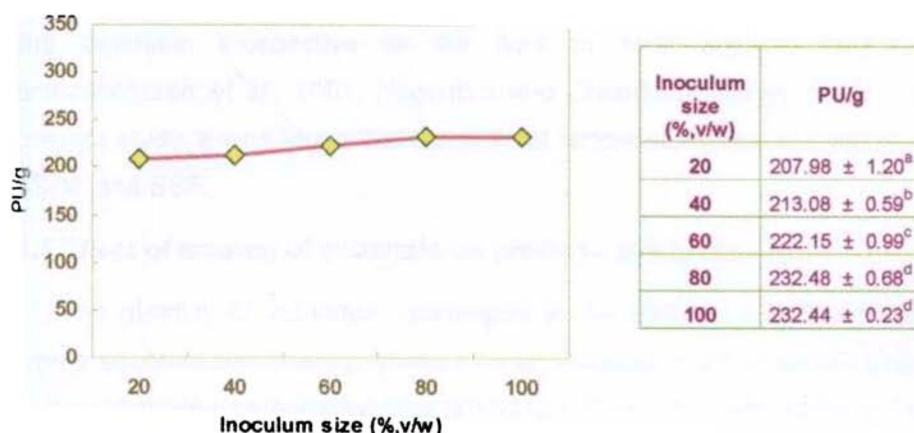
phase and thereafter, there was a decline in the production which can be related to the death phase of the growth curve. The decline in enzyme activity might be due to denaturation and/or decomposition of protease as a result of interactions with other compounds in the fermented medium(Cui *et al.*, 1998).

In a similar study, the protease production in *Pseudomonas* 1-6 was reported (Sakata *et al.*, 1977) to be very high in the logarithmic phase and then decreased. In some instances, there is little or no enzyme production during the exponential growth phase (Frankena *et al.*, 1985). In a number of cases, the synthesis and secretion of proteases was initiated during the exponential growth phase with a substantial increase near the end of the growth phase and with maximum amounts of protease produced in the stationary growth phase (Durham *et al.*, 1987; Tsai *et al.*, 1988, Takii *et al.*, 1990; Moon and Parulekar, 1991; Ferrero et al., 1996; Manachini *et al.*, 1998).

In SmF maximum production was attained at a shorter time than in SSF. The exclusive features of availability of excess water in the medium and agitation in SmF might be the reason. Vecht-Lifshitz *et al.* (1990) observed that viscosity of the medium influenced the process parameters in submerged fermentation.

3.3.1.3 Effect of inoculum size on protease production

Higher inoculum levels gave greater yields until a level of 80 %(v/w) beyond which there was no increase in the amount of enzyme produced (Fig.3.3), the pattern being the same as that of SSF.

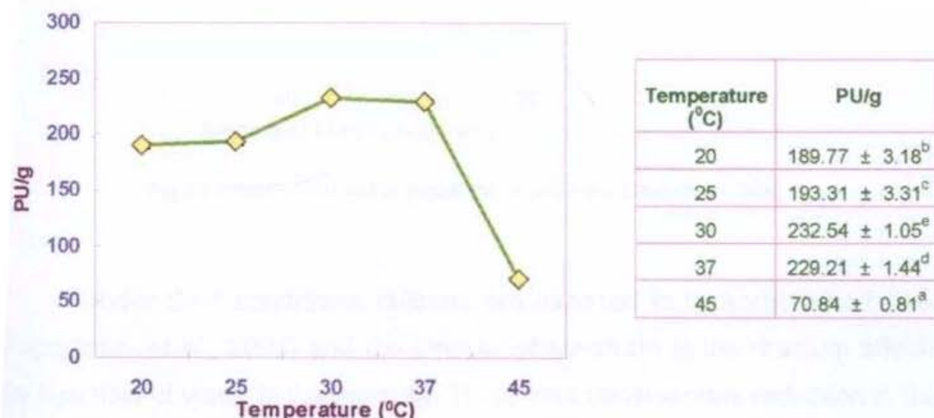


Inoculum size (%,v/w)	PU/g
20	207.98 ± 1.20[a]
40	213.08 ± 0.59[b]
60	222.15 ± 0.99[c]
80	232.48 ± 0.68[d]
100	232.44 ± 0.23[d]

Fig.3.3 Effect of inoculum size on protease production: SmF

3.3.1.4 Effect of temperature on protease production under SmF

The temperature preference of *Vibrio* sp. V26 was the same as that of SSF. All the temperatures selected for study supported growth and enzyme production except at 45^{0}C. The optimum was found to be 30^{0}C. Production declined sharply when the temperature of incubation was higher (Fig.3.4).



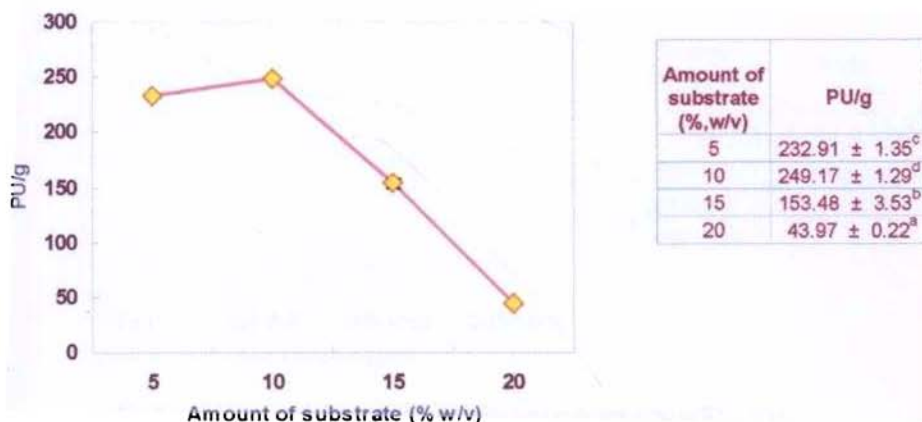
Temperature (^{0}C)	PU/g
20	189.77 ± 3.18[b]
25	193.31 ± 3.31[c]
30	232.54 ± 1.05[e]
37	229.21 ± 1.44[d]
45	70.84 ± 0.81[a]

Fig.3.4 Effect of temperature on protease production: SmF

Optimum temperature for enzyme production is largely characteristic of the organism irrespective of the type of solid support involved (Chandrashekaran *et al.*, 1991; Nagendra and Chandrashekaran, 1996). In the present study, it was found that the optimal temperature was the same in both SmF and SSF.

3.3.1.5 Effect of amount of substrate on protease production

The quantity of substrate submerged in the medium influenced the enzyme production significantly. There was an increase in the protease yield when the substrate concentration was raised to 10%w/v.This was found to be the optimum as there was considerable reduction in the yield when the substrate concentration was increased further as depicted in Fig.3.5.



Amount of substrate (%,w/v)	PU/g
5	232.91 ± 1.35[c]
10	249.17 ± 1.29[d]
15	153.48 ± 3.53[b]
20	43.97 ± 0.22[a]

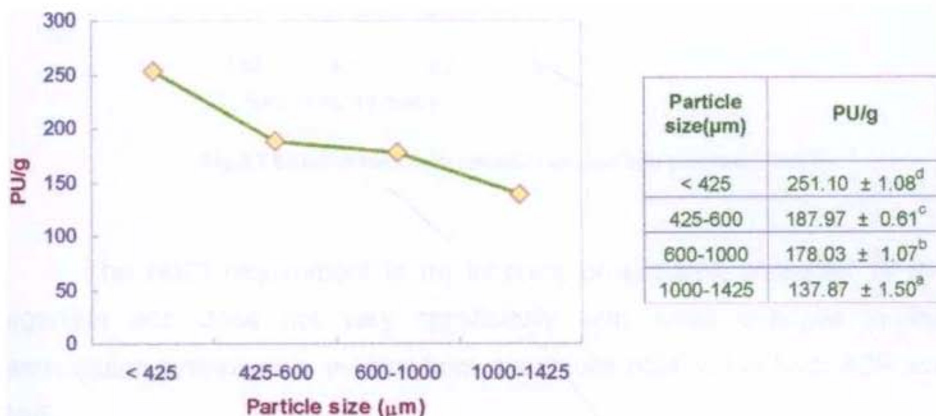
Fig.3.5 Effect of amount of substrate on protease production: SmF

Under SmF conditions, cultures are exposed to hydrodynamic forces (Papagianni *et al.*, 2001) and the amount of substrate in the medium affects the free flow of water in the medium. There was considerable reduction in the yield when the substrate concentration was increased beyond the optimum, may be due to restricted mobility of the substrate in the medium. There is

scarcely any report available on the optimisation of the amount of substrate under SmF but its impact in the present study was reasonable.

3.3.1.6 Effect of particle size of substrate on protease production

The influence of particle size of the substrate on the production of protease under SmF appeared to be very much similar to that of SSF. Maximum protease production was obtained with substrate particles of average size < 425 µm. Fig. 3.6 shows the results obtained when fermentation was carried out with different particle sizes of the substrate of wheat bran. Larger particles exhibited a decreasing trend towards enzyme yield according to the ANOVA results shown below.



Particle size(µm)	PU/g
< 425	251.10 ± 1.08[d]
425-600	187.97 ± 0.61[c]
600-1000	178.03 ± 1.07[b]
1000-1425	137.87 ± 1.50[a]

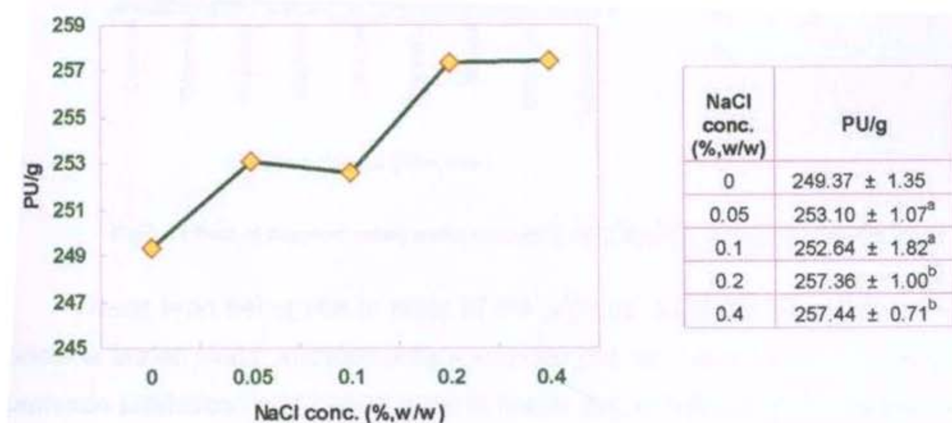
Fig.3.6 Effect of particle size of substrate on protease production: SmF

The experimental results showed that the effect of particle size of substrate did not vary with SmF and SSF.

3.3.1.7 Effect of NaCl concentration on protease production under SmF

The NaCl requirement of *Vibrio* sp. V26 for maximal yield of protease production under SmF conditions is shown in Fig.3.7. Addition of NaCl in the medium was found to be favourable for improving protease production. A

concentration of 0.2% of NaCl was optimum beyond which there was no increase in production levels. However, there were no considerable variations in the enzyme production at various concentrations of NaCl.



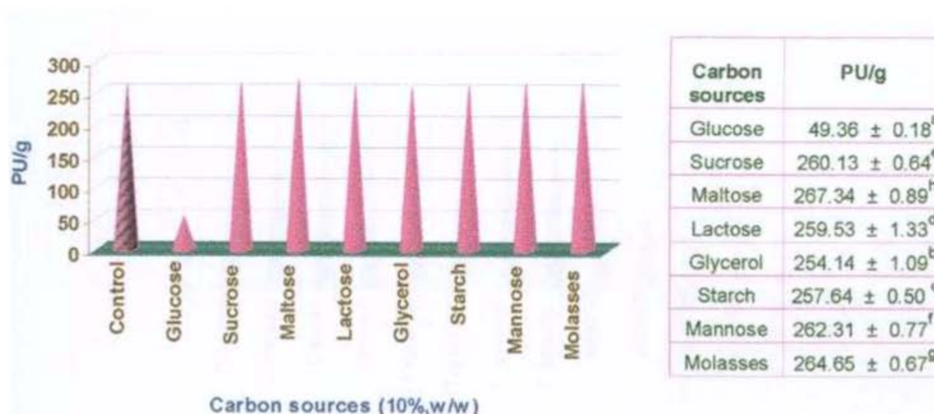
NaCl conc. (%,w/w)	PU/g
0	249.37 ± 1.35
0.05	253.10 ± 1.07[a]
0.1	252.64 ± 1.82[a]
0.2	257.36 ± 1.00[b]
0.4	257.44 ± 0.71[b]

Fig.3.7 Effect of NaCl concentration on protease production: SmF

The NaCl requirement is an inherent or acquired character of the organism and does not vary significantly with small changes in the fermentation system as is evident from the results obtained in both SSF and SmF.

3.3.1.8 Effect of supplementary carbon sources on protease production

Vibrio sp. V26 exhibited the same pattern of preference for carbon sources in SmF as that of SSF. Maltose was found to induce maximum protease production. The yield was found to be much impeded by glucose (Fig. 3.8). ANOVA results showed that maltose was giving significantly higher production followed by molasses, mannose and sucrose.



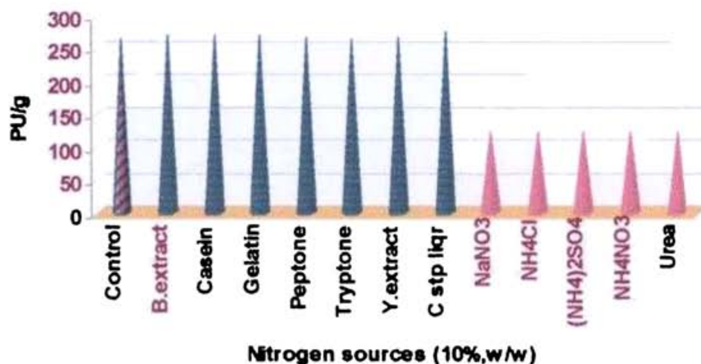
Carbon sources	PU/g
Glucose	49.36 ± 0.18^{a}
Sucrose	260.13 ± 0.64^{e}
Maltose	267.34 ± 0.89^{h}
Lactose	259.53 ± 1.33^{d}
Glycerol	254.14 ± 1.09^{b}
Starch	257.64 ± 0.50^{c}
Mannose	262.31 ± 0.77^{f}
Molasses	264.65 ± 0.67^{g}

Fig.3.8 Effect of supplementary carbon sources (10% w/w) on protease production: SmF

Wheat bran being rich in most of the primary nutrients required by the bacteria under study, supplementary sources did not have major effects on protease production and hence there is hardly any difference in the choice of these supplements between SmF and SSF.

3.3.1.9 Effect of supplementary nitrogen sources on protease production

The choice of nitrogen sources preferred by the present isolate in SmF and SSF remarkably resembled each other. Unlike inorganic nitrogen sources, the supplement of organic nitrogen sources favoured an increase in protease production. Addition of corn steep liquor marginally increased protease production followed by beef extract and casein. The addition of inorganic sources had an adverse effect on protease production as demonstrated by the reduced enzyme titres. The graphical representation of the results is presented in Fig.3.9.



● Organic N sources ● Inorganic N sources

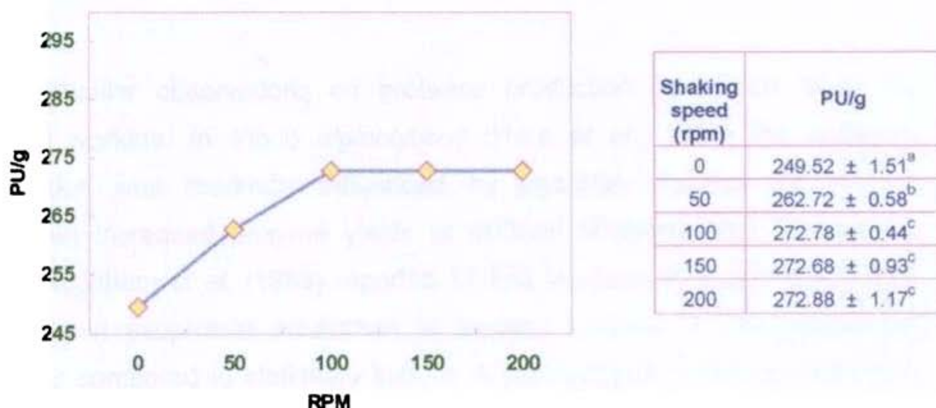
Nitrogen sources	PU/g
Beef extract	268.11 ± 0.06^{f}
Casein	268.36 ± 0.12^{f}
Gelatin	267.19 ± 2.21^{e}
Peptone	263.79 ± 0.24^{c}
Tryptone	262.56 ± 1.96^{b}
Yeast extract	265.48 ± 0.41^{d}
Corn steep liquor	272.70 ± 0.79^{g}
$NaNO_3$	121.57 ± 0.56^{a}
NH_4Cl	121.72 ± 0.66^{a}
$(NH_4)_2SO_4$	121.36 ± 0.51^{a}
NH_4NO_3	121.59 ± 0.51^{a}
Urea	121.65 ± 0.79^{a}

Fig.3.9 Effect of supplementary nitrogen sources (10% w/w) on protease production: SmF

As described in the previous section, the choice of supplementary nitrogen sources also did not differ from SSF.

3.3.1.10 Effect of shaking speed on protease production under SmF

There was a significant increase in enzyme production when the culture medium was agitated than when it was kept stationary. The production was maximal at shaking speeds above 100 rpm. The results are presented in Fig.3.10.



Shaking speed (rpm)	PU/g
0	249.52 ± 1.51^{a}
50	262.72 ± 0.58^{b}
100	272.78 ± 0.44^{c}
150	272.68 ± 0.93^{c}
200	272.88 ± 1.17^{c}

Fig.3.10 Effect of shaking speed on protease production: SmF

For the successful operation of aerobic fermentations, it is essential to supply the microorganisms with sufficient oxygen to meet their requirements at any stage in the process. Failure to supply oxygen may lead to undesirable changes in enzymatic makeup (Rolinson,1952) or death of the microorganisms (Hromatka *et al.*, 1951). Agitation of the fermenting mass has beneficial effects (Takamine, 1914; Lindenfelser and Ciegler, 1975; Knapp and Howell, 1980) like providing homogeneity through out the fermentation period, promotion of growth on individual particles of the substrate, prevention of aggregate formation, exposure of individual substrate particle to the fermenter's atmosphere, promotion of gas transfer, facilitation of heat exchange, prevention of localised changes and the effective distribution of inoculum.

In the present study, agitation of the medium was found to enhance enzyme production considerably, as it greatly influences the availability of nutrients as well as dissolved oxygen to the organism. After 48 h of incubation, the enzyme production was very low when the culture was kept

stationary. The enzyme production was high at shaking speeds above 100 rpm.

Similar observations on protease production had been made by several workers. In *Vibrio alginolyticus* (Hare *et al.*, 1981) the protease production was markedly influenced by agitation. *Bacillus* sp. B-21-2 produced increased enzyme yields at 600rpm (Fujiwara and Yamamoto, 1987). Donham *et al.* (1988) reported 17-fold increase in growth and 7-fold increase in exoprotein production in aerated cultures of *Staphylococcus stimulus* compared to stationary culture. A high alkaline protease production was reported in a *Bacillus* sp. at 300 rpm (Takami *et al.*, 1989). Similarly, *Bacillus firmus* exhibited maximum production at agitation rate of 360 rpm and aeration rate of 7.0 l min^{-1}. However, lowering the aeration rate to 0.1 l min^{-1} caused a drastic reduction in the protease yields (Moon and Parulekar,1991). Optimum yields of alkaline proteases were obtained at 200 rpm for *Bacillus subtilis* ATCC 14416 (Chu *et al.*, 1992) and *Bacillus licheniformis* (Sen and Satyanarayana, 1993). Matta *et al.* (1994) reported better protease production by *Pseudomonas sp.* AFT-36 under continuous agitation (180rpm) than that under intermittent or no agitation. In standing cultures protease levels were very low compared to the shaking cultures. The positive effect of aeration was reported by Madan *et al.* (2000), as aerated cultures gave better protease production than that by stationary culture.

The aeration rates are governed by the nature of the microorganism used, the degree of O_2 requirement for the synthesis of the product, the amount of metabolic heat to be dissipated from the mass, the thickness of the substrate layer employed, the degree to which CO_2 and other volatile metabolites are to be eliminated and the degree of air spaces available in the substrate (Chahal, 1983). For example, a larger gas volume is required by lignocellulosic saprophytic fungi than by the parasitic fungi (Schanel and

Rypacek, 1958). A higher aeration rate is essential for increased production of aflatoxin, β-galactosidase and invertase (Silman *et al.*, 1979; Silman, 1980; Chahal, 1983) but it inhibits production of achratoxin (Lindenfelser and Ciegler, 1975).

The variation in the agitation speed influences the extent of mixing in the shake flask and will also affect the nutrient availability. This is also one of the means to obtain different dissolved oxygen profiles. During fermentation, the aeration rate indirectly indicates the dissolved oxygen level in the fermentation broth. Therefore, the reduction in oxygen supply is an important limiting factor for growth as well as protease synthesis.

It has been well demonstrated that the various physico-chemical factors had a profound influence on the alkaline protease production in SmF and maximum production could be obtained by the strategic manoeuvring of the culture medium in accordance with the above results.

4

Production of alkaline protease under biphasic fermentation

The techniques of increasing the bacterial mass can be simplified by growing them in a confined environment with diffusional access to a reservoir of nutrients. Culturing them on an agar or membrane surface accomplishes this; however, the total cell yield often is low, the cells probably are not physiologically uniform, and difficulty may occur in dislodging them. The concentration of cells or their products also may be obtained in a liquid medium by using a cellophane sac to separate the culture from a large nutrient supply (Hestrin *et al.*, 1943; Gorelick *et al.*, 1951; Vinet and Fredette, 1951). A combination of these principles led to the idea of concentrating bacteria in a biphasic growth system that consists simply of a layer of solid nutrient medium overlaid with a small volume of nutrient broth.

Hestrin *et al.* (1943) briefly mentioned the use of aqueous sucrose over a nutrient agar for the production of bacterial levansucrase and Roderiguez *et al.* (1998) succeeded in the removal of heavy metals and pathogens during biphasic fermentation of solid wastes.

Gorelick *et al.* (1951) grew their cultures in charcoal-saline, with the broth contained in a cellophane sac. Nutrient broth was found to be superior to saline as the overlay in the biphasic system; and charcoal did not offer any advantage whether incorporated into the agar, the broth, or both layers.

It is a well established fact that the biomass yield can be increased upto 30 fold in a biphasic growth system (Tyrell *et al.*, 1958). In this method there is a slow release of nutrients from the lower solid phase of the medium, which support the growth of the organism when the medium is exhausted just as in batch culture (Kaur *et al.*, 2001).

Against this background an attempt has been made to enhance the protease yield using a biphasic growth system employing the optimum process parameters obtained in submerged fermentation system.

4.1 Materials and methods

4.1.1 Inoculum preparation

Inoculum was prepared as described in section 2.1.2.

4.1.2 Alkaline protease production in biphasic fermentation

This type of growth system consisted of two phases of medium, the lower one being a solid phase which was overlayered with liquid phase medium. The solid phase consisted of optimised submerged fermentation medium with 2% agar (50 ml in 250ml Erlenmeyer flask giving a thickness of 14 mm) and the liquid phase (12.5 ml) consisted of the same optimised medium devoid of agar. The medium for the two phases were prepared and

sterilized separately. After autoclaving, the solid medium was allowed to cool and set after which it was overlaid with liquid medium under aseptic conditions. The medium was inoculated with the optimum inoculum size and was incubated for 120 h at 30^{0}C under static conditions. The enzyme was extracted and the protease yield was determined. The photograph of a biphasic fermentation set up is given in Plate 4.

4.1.3 Enzyme extraction

Enzyme produced was extracted as described in section 3.1.3.

4.1.4 Alkaline protease assay

Alkaline protease was assayed as described in section 2.1.5

4.1.5 Optimisation of fermentation process parameters under biphasic fermentation

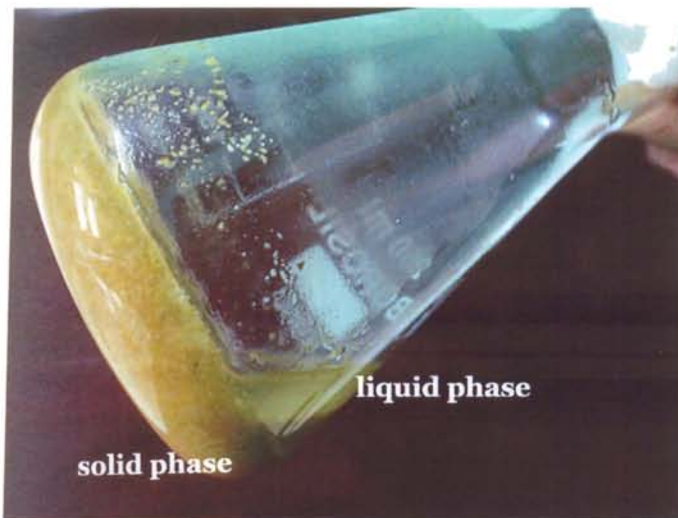
The approach was to optimise each parameter, independent of the others and consequently optimal conditions were adopted for all the experiments. The various process parameters optimised for maximal protease production were as follows:

4.1.5.1 Effect of incubation period on protease production

The biphasic culture medium was incubated for varying time periods of 24, 48, 72 96 and 120h to find the optimum time necessary for maximum enzyme production.

4.1.5.2 Effect of agar to broth ratio on protease production

The influence of agar to broth ratio on protease production in a biphasic system was studied by altering the volume of broth keeping the level of agar at a constant volume of 50 ml. The different ratios experimented were 10, 5, 4, 2 and 1.



Biphasic fermentation set up in a conical flask

Plate 4

4.1.5.3 Effect of shaking on protease production

Since there was a liquid phase in the system it was expected that shaking could enhance protease production. Accordingly the culture medium was subjected to shaking on a rotary shaker at 50 and 100 rpm (above which the agar breaks), and was compared to the enzyme yield at static conditions.

4.1.5.4 Effect of percentage of agar on protease production

The appropriate amount of agar required to solidify the lower solid layer in a biphasic system for maximum protease production was studied by varying the percentage of agar as 0.5, 1.0, 1.5, 2.0 and 2.5.

All the experiments were carried out in triplicates in order to corroborate the validity of the results obtained.

4.2 Statistical analysis

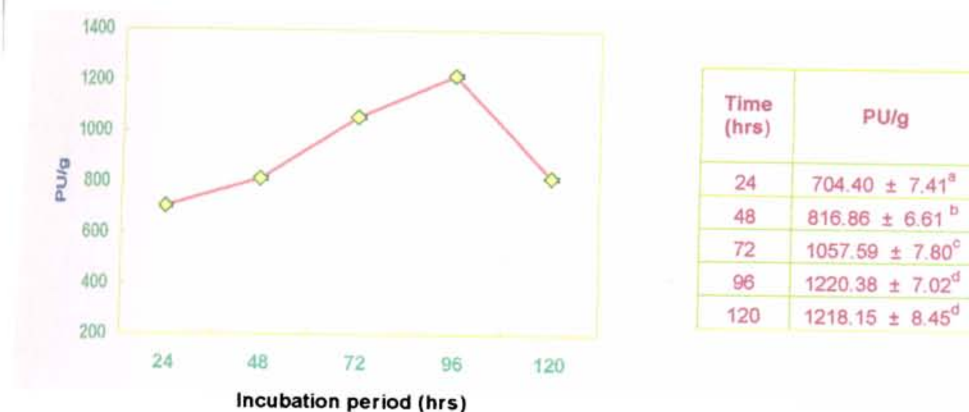
Data generated from the above experiments were analysed by One-way Analysis of Variance (ANOVA). Mean of the results was compared using SPSS 10.0 for windows at a significance level of $p<0.05$.

4.3 Results

The results of the optimisation studies of the fermentation process parameters under biphasic fermentation are shown as graphs and tables. The ANOVA results are also given in the corresponding tables as superscript alphabets. Values with the same superscripts do not vary significantly.

4.3.1 Effect of incubation period on protease production

Enzyme assay results proved that 96 h of incubation was enough for maximum protease production in a biphasic system and longer incubation did not have a significant effect on enzyme yield (Fig.4.1). ANOVA results also substantiated the same findings.

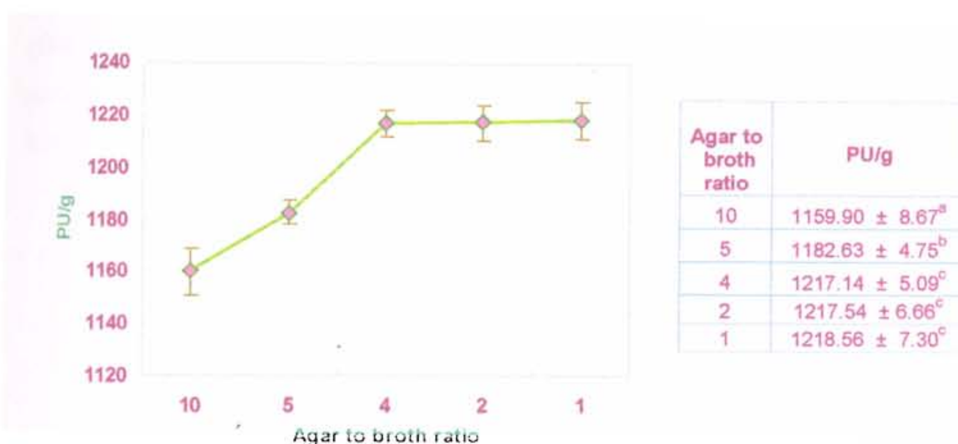


Time (hrs)	PU/g
24	704.40 ± 7.41[a]
48	816.86 ± 6.61[b]
72	1057.59 ± 7.80[c]
96	1220.38 ± 7.02[d]
120	1218.15 ± 8.45[d]

Fig.4.1 Effect of incubation period on protease production: Biphasic system

4.3.2 Effect of agar to broth ratio on protease production

Greater volumes of broth increased the protease production until an agar to broth ratio of 4, beyond which there was no increase in yield (Fig.4.2).

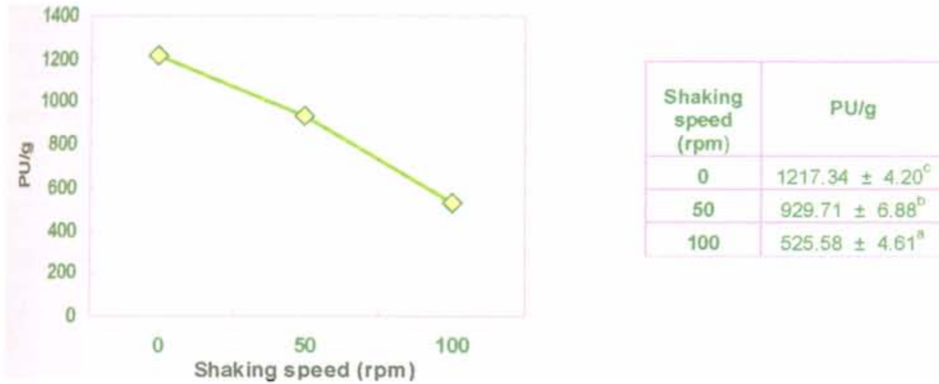


Agar to broth ratio	PU/g
10	1159.90 ± 8.67[a]
5	1182.63 ± 4.75[b]
4	1217.14 ± 5.09[c]
2	1217.54 ± 6.66[c]
1	1218.56 ± 7.30[c]

Fig.4.2 Effect of agar to broth ratio on protease production: Biphasic system

4.3.3 Effect of shaking on protease production

There was a significant decrease in the enzyme production when the culture was agitated when compared to the stationary culture (Fig.4.3).

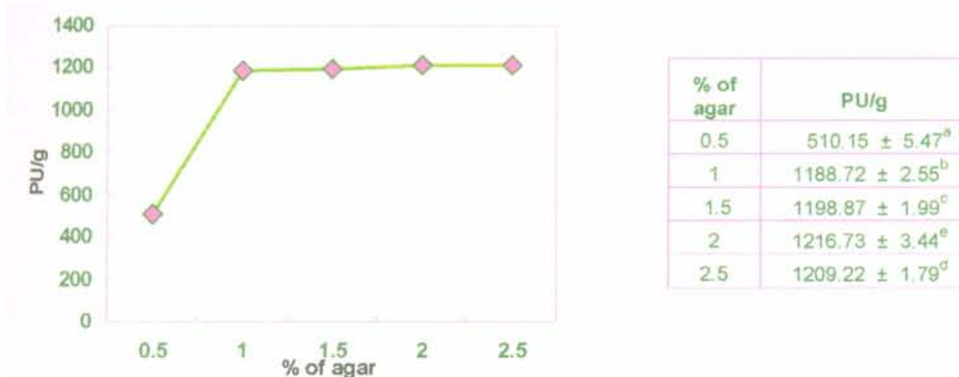


Shaking speed (rpm)	PU/g
0	1217.34 ± 4.20^{c}
50	929.71 ± 6.88^{b}
100	525.58 ± 4.61^{a}

Fig.4.3 Effect of shaking speed on protease production: Biphasic system

4.3.4 Effect of percentage of agar on protease production

The percentage of agar in the solid stratum of the biphasic medium influenced protease production as there was a corresponding increase in the protease yield when the percentage of agar was increased (Fig 4.4). Enzyme production was maximum at 2% agar and further increase in the amount of agar caused considerable reduction in the yield.



% of agar	PU/g
0.5	510.15 ± 5.47^{a}
1	1188.72 ± 2.55^{b}
1.5	1198.87 ± 1.99^{c}
2	1216.73 ± 3.44^{e}
2.5	1209.22 ± 1.79^{d}

Fig.4.4 Effect of % of agar on protease production: Biphasic system

4.4 Discussion

Biphasic fermentation was found to be a very promising technique as the yield of the enzyme was comparatively very higher than SSF and SmF. Optimisation of the medium is done to maintain a balance between the various medium components, thus minimizing the amount of unutilized components at the end of fermentation.

4.4.1 Effect of incubation period on protease production

The optimum incubation period required for maximum enzyme production is governed by the characteristics of the culture and is based on growth rate and enzyme production. In biphasic fermentation system there is a slow release of nutrients from the lower solid phase of the medium, which support the growth of the organism when the nutrients in the liquid medium is exhausted just as in batch culture (Kaur *et al.*, 2001).

In the present study, a longer incubation period is required for maximum enzyme production as compared to SmF. An incubation period of 96 h was required for maximum protease production in a biphasic system and further incubation did not have a significant effect on enzyme yield. The extra time taken might be for the slow diffusion of nutrients. It is worth mentioning that the enzyme yield obtained was very good compared to SmF.

4.4.2 Effect of agar to broth ratio on protease production

The ratio between the volumes of agar medium and broth was found to be a critical factor in the biphasic system. It was found that greater volumes of broth increased the protease production until an agar to broth ratio of 4, beyond which there was no increase in yield. The quantities of 50 ml of agar and 12.5 ml of broth were found to be most suitable.

An exactly similar observation was made by Tyrell *et al.* (1958). When 50ml of glucose nutrient agar was used with various amounts of broth, the optimum concentration index of *Escherichia coli* was coupled with a favourable yield index at an agar to broth ratio of 4.

4.4.3 Effect of shaking on protease production

Agitation of the fermenting mass has beneficial effects (Knapp and Howell, 1980; Takamine, 1914; Lindenfelser and Ciegler, 1975) like providing homogeneity throughout the fermentation period, promotion of growth on individual particles of the substrate, prevention of aggregate formation, exposure of individual substrate particles to the fermenter's atmosphere, promotion of gas transfer, facilitation of heat exchange, prevention of localised changes and the effective distribution of inoculum. Failure to supply oxygen may lead to undesirable changes in enzymatic makeup (Rolinson, 1952) or death of the microorganisms (Hromatka *et al.*, 1951). The positive effect of aeration was reported by Madan *et al.* (2000), as aerated cultures gave better protease production than that by stationary culture.

In contrast to the above observations, there was a significant decrease in the enzyme production in the present study when the culture medium was agitated, compared to when kept stationary. Shaking might have damaged the agar gel. Tyrell *et al.* (1958) suggested that this could be minimised by layering the agar above the level of maximum diameter of the flask, by invaginating the flask, or by using a rectangular shaped bottle. Lindenfelser and Ciegler (1975) reported that shaking inhibits production of achratoxin.

4.4.4 Effect of percentage of agar on protease production

In the present study, the percentage of agar in the solid stratum of the biphasic medium influenced the protease production as there was a parallel increase in the protease yield when the percentage of agar was increased. The output was maximum at 2% agar and further increase in the amount of agar caused considerable reduction in the yield. The lower agar content might have discouraged the concentration processes and higher agar might have impaired diffusional facilities in the medium.

The simple principle of this biphasic growth system appears applicable to a larger scale, to other uses, and to other organisms. Bench scale experiments indicate that high yields are possible from a two-phase process. Hestrin's application of the biphasic principle to levan synthesis (Hestrin *et al.*, 1943) indicates that it may be useful in concentrating products of growth. In conclusion, biphasic fermentation is an economically feasible technology which enhances the overall output of the system.

5

Comparative techno-economic implications of solid state, submerged and biphasic fermentations

The overall cost of enzyme production and downstream processing are the major obstacles against the successful application of any technology in the enzyme industry. Researchers have used several methods to increase the yields of alkaline proteases to meet various industrial requirements. In order to scale up protease production from microorganisms on a commercial level, biochemists and process engineers use several techniques to obtain high yields of protease in a fermenter. Controlled batch and fed-batch fermentations using simultaneous control of glucose, ammonium ion concentration, oxygen tension, pH and salt availability (Mao *et al.*, 1992; Hubner *et al.*, 1993; Van Putten *et al.*, 1996; Hameed *et al.*, 1999) and chemostat cultures (Frankena *et al.*, 1985; 1986) have been successfully used for improving protease production. In a recent study by Beg *et al.* (2002)

the over all alkaline protease yield from *B.mojavensis* was improved upto four fold under semi-batch and fed batch operations by separating biomass and protease production phases, using intermittent de-repression and induction during the growth of the organism. Current approaches for increasing protease yield include screening for hyper-producing strains, cloning and over-expression, fed-batch and chemostat fermentations, and optimisation of the fermentation medium through a statistical approach, such as response surface methodology.

The adoption of properly designed approaches with multi-models allows process and biochemical engineers to devise scale up strategies for increasing enzyme production. In view of the impending uses of proteases and the need for development of economical methods for maximum enzyme production with the aim of reducing the over all cost of the industrial process, the use of solid state, submerged and biphasic systems using cheaper industrial and agro-byproducts becomes an excellent alternative in achieving higher enzyme yields.

Despite the increasing number of publications dealing with solid-state (substrate) fermentation (SSF), it is very difficult to draw a general conclusion from the data presented. This is due to the lack of proper standardisation that would allow an objective comparison with other processes. Research work has so far, focussed on the general applicability of SSF for the production of enzymes, metabolites and spores, in that different solid substrates (agricultural waste) have been combined with many kinds of fungi and the productivity of each fermentation reported.

Raimbault (1998) compared the advantages of SSF over liquid substrate fermentation (LSF). The consumption of water is less in SSF and no effluents are discarded. Low energy consumption and low cost of equipments

are the main advantages of SSF. Nevertheless, SSF has several important limitations. The monitoring of process parameters is difficult and there is a risk of contamination for low growth rate fungi.

Most of the recent research on SSF is carried out in developing nations as a possible alternative for conventional submerged fermentations, which are the main process in pharmaceutical and food industry in industrialised nations. On a bench-scale, SSF appears to be superior to submerged fermentation technology (SmF) in several aspects. However, SSF scaling up, necessary for use on a commercial scale, raises severe engineering problems due to the build-up of temperature, pH, O_2, substrate and moisture gradients. The role of the physiological and genetic properties of the microorganisms used during growth on solid substrates compared with aqueous solutions has so far been all but neglected, despite the fact that it may be the microbiology that makes SSF advantageous against the SmF biotechnology. (Holker *et al* ., 2004).

Environmental factors such as temperature, pH, water activity, oxygen levels and concentrations of nutrients and products significantly affect microbial growth and product formation. In submerged stirred cultures, environmental control is relatively simple because of the homogeneity of the suspension of microbial cells and of the solution of nutrients and products in the liquid phase.

The low moisture content of SSF enables a smaller reactor volume per substrate mass than LSF and also simplifies product recovery (Moo-Young *et al*., 1983). However, serious problems arise with respect to mixing, heat exchange, oxygen transfer, moisture control and gradients of pH, nutrient and product as a consequence of the heterogeneity of the culture. The latter characteristic of SSF makes the measurement and control of the

above mentioned parameters difficult, laborious and often inaccurate, thereby limiting the industrial potential of this technology (Kim *et al.*, 1985). The microorganisms that have been selected for SSF are tolerant to a wide range of cultivation conditions (Mudgett, 1986).

In this chapter an attempt has been made to compare the fermentation profiles of the three fermentation systems in terms of time and yield. Since SSF and SmF differ only in the amount of moisture content, they share additional comparable aspects exclusive of biphasic system and a separate comparative account has been given.

5.1 Results and discussion

Though the same microorganism *Vibrio* sp. V 26 has been employed for all the three fermentations, the product yield and time showed significant differences from each other whereas, nutritional and temperature preferences did not demonstrate much variation.

5.1.1 Comparative analysis of solid state, submerged and biphasic fermentations

Eventhough protease production was accomplished by three different fermentation systems, their technical aspects which can qualitatively be compared are only a few. Moreover, the major objective of the study has been the enhancement of protease production and hence it is logical to compare the quantitative aspects like total yield and time impended for the different fermentation systems.

5.1.1.1 Protease yield under solid state, submerged and biphasic fermentations

The process parameters were optimised for each of the solid state, submerged and biphasic fermentations by evaluating the effect of each individual parameter and incorporating it at the standardised level before

optimising the next. Consequently, the yield obtained at the optimal magnitude of the final parameter gives the maximum output at the combination of the most favourable factors suitable for each of the three fermentations. The best results thus obtained were used for comparing the performance of solid state, submerged and biphasic fermentations in terms of protease productivity.

Solid state fermentation is the growth of microorganisms on moist substrates in the absence of free flowing water. In SSF using wheat bran as the prime solid substrate, the *Vibrio* sp. V26 produced a maximum protease yield of 62.88 PU/g. In SmF, where there is free flowing water available in the medium, the protease yield was enhanced upto 4.34 fold (272.88 PU/g) under optimum conditions of fermentation. In the biphasic system, there was great increase in protease production, upto 19.35 fold (1216.73 PU/g) compared to SSF and upto 4.46 fold compared to SmF. The graphical representations of the comparison have been given in Fig.5.1a,b&c and the data has been presented in Table 5.1 (appendix).

As for the economic feasibility of these fermentation systems, SmF seems to be the one recommendable to the industry. Though biphasic system accounts for a large output, the setting up of the system is quite difficult and the solidifying agent, agar used in the solid phase escalates the cost of production. SSF is the most cost-effective method but the comparatively higher yield in SmF makes it preferable to the industry.

Similar experiments conducted by Kaur *et al.* (2001) proved that, in SSF using wheat bran, rice bran and sunflower seed cake as the substrates, the *Bacillus* P-2 produced maximum protease yields of upto 1210, 1040, and 800 PU/g of dry solid substrates respectively. In SmF, the protease yield was enhanced upto 2.3-fold, 1.3–fold and 1.4-fold, using wheat bran, rice bran and

sunflower seed cake, respectively. In the biphasic system, the production was increased upto 3.94-fold, 3.21-fold and 2.2-fold. Tyrell *et al.* (1958) reported that the biomass yield could be increased upto 30-fold in a biphasic growth system.

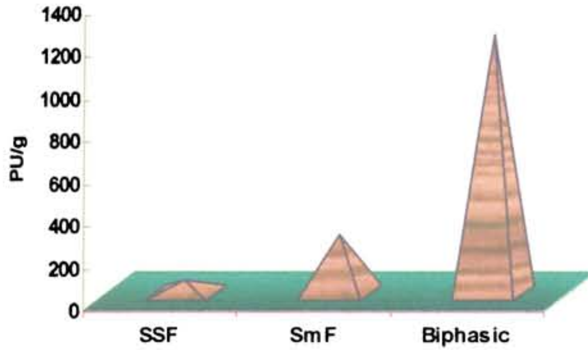


Fig.5.1a Maximum protease yield under SSF. SmF and biphasic fermentations

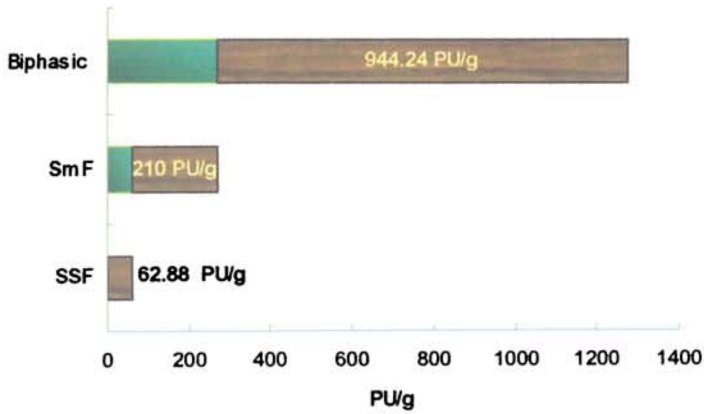


Fig.5.1b Enhanced protease yield under SSF, SmF and biphasic fermentations

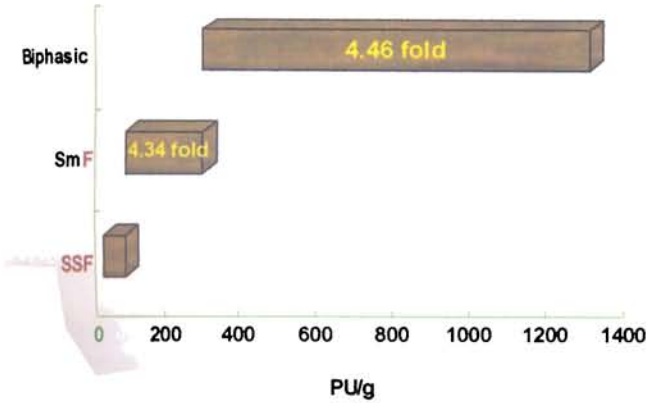


Fig.5.1c Scale of enhancement of protease yield under SSF, SmF and biphasic fermentations

The absence of free flowing water makes SSF distinct from the other two fermentations studied. Both SmF and biphasic systems have a higher moisture content in the medium. Obviously the lower output of SSF compared to others could be correlated to the lower amounts of water in the system. The role of the water content of the substrate has been widely described and reviewed by different authors (Cannel and Moo-Young, 1980a; Oriol, 1987; Oniol *et al.*, 1988; Acuna-arguelles *et al.*, 1994; Xavier and Lonsane, 1994;; Bellon-Maurel *et al.*, 2003; Gervais and Molin, 2003). As the optimal value of moisture content depends on both the microorganism and the solid matrix used, for economical production, the microorganism should be grown in optimal moisture levels either for maximising the growth or metabolite production depending on the application. Bacteria generally grow at higher water activity (a_w) levels, especially vibrios are hydrophilic ones. The water activity affects the biomass development, metabolic reactions and the mass transfer processes (Gervais and Molin, 2003 and Bellon-Maurel *et al.*,

2003).The adequate value of a_w depends on both the product and the requirements of the microorganism.

In addition, shaking of the fermenting medium in SmF, contribute to the increased yield compared to SSF. Agitation of the fermenting mass has beneficial effects such as providing homogeneity through out the fermentation period, promotion of growth on individual particles of the substrate, prevention of aggregate formation, exposure of individual substrate particles to the fermenter's atmosphere, promotion of gas transfer, facilitation of heat exchange, prevention of localised changes and the effective distribution of inoculum (Takamine, 1914; Lindenfelser and Ciegler, 1975; Knapp and Howell, 1980).

In biphasic fermentation there is a slow release of nutrients from the lower solid phase of the medium, which supports the growth of the organism when the liquid medium is exhausted just as in batch culture (Kaur *et al.*, 2001). This might lead to the concentration and scaling up of the products shooting up the productivity.

5.1.1.2 Comparison of the time impended for maximum protease yield under solid state, submerged and biphasic fermentations

The optimal incubation period for maximum protease production is characteristic of the microorganism involved and is largely based on growth kinetics. The time employed may vary from 48h to 8-9 days for bacteria or fungus (Aikat and Bhattacharya, 2000; Puri *et al.*, 2002).

In the present study, it was observed that in SmF, maximum production was attained at a shorter time than in SSF. The organism required a minimum of 96 h for maximum enzyme production in SSF whereas, it took only 48 h for best results in SmF. Availability of excess water in the medium

and agitation in SmF might be the reason for this. Vecht-Lifshitz *et al.* (1990) observed that viscosity of the medium influenced the process parameters in submerged fermentation. Conversely, a longer incubation period was required for maximum production in biphasic fermentation system compared to SmF. An incubation period of 96 h was required for maximum protease production in a biphasic system and further incubation did not have a significant effect on enzyme yield. The extra time taken might be due to slow diffusion of nutrients. The results are presented in Fig. 5.2 and Table 5.2 (appendix).

The results can also be ascribed to the ability of the organism to degrade the substrate under different fermentation conditions. At 48h the easily degradable part of the substrate must have been used in SmF, which took 96 h in SSF and biphasic system.

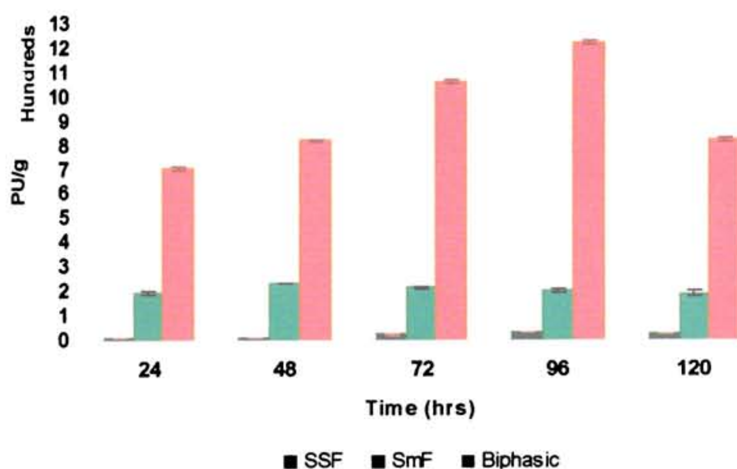


Fig.5.2 Comparison of the time impended for maximum protease yield under solid state, submerged and biphasic fermentations

5.1.2 Comparative analysis of nutritional preferences of *Vibrio* sp. V 26 under solid state and submerged fermentations

Bacteria differ radically with respect to the conditions that allow their optimal growth. In terms of nutritional needs, all cells require carbon, nitrogen, sulphur, phosphorus, numerous inorganic salts (potassium, magnesium, sodium, calcium, and iron), and a large number of other elements called micronutrients (eg., zinc, copper, manganese, selenium, tungsten, and molybdenum). There is a great deal of nutritional diversity among microorganisms; therefore, microbial growth is greatly affected by the nutrients that are available in their environment.

The media commonly used in a laboratory setting are composed of extracts from plants, animals or yeast and, therefore, are rich in nutrients. As these media contain a wide range of nutrients that are well above the minimal nutritional requirements of the organism being cultured, these media support the growth of a wide range of organisms. Depending on the fastidiousness of an organism, these media can be supplemented with vitamins, nucleic acids, cofactors and amino acids. The major organic materials available in nature are polymeric and in general all these can be used by microorganisms as substrate. These natural polymers can be suitably implemented for laboratory setting under different fermentations for better and low cost productions.

5.1.2.1 Comparison of choice of substrates by *Vibrio* sp. V26 for maximum protease production under solid state and submerged fermentations

All solid substrates have a common feature: their basic macromolecular structure. In general, substrates for SSF are composite and heterogenous products from agriculture or by-products of agro-industry. This basic macromolecular structure (eg. cellulose, starch, pectin, lignocellulose,

basic macromolecular structure (eg. cellulose, starch, pectin, lignocellulose, fibres etc.) confers the properties of a solid to the substrate. The structural macromolecule may simply provide an inert matrix (sugarcane bagasse, inert fibres, resins) within which the carbon and energy source (sugars, lipids, organic acids) are adsorbed. But generally, the macromolecular matrix represents the substrate and provides the carbon and energy source. Although the moist solids are constantly degraded in nature by microorganisms, little information is available on the factors affecting the uptake of solid substrates. In general, growth is attributed to the action of enzymes in breaking down the solids to the components that are capable of permeating into the microbial cells.

In the present study it was found that different substrates performed quite differently in SmF and SSF, except for wheat bran, which proved to be the best substrate in SmF as well. Rice bran and ragi which yielded the least in SSF produced good results in SmF, while rawa and maize bran which gave good results in SSF were poor producers in SmF. The comparative preferences of different substrates under SSF and SmF are given in Fig.5.3 and Table 5.3 (appendix).

Decreased moisture content might have impaired the accessibility of rice and ragi to the microorganism in SSF. With free flowing water in the medium, both these substrates yielded higher amounts of enzyme in SmF whereas, rawa and maize bran favoured decreased amounts of water in the medium as demonstrated by better performance under SSF conditions. Wheat bran is suitable for both SSF and SmF with better productivity under SmF which itself indicates that the microorganism under study prefers higher water content in the medium. While in SmF, the microorganism is exposed to hydrodynamic forces, in SSF, growth is restricted to the surface of the solid

availability of nutrients and the geometric configuration of the matrix (Moo-Young *et al.*, 1978).

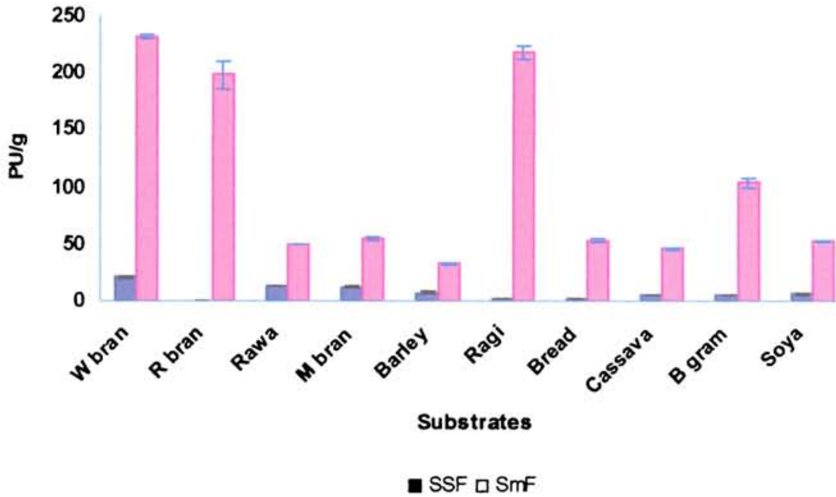


Fig.5.3 Comparison of choice of substrates by *Vibrio* sp. V26 for maximum protease production under solid state and submerged fermentations

The microbial modification of the substrate resulting in partial hydrolysis is clearly governed by a variety of factors such as production by the cells of enzymes involved, uptake of permeable products by the cells, diffusion of enzyme in mass of solids, accessibility of solids to cells, heterogeneity of solid surfaces, organism proximity, requirement of the presence of more readily degradable carbon sources in the system and overall enzyme kinetics (Pollock, 1962; Erickson, 1978; Knapp and Howell, 1980).

5.1.2.2 Comparison of choice of particle size of the substrate by *Vibrio* sp. V26 for maximum protease production under solid state and submerged fermentations

The present investigation illustrated that the effect of particle size of substrate did not vary with SmF and SSF. Maximum protease production was obtained with substrate particles of average size < 425 µm for both types of fermentations as evident in Fig.5.4 and Table 5.4 (appendix).

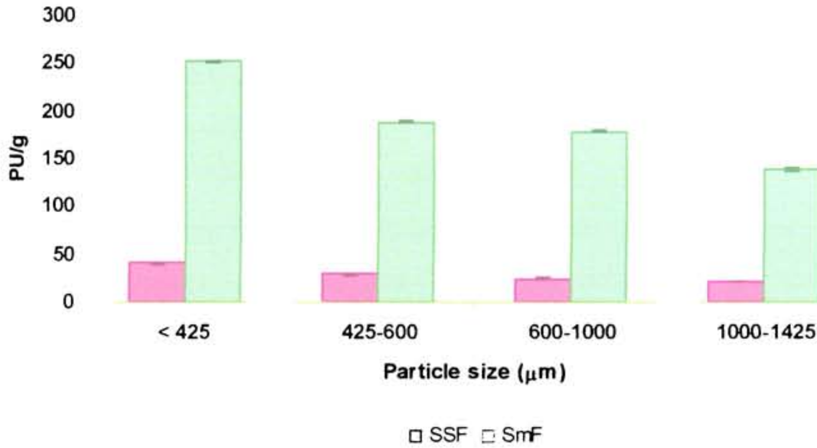


Fig.5.4 Comparison of choice of particle size of the substrate by *Vibrio* sp. V26 for maximum protease production under solid state and submerged fermentations

Usually smaller particles stimulate greater growth (Hunang *et al.*, 1985). With smaller particle, the surface area for growth was greater but the inter-particle porosity was less. With the larger size, the porosity was greater but the saturated surface area was less. These two opposing factors probably interacted to give the value corresponding to optimum growth and product formation (Muniswaran and Charryalu, 1994). Huang *et al.* (1984) reported that greater growth of fungal cultures was stimulated by smaller particle size substrate. However, too small substrate particles may result in substrate

agglomeration in most of the cases, which may interfere with microbial respiration/aeration and at the same time, larger particles provide better respiration/ aeration efficiency.

The physical morphology, especially, the porosity and particle size of the substrate, governs the accessible surface area of the organism. The influence of substrate particle size, which determines the accessible surface area to the microorganisms on product formation has been emphasised earlier (Hesseltine, 1972).

5.1.2.3 Comparison of choice of supplementary carbon sources by *Vibrio* sp. V26 for maximum protease production under solid state and submerged fermentations

As wheat bran is well loaded with most of the principal nutrients of the bacteria under study, supplementary sources proved to have no major effect on protease production. Maximum protease yield was obtained when maltose was supplemented in the medium. Protease production was very low when glucose was added to the medium (Fig. 5.5). Earlier studies have indicated a reduction in protease production due to catabolite repression by glucose (Hanlon *et al.*, 1982; Frankena *et al.*, 1986; Kole *et al.*, 1988; Secades and Guijarro, 1999).

High carbohydrate concentrations were found to repress enzyme production. Therefore, carbohydrate was added continually or in aliquots throughout the fermentation to supplement the exhausted component and keep the volume limited thereby reducing the power requirements (Aunstrup, 1980).

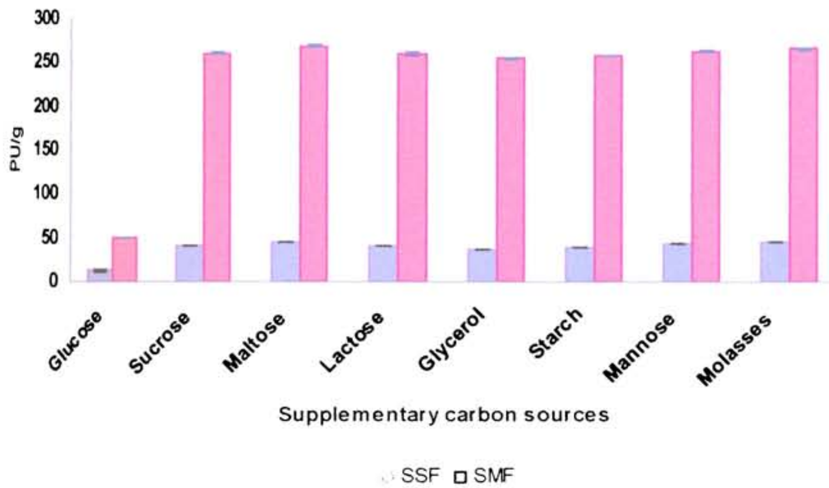


Fig.5.5 Comparison of choice of supplementary carbon sources by *Vibrio* sp. V26 for maximum protease production under solid state and submerged fermentations

5.1.2.4 Comparison of choice of supplementary nitrogen sources by *Vibrio* sp. V26 for maximum protease production under solid state and submerged fermentations

As in the case of carbon sources, the choice of supplementary nitrogen sources also did not differ between SSF and SmF. Corn steep liquor was found to be a more suitable supplement though beef extract and casein gave good results. Inorganic nitrogen sources showed unsatisfactory results regarding the total protease yield while organic sources proved good (Fig. 5.6 and Table 5.6 (appendix)).

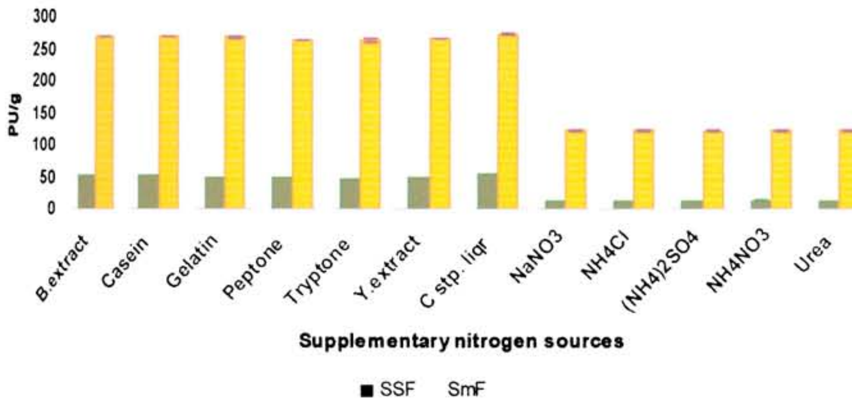


Fig.5.6 **Comparison of choice of supplementary nitrogen sources by *Vibrio* sp. V26 for maximum protease production under solid state and submerged fermentations**

Corn steep liquor was found to be a cheap and suitable source of nitrogen by some workers (Fujiwara and Yamamoto, 1987; Malathi and Chakraborty, 1991; Sen and Satyanarayana, 1993). Similar to the present study, low levels of alkaline protease production were reported with the use of inorganic nitrogen sources in the production medium (Chandrashekaran and Dhar, 1983; Sen and Satyanarayana 1993; Chaphalkar and Dey, 1994). The presence of ammonium significantly reduced protease production in *Vibrio* strain SA1 (Wiersma *et al.*, 1978).

5.1.3 Comparison of optimum temperature required by *Vibrio* sp. V26 for maximum protease production under solid state and submerged fermentations

Ramesh and Lonsane (1987) observed that incubation temperature has a profound effect on the enzyme yield and the duration of the enzyme synthesis phase. Optimum temperature for enzyme production is largely

characteristic of the organism irrespective of the type of solid support involved in SSF (Chandrashekaran *et al.*, 1991; Nagendra and Chandrashekaran, 1996). In the present study, it was found that the optimum temperature for maximum protease production was the same, 30^{0}C in both SmF and SSF, as illustrated in the Fig.5.7 and Table 5.7 (appendix).

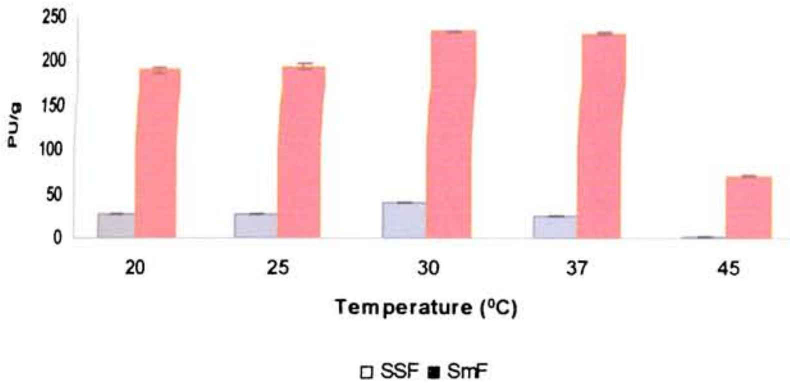
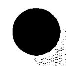


Fig.5.7 Comparison of optimum temperature required by *Vibrio* sp. V26 for maximum protease production under solid state and submerged fermentations

In a fermentation system, the temperature level reached is a function of the type of microorganism, the porosity, the particle diameter and the depth of the substrate (Raimbault, 1998; Gervais and Molin, 2003; Raghavarao, 2003). The overall rate of heat transfer may be limited by the rates of intra- and inter-particle heat transfer and by the rate at which heat is transferred from the particle surface to the gas phase. However, in the present study, the optimum temperature requirement remained the same in low moisture containing SSF and SmF where there is free flowing water. Therefore, in this case the temperature preference could largely be a characteristic of the organism rather than the moisture alterations in the environment. But, one should not exclude the problems of heat build up in a big capacity bioreactor

employed at the industrial level. Even then, the temperature for optimum should not change, but steps have to be taken to maintain the same.

The basic requirements for solid state, submerged and biphasic fermentations do not vary much according to the present study and the cost of production was almost equal, though it may have practical differences going to an industrial level. However, the use of agro-industrial and other solid waste substrates in SSF, SmF and biphasic fermentations helps to provide an excellent substitute in conserving the already dwindling global energy sources.



6

Production of alkaline protease using immobilised cell technology

Modern biotechnology is developing fast into a major process industry. On this way it is confronted with the requirements of large scale industrial processes, mainly economic and ecologic limitations. Classical biotechnological processes are usually linear processes, where the mass flows proceed from raw material treatment to conversions and to product separation and purification. The relatively simple process morphology more often than not results in low productivities, low product concentrations and large amounts of wastes which make biotechnology ill equipped to rival other process technologies on economic and ecologic terms. This calls for an overall process optimisation, including the upstream processes, conversion and downstream operations.

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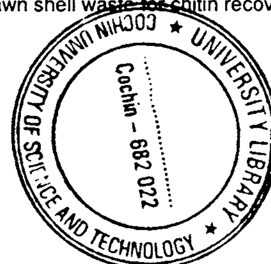
One of the major possibilities of optimising biotechnological processes lies in immobilisation technologies, which may increase productivity, product concentrations, and to some extent, conversion of raw materials. Immobilisation is the technique by which enzymes and cells are transformed into heterogeneous catalysts (Walker and Gingold, 1988). The biocatalyst is confined to a restricted region through which the substrate solution is passed, to emerge as a catalyst-free product. This approach makes it physically easier to control the environment in the immediate vicinity of the reaction and can dramatically increase the process intensity by concentrating cell activity into a limited space.

Initial research into immobilisation technology concentrated on single enzyme systems requiring no cofactors, the enzymes studied being predominantly those that catalyse degradative reactions (Crook, 1968). The limitations of the practical applicability of such systems have led workers to study the immobilisation of those enzymes requiring cofactors (Wilson *et al.*, 1968), and interest has been shown in immobilizing cofactors (Weibel *et al.*, 1971). Some research has also been reported on multistep cofactors (Mosbach and Mattiason, 1970; Mattiason and Mosbach, 1971). Such systems do not appear attractive as expensive purified cofactor preparations are required and, for a multistep reaction, a complex utilization procedure would be needed to achieve an efficient interaction between components of the system. Immobilised whole cell preparations have been used for the catalysis of single step reactions, in particular, for the isomerisation of glucose (Vieth *et al.,* 1973).

At present, cell immobilisation technology is often studied for its potential to improve fermentation processes and bioremediation (Beshay *et al.*, 2002; Abd-EL-Haleem *et al.*, 2003). Immobilisation of whole cells for the



production of extracellular enzymes offers many advantages such as, increasing the productivity and the operational stability or reducing the delays involved in enzyme production (Kokubu *et al.*, 1981, Fujimura *et al.*, 1984), eliminating the contaminants from the stream in continuous fermentations with out loss of biomass (Helmo *et al.*, 1985), the ability to separate cell mass from the bulk liquid for possible reuse, facilitating continuous operation over a prolonged period and enhanced reactor productivity (Zhang *et al.*, 1989; Galazzo and Bailey, 1990).

Currently attention is being drawn to the immobilisation of bacteria in order to achieve a higher cell density in bioreactors; based on this, smaller reactors, shorter residence/retention time or higher flow rates can be employed. Several important advantages of immobilised cell technology over traditional batch free-cell systems such as possibility of continuous operation, high cell densities, improved resistance against contamination and high fermentation rates have been explained by Pilkington *et al.* (1998), Willaert (2000), Nedovic *et al.* (2001, 2002) and Virkajarvi (2002). Major advantages were demonstrated with lactic acid bacteria and bifidobacteria for this technology compared with free-cell fermentations, including stable strain ratios with mixed cultures and prevention of washout during long-term continuous culture, reduction of susceptibility to contamination and bacteriophage attack, enhancement of plasmid stability, protection of cells from shear forces in the stirred reactor, and continuous and uniform inoculation of the bulk medium (Champagne *et al.*, 1994 and Lacroix *et al.*, 2003).

Cell immobilisation has been applied to numerous food fermentations using a wide variety of bacteria, yeasts, molds and plant cells (Groboillot *et al.*, 1994; Lacroix *et al.*, 2003). Shapiro and Dworkin (1997) demonstrated

enhanced cell-to-cell signalling and cell-matrix interactions leading to co-ordinated behaviour of immobilised microorganisms. Immobilisation of *Acinetobacter* sp. has been investigated using alginate (Muyima and Cloete, 1995) or ceramic (Kariminiaae-Hamedaani *et al., 2003*) carriers. Calcium and aluminium alginate gels have previously been used for the immoblisation of cells and enzymes (U.S. Patent 3,733,205, May 15th, 1973; Hackel *et al.*, 1975). Besides the synthetic carriers, natural zeolite has been shown as a promising material for the immobilisation of microorganisms (Shindo *et al.*, 2001) due to its high porosity and large surface area. The extent of bacterial colonisation depends on the chemical properties and particle size of zeolite.

In the immobilisation of microbial biomasses, porous and microporous carriers are frequently used. Their advantages include great porosity, mechanical stability, potentially high endurance of the carrier to immobilised mass and a short diffusion distance between the outer and the inner surface of the carrier. Carriers enhance the activity of intracellular enzymes eg. lipase, proteases etc. In addition, such enzymes retain the substrate specificity and enzymatic activity for a longer time and the immobilised biocatalysts are easier to handle in continuous processes. Commonly used carriers include DEAE cellulose, porous glass, silicon carbide and recently wood chips. These carriers provide simple immobilisation procedure and good mechanical properties but are limited by relatively low cell concentrations and significant cell leakage. Porous matrices provide an alternative solution providing higher cell concentrations and better cell retention.

The most crucial stages in the process of obtaining immobilised biocatalysts include: the selection of strains with specified substrate activity, the selection of immobilisation technique and the carrier properties eg. porosity, charge etc. However, proper selection of immobilisation techniques

and supporting materials is primarily required to minimise the disadvantages of immobilisation. One of the most suitable methods for immobilisation is entrapment in calcium alginate, because this technique is simple and cheap. Sodium alginate is readily available and it is a non-toxic biological material. Therefore, it is suitable as an immobilisation matrix for biomolecules and microorganisms (Mattiasson, 1983). Beads of calcium alginate are prepared under mild conditions and have been used extensively for microencapsulating and entrapping cells.

Owing to their high affinity for water, total lack of toxicity and the ability to form viscous solutions and gels, alginates have long been widely used in the food industry. Their ability to form gels very rapidly in the presence of calcium ion under extremely mild conditions has been exploited for immobilisation of particulate enzymes (Hussain *et al.*, 1985) and many different kinds of living cells (Nilsson and Mosbach, 1980; Kopp and Rehm, 1983; Draget *et al.*, 1988; Jain and Ghose, 1984; Smidsrod and Skjak-Braek, 1990). Alginates are glycuronans extracted from seaweeds. The molecules are linear chains of (1-4)-linked residues of β-D-mannuronic acid and α-L-guluronic acid in different proportions and sequential arrangements. Martinsen *et al.* (1989) and Smidsrod and Skjak-Braek (1990) reported certain properties of calcium alginate gels including mechanical rigidity, swelling and shrinking characteristics and resistance to interference by monovalent cation.

Protease production in free- cell cultures has been extensively studied. However, only a few microorganisms have been immobilised for protease production such as *Streptomyces fradae* in polyacrylamide gel (Kokubu *et al.*, 1981), *Myxococcus xanthus* in carrageenan gel beads (Younes *et al.*, 1984;1987), *Bacillus firmus* on Luxopor (Helmo *et al.*, 1985),

Serratia marcescens and *Myxococcus xanthus* (Jean-Christophe *et al.*, 1988) and *Teredinobacter turnirae* (Beshay, 2003) in calcium alginate beads.

In this perspective, an attempt was made to find out the possibility of immobilising cells of the potent protease producing strain of *Vibrio* sp. V 26 in calcium alginate gel beads and to investigate the effect of immobilisation conditions on alkaline protease production. The operational stability and storage life of the resulting biocatalyst as well as the release of free cells into the medium and their subsequent growth were also studied. Furthermore, comparisons of alkaline protease production by *Vibrio* sp. V26 as free and immobilised cells were made.

6.1 Materials and methods

6.1.1 Calcium alginate entrapment of cells

The calcium alginate entrapment of cells was performed according to the method previously described by Johnsen and Flink (1986). Sodium alginate was dissolved in boiling water (1.5%, w/v) and autoclaved at 121^{0}C for 15 min. Cells of *Vibrio* sp. were harvested during the mid-logarithmic phase of growth by centrifugation (5000 g, for 10 min) and the cell pellet containing approximately 4x10^{11} live cells/g was added to the sterilised alginate solution to give a final concentration of 5% (w/v). The resulting suspension was extruded drop by drop through a hypodermic syringe into a gently stirred 3% (w/v) cold sterile $CaCl_2$ solution to obtain gel beads of approximately 2mm in diameter. The gel beads of calcium alginate with entrapped bacterial cells were cured and hardened in the same solution for 30 to 60 min. ensuring the completion of gelling process. The beads were then thoroughly rinsed with sterile physiological saline to remove excess calcium ions and untrapped cells and, stored at 4^{0}C in fresh sterile saline until

used. Plate 5 shows the photograph of calcium alginate beads with entrapped cells.

6.1.2 Production of alkaline protease by immobilised cells in calcium alginate beads

A volume of 5% (v/v) of gel beads were inoculated into sterile nutrient broth as detailed in section 2.2.2 (Plate 6) and was incubated at 30^{0}C on a rotary shaker at 100 rpm. Samples were aseptically withdrawn at 2h intervals upto 60 *hours for estimation of alkaline protease production and free cell density.*

6.1.3 Recovery of calcium alginate beads and enzyme extraction

After fermentation the whole contents were filtered through a sterile muslin cloth. [illegible] stored at 4^{0}C for future use. A portion of the culture filtrate was drawn to determine the free cell density and the remaining was centrifuged at 10000 g for 15 min. at 4^{0}C to remove the cells. The cell free supernatant containing the enzyme was assayed for protease activity.

6.1.4 Determination of cell leakage

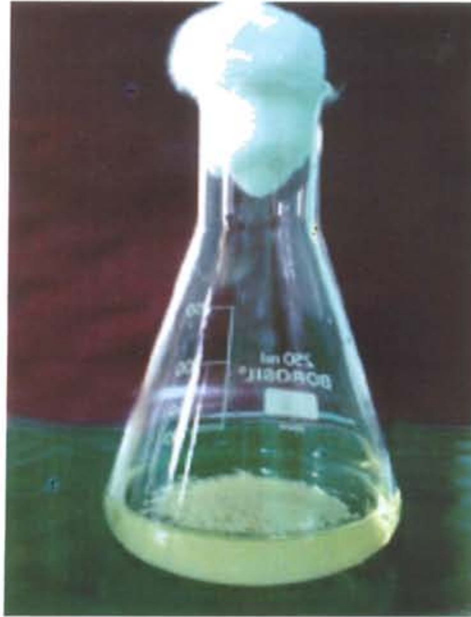
Cell leakage was measured as free cell density by the absorbance of culture samples at a wavelength of 600 nm and was expressed in units of optical density (OD).

6.1.5 Alkaline protease assay

Alkaline protease was assayed as described in section 2.1.5. In the immobilisation studies one unit of proteolytic activity (PU) was defined as that amount of enzyme, which liberated 1 μg of tyrosine per ml per minute under the specific conditions of the assay.

Calcium alginate beads

Plate 5



Ca alginate beads inoculated in nutrient medium

Growth in medium inoculated with Ca alginate beads

Calcium alginate beads in medium

Plate 6

Aseptic recovery of beads

Residual beads after filtration

Recovery of beads from fermented broth

Plate 7

6.1.6 *Effect of immobilisation conditions on alkaline protease* production and cell leakage

The method followed was to optimise each factor, free of others and the optimal level was employed in all the subsequent experiments. The various immobilisation conditions studied were as follows:

6.1.6.1 Effect of alginate concentration on alkaline protease production and cell leakage

In order to find out the optimum alginate concentration for *Vibrio* sp. V26 cell immobilisation, alginate solutions of different concentrations such as 1.5, 2.0, 2.5 and 3.0 %, w/v were used for the preparation of beads.

6.1.6.2 Effect of initial cell loading on alkaline protease production and cell leakage

Gel beads containing 0.2%,1.0%, 5.0%, 10.0%, 15.0%, and 20.0% (w/v) of wet cells were added to the production medium and the influence of initial cell loading of the beads was tested.

6.1.6.3 Effect of bead inoculum on alkaline protease production and cell leakage

Various volumes of gel beads were added to the growth medium giving a bead inoculum of 1.0%, 5.0%, 10.0%, 20.0%, 30.0%, and 50.0% (v/v), to find out the appropriate measure of beads that has to be supplemented for maximum protease production.

6.1.7 Alkaline protease production and cell density of free and immobilised cells

Calcium alginate beads prepared under optimal conditions were inoculated into fresh growth medium and the results were compared with free cells cultured in parallel.

6.1.8 Estimation of operational stability of beads

Alkaline protease was produced in repeated batch shake cultures of 12 reaction cycles. After each cycle, the beads were recovered, thoroughly rinsed in sterile saline and were resuspended in a fresh medium of the next cycle. At each cycle the culture filtrate was analysed for protease production. The operational stability was determined as half life of the beads which is the time required for enzyme production to come down to half after attaining the maximum value.

6.1.9 Estimation of shelf life of beads

The beads produced under standard conditions were stored at 4^{0}C in physiological saline for five weeks and the proteolytic activity was determined at one-week intervals, in order to ascertain the amenability of the beads for use after storage.

6.2 Statistical analysis

With a view to determining significant difference, if any, among the studied parameters, the results were analysed using one way ANOVA and Duncan's multiple comparisons of the means using SPSS 10.0 for windows. Significant differences were indicated at $p<0.05$.

All the experiments were carried out in triplicates in order to substantiate the validity of the results obtained.

6.3 Results

The results of the immobilisation studies are shown as graphs and tables. The ANOVA results are also given in the tables as superscript alphabets. Values with the same superscripts do not vary significantly.

6.3.1 Effect of alginate concentration on alkaline protease production and cell leakage

The production of alkaline protease enhanced with increasing alginate concentration and reached its maximum at 2.5%. Further increase in concentration was accompanied by a decrease in the enzyme yield (Fig.6.1a and Table 6.1a). The trend was the same at all hours of incubation; the maximum production occurred at 36h and further incubation had no effect on the yield. As for the cell leakage, free cell density increased with alginate concentration reaching the maximum at 2.5%, at an incubation period of 48 h and decreased sharply towards 60 h (Fig.6.1b and Table 6.1b). Statistical analyses supported the results.

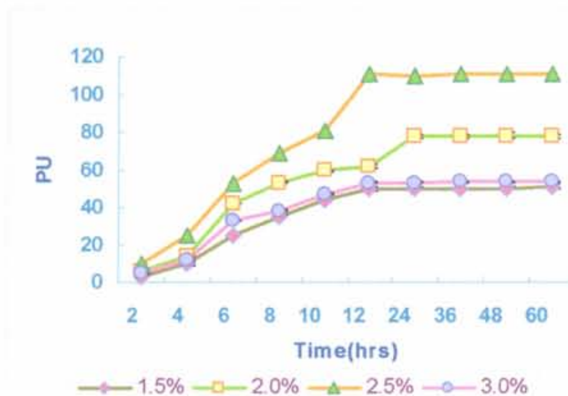


Fig.6.1a Effect of Alginate concentration on protease production

Time (hrs)	Protease units (PU) at different alginate concentrations			
	1.5%	2.0%	2.5%	3.0%
2	3.25±0.16^{Aa}	6.44 ±0.15^{Ca}	10.31±0.12^{Da}	4.53±0.08^{Ba}
4	9.98±0.53^{Ab}	14.07±0.08^{Cb}	25.13±0.2^{Db}	11.55±0.20^{Bb}
6	*25.13±0.20^{Ac}*	*42.19±0.11^{Cc}*	*53.27±0.30^{Dc}*	*33.32±0.17^{Bc}*
8	35.08±0.49^{Ad}	53.33±0.29^{Cd}	68.68±0.44^{Dd}	38.43±0.30^{Bd}
10	43.94±0.38^{Ae}	59.58±0.57^{Ce}	81.27±0.34^{De}	47.44±0.14^{Be}
12	49.79±0.93^{Af}	61.89±0.29^{Cf}	110.87±1.00^{Df}	53.44±0.40^{Bf}
24	50.09±0.48^{Ag}	77.95±0.49^{Cg}	110.23±1.06^{Dg}	53.47±0.41^{Bg}
36	50.13±0.17^{Ah}	78.03±0.74^{Ch}	111.39±1.39^{Dh}	53.59±0.13^{Bh}
48	50.40±0.36^{Ah}	78.40±0.74^{Ch}	110.67±1.39^{Dh}	53.59±0.13^{Bh}
60	50.58±0.39^{Agh}	77.96±0.25^{Cgh}	110.61±0.85^{Dgh}	53.59±0.26^{Bgh}

Table 6.1a Effect of Alginate concentration on protease production

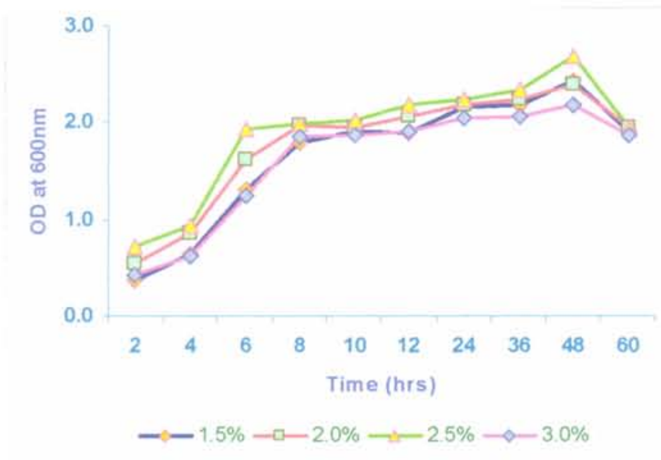


Fig.6.1b Effect of Alginate concentration on free cell density

Time (hrs)	OD (600 nm) at different alginate concentrations			
	1.5%	2.0%	2.5%	3.0%
2	0.38 ± 0.04^{Ba}	0.54 ± 0.03^{Ca}	0.71 ± 0.02^{Da}	0.42 ± 0.02^{Aa}
4	0.64 ± 0.04^{Bb}	0.86 ± 0.05^{Cb}	0.94 ± 0.01^{Db}	0.62 ± 0.06^{Ab}
6	1.31 ± 0.00^{Bc}	1.62 ± 0.03^{Cc}	1.93 ± 0.03^{Dc}	$1.25\pm0.02^{A c}$
8	1.79 ± 0.04^{Bd}	1.97 ± 0.01^{Cd}	1.98 ± 0.01^{Dd}	1.84 ± 0.03^{Ad}
10	1.91 ± 0.01^{Be}	1.96 ± 0.04^{Ce}	2.04 ± 0.04^{De}	1.88 ± 0.01^{Ae}
12	1.89 ± 1.89^{Bf}	2.07 ± 2.18^{Cf}	2.18 ± 2.07^{Df}	1.90 ± 1.90^{Af}
24	2.16 ± 0.01^{Bg}	2.19 ± 0.01^{Cg}	2.25 ± 0.04^{Dg}	2.05 ± 0.03^{Ag}
36	2.19 ± 0.01^{Bh}	2.25 ± 0.01^{Ch}	2.35 ± 0.06^{Dh}	2.07 ± 0.02^{Ah}
48	2.44 ± 0.23^{Bi}	2.39 ± 0.18^{Ci}	2.69 ± 0.0^{Di}	2.17 ± 0.02^{Ai}
60	1.91 ± 0.03^{Bde}	1.96 ± 0.01^{Cde}	1.95 ± 0.04^{Dde}	1.86 ± 0.04^{Ade}

Table 5.1b Effect of Alginate concentration on free cell density

6.3.2 Effect of initial cell loading on alkaline protease production and cell leakage

Fig.6.2a and Table 6.2b show the extracellular proteolytic activity outside the beads produced by *Vibrio* sp., from beads of varying initial cell loading. It was observed that the amount of cells that go into the beads have profound influence on protease production as productivity was lower with lower amounts of cells. The optimum cell loading was found to be 10, %w/v ; higher or lower inoculum levels resulted in reduced enzyme yield as evident from the ANOVA results. The optimum period of incubation was 36 h after which the yield remained the same. Conversely, an initial cell loading of 15%w/v gave the maximum cell density. As per the statistical analysis the maximum density was at 24h, which remained static even at 48h and came down towards 60h (Fig.6.2 b and Table 6.2b).

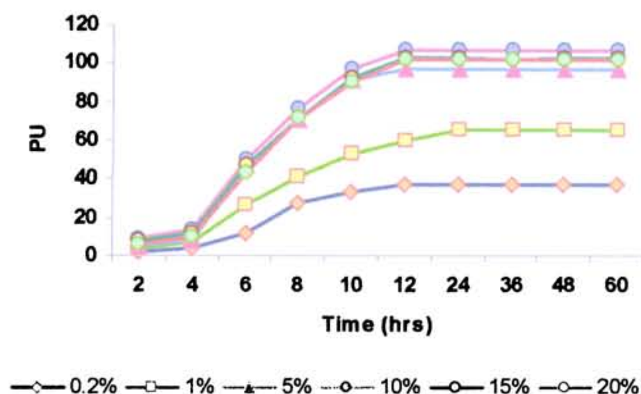


Fig.6.2a Effect of Initial Cell Loading on protease production

Time (hrs)	Protease units (PU) at different Initial Cell Loading					
	0.2%	1%	5%	10%	15%	20%
2	1.51±0.06^{Aa}	3.51±0.08^{Ba}	5.15±0.07^{Ca}	8.88±0.07^{Fa}	7.58±0.11^{Ea}	6.27±0.16^{Da}
4	3.53±0.08^{Ab}	6.51±0.21^{Bb}	8.11±0.05^{Cb}	13.74±0.29^{Fb}	11.67±0.29^{Eb}	10.27±0.11^{Db}
6	11.60±0.32^{Ac}	26.24±0.25^{Bc}	47.41±0.63^{Cc}	50.02±0.26^{Fc}	47.61±0.36^{Ec}	43.51±0.33^{Dc}
8	27.41±0.26^{Ad}	41.23±0.33^{Bd}	71.17±0.64^{Cd}	76.75±0.21^{Fd}	72.12±0.50^{Ed}	71.35±0.20^{Dd}
10	33.06±0.34^{Ae}	53.59±0.34^{Be}	91.83±0.40^{Ce}	97.58±0.54^{Fe}	92.12±0.36^{Ee}	90.11±0.31^{De}
12	37.70±0.43^{Af}	59.53±0.70^{Bf}	97.39±0.47^{Cf}	105.84±0.46^{Ff}	103.60±0.41^{Ef}	102.06±0.15^{Df}
24	37.68±0.30^{Ag}	65.89±0.79^{Bg}	97.33±0.45^{Cg}	105.81±0.30^{Ff}	103.02±0.40^{Eg}	102.34±0.49^{Dg}
36	37.68±0.30^{Ag}	66.38±1.04^{Bg}	97.75±0.36^{Cg}	106.78±0.41^{Fg}	102.44±2.11^{Eg}	102.22±0.30^{Dg}
48	37.27±0.85^{Ag}	65.77±1.03^{Bg}	97.41±0.47^{Cg}	107.08±0.62^{Fg}	103.18±0.60^{Eg}	102.58±0.10^{Dg}
60	37.86±0.33^{Ag}	66.28±0.62^{Bg}	97.11±0.71^{Cg}	106.72±0.46^{Fg}	103.30±0.77^{Eg}	102.44±0.32^{Dg}

Table 6.2a Effect of Initial Cell Loading on protease production

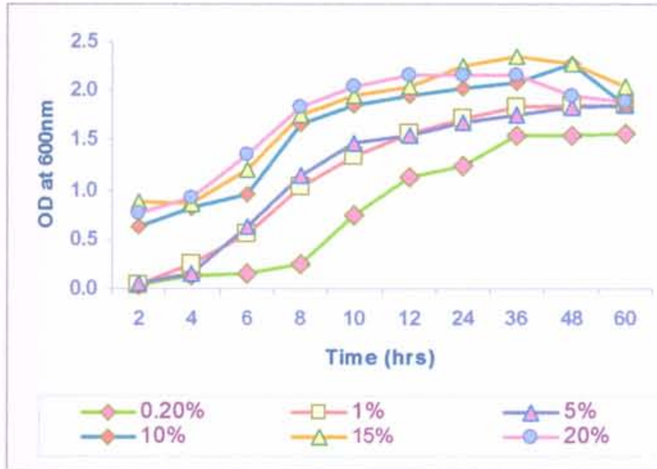


Fig.6.2b Effect of Initial Cell Loading on cell leakage

Time (hrs)	OD (600 nm) at different Initial Cell Loading					
	0.20%	1%	5%	10%	15%	20%
2	0.05±0.00^{Aa}	0.04±0.00^{Ba}	0.05±0.00^{Ba}	0.63±0.04^{Ca}	0.88±0.02^{Ea}	0.77±0.02^{Da}
4	0.12±0.02^{Ab}	0.25±0.00^{Bb}	0.16±0.01^{Bb}	0.82±0.04^{Cb}	0.86±0.02^{Eb}	0.92±0.01^{Db}
6	0.16±0.00^{Ac}	0.55±0.02^{Bc}	0.63±0.02^{Bc}	0.95±0.01^{Cc}	1.21±0.01^{Ec}	1.36±0.02^{Dc}
8	0.24±0.01^{Ad}	1.03±0.00^{d}	1.15±0.02^{Bd}	1.67±0.03^{Cd}	1.76±0.04^{Ed}	1.83±0.05^{Dd}
10	0.75±0.01^{Ae}	1.35±0.02^{Be}	1.46±0.05^{Be}	1.85±0.03^{Ce}	1.94±0.04^{Ee}	2.05±0.02^{De}
12	1.13±0.01^{Af}	1.56±0.04^{Bf}	1.55±0.03^{Bf}	1.94±0.04^{Cf}	2.05±0.04^{Ef}	2.15±0.02^{Df}
24	1.25±0.03^{Ag}	1.73±0.04^{Bg}	1.69±0.04^{Bg}	2.02±0.01^{Cg}	2.26±0.02^{Eg}	2.16±0.03^{Dg}
36	1.54±0.01^{Ah}	1.83±0.0^{Bh}	1.75±0.04^{Bh}	2.07±0.01^{Ch}	2.34±0.03^{Eh}	2.16±0.03^{Dh}
48	1.54±0.01^{Ah}	1.86±0.02^{Bh}	1.84±0.04^{Bh}	2.27±0.04^{Ch}	2.27±0.02^{Eh}	1.95±0.05^{Dh}
60	1.57±0.02^{Ag}	1.85±0.05^{Bg}	1.86±0.03^{Bg}	1.86±0.02^{Cg}	2.05±0.00^{Eg}	1.89±0.05^{Dg}

Table 6.2b Effect of Initial Cell loading on cell leakage

6.3.3 Effect of bead inoculum on alkaline protease production and cell leakage

With immobilised *Vibrio* sp., protease production was maximum with 5% bead inoculum starting from the first 12h of incubation as shown in Fig.6.3.a and Table 6.3.a. Beyond 5% bead inoculum the production came

down. A bead inoculum of 10% favoured cell density to the maximum, which was at its peak at 24h and decreased thereafter (Fig.6.3.b and Table 6.3.b).

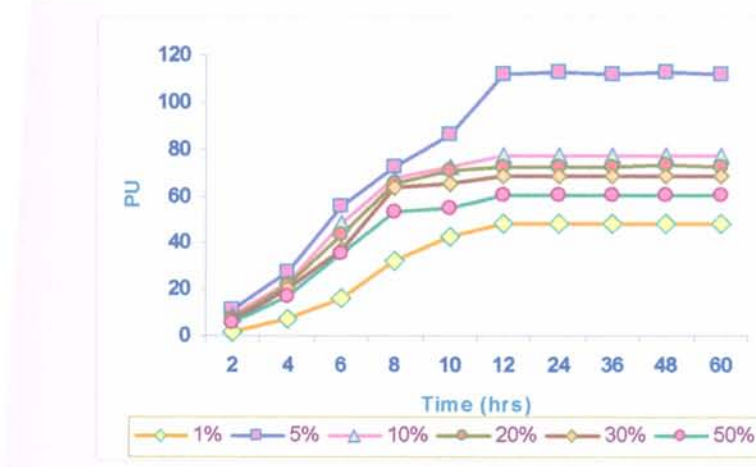


Fig.6.3a Effect of bead inoculum on protease production

Time (hrs)	Protease units (PU) at different bead inocula					
	1%	5%	10%	20%	30%	50%
2	1.50±0.05^{Aa}	11.42±0.20^{Fa}	8.80±0.05^{Ea}	7.63±0.10^{Da}	6.24±0.12^{Ca}	5.33±0.14^{Ba}
4	7.63±0.10^{Ab}	27.44±0.18^{Fb}	22.55±0.08^{Eb}	21.46±0.22^{Db}	20.37±0.15^{Cb}	17.09±0.07^{Bb}
6	15.82±0.10^{Ac}	55.27±0.14^{Fc}	48.45±0.27^{Ec}	43.41±0.11^{Dc}	37.38±0.18^{Cc}	35.40±0.24^{Bc}
8	32.41±0.24^{Ad}	72.73±0.19^{Fd}	67.71±0.32^{Ed}	65.41±0.30^{Dd}	63.45±0.56^{Cd}	52.87±0.08^{Bd}
10	42.51±0.14^{Ae}	86.57±0.18^{Fe}	72.73±0.19^{Ee}	70.89±0.12^{De}	65.13±0.56^{Ce}	54.86±0.12^{Be}
12	48.03±1.71^{Af}	112.11±0.40^{Ff}	77.52±0.17^{Ef}	72.81±0.22^{Df}	68.66±0.44^{Cf}	60.39±0.25^{Bf}
24	48.55±0.19^{Af}	112.50±0.51^{Ff}	77.49±0.17^{Ef}	72.72±0.30^{Df}	68.29±0.22^{Cf}	60.55±0.31^{Bf}
36	48.44±0.24^{Af}	112.28±0.30^{Ff}	77.52±0.28^{Ef}	72.56±0.15^{Df}	68.49±0.33^{Cf}	60.43±0.25^{Bf}
48	48.43±0.25^{Af}	112.43±0.31^{Ff}	77.41±0.24^{Ef}	72.89±0.14^{Df}	68.41±0.36^{Cf}	60.59±0.292^{Bf}
60	48.49±0.25^{Af}	112.19±0.22^{Ff}	77.17±0.36^{Ef}	72.69±0.09^{Df}	68.25±0.30^{Cf}	60.43±0.28^{Bf}

Table 6.3a Effect of bead inoculum on protease production

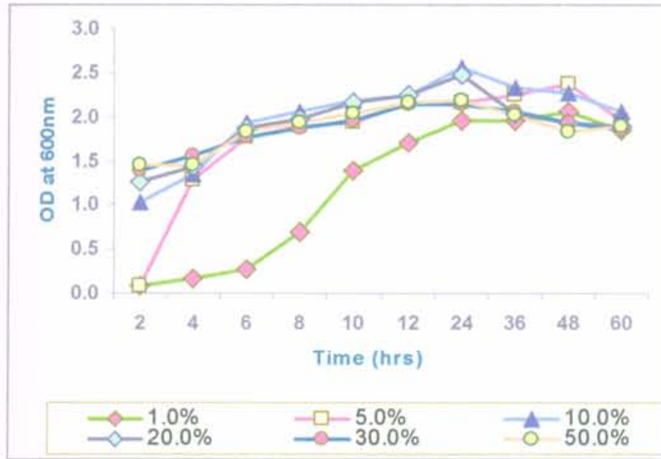


Fig.6.3b Effect of bead inoculum on cell leakage

Time (hrs)	OD (600 nm) at different bead inocula					
	1%	5%	10%	20%	30%	50%
2	0.08±0.01 Aa	0.08±0.01 Ba	1.03±0.01 Fa	1.25±0.02 Ea	1.38±0.03 Ca	1.45±0.04 Da
4	0.18±0.01 Ab	1.28±0.03 Bb	1.34±0.02 Fb	1.43±0.01 Eb	1.55±0.03 Cb	1.46±0.03 Db
6	0.27±0.02 Ac	1.76±0.03 Bc	1.94±0.05 Fc	1.86±0.05 Ec	1.76±0.03 Cc	1.82±0.05 Dc
8	0.69±0.03 Ad	1.88±0.02 Bd	2.06±0.04 Fd	1.96±0.02 Ed	1.86±0.06 Cd	1.93±0.05 Dd
10	1.38±0.05 Af	1.93±0.05 Bf	2.17±0.04 Ff	2.16±0.03 Ef	1.94±0.04 Cf	2.04±0.02 Df
12	1.71±0.04 Ah	2.16±0.05 Bh	2.25±0.02 Fh	2.25±0.02 Eh	2.15±0.03 Ch	2.16±0.02 Dh
24	1.94±0.03 Ai	2.16±0.02 Bi	2.57±0.07 Fi	2.48±0.06 Ei	2.15±0.01 Ci	2.18±0.05 Di
36	1.95±0.03 Ah	2.25±0.02 Bh	2.34±0.09 Fh	2.04±0.03 Eh	2.06±0.02 Ch	2.02±0.06 Dh
48	2.06±0.02 Ag	2.36±0.05 Bg	2.28±0.03 Fg	1.92±0.05 Eg	1.92±0.06 Cg	1.82±0.09 Dg
60	1.84±0.05 Ae	1.95±0.03 Be	2.07±0.04 Fe	1.88±0.01 Ee	1.85±0.12 Ce	1.89±0.07 De

Table 6.3b Effect of bead inoculum on cell leakage

6.3.4 Alkaline protease production and cell density of free and immobilised cells

As illustrated in Fig.6.4 a & b and Table 6.4, alkaline protease production in the medium with immobilised *Vibrio* sp. stayed well above the free cells at all hours of incubation. It was found that protease production increased 2.3 fold, using immobilised cells. The cell densities of the two were

similar at the initial and final hours of the experiment and in between, the cell density of the medium with immobilised cells was higher than that of free cells (Fig 6.4c). Protease production and cell density kept increasing with time in the case of free cells; in the case of immobilised cells also, enzyme production increased with time until reaching a maximum at 12 h, thereafter remaining the same. As for cell density the values declined from 48 h. ANOVA was performed independently for free and immobilised cells both for protease production and cell density, and is given in Table 6.4.

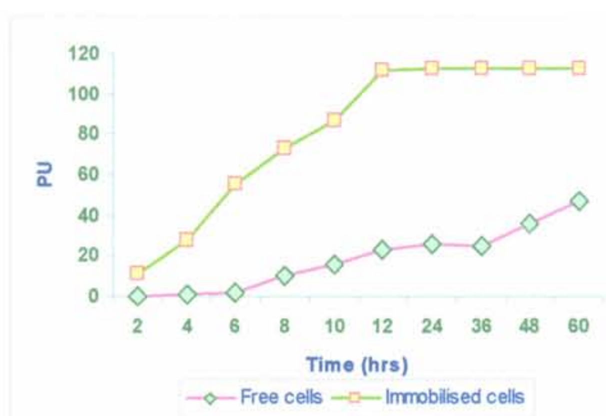


Fig.6.4a Protease production by free and immobilised cells

Time (hrs)	a. Protease production (PU)		b. Cell density (OD at 600nm)	
	Free cells	Immobilised cells	Free cells	Immobilised cells
2	0.11 ± 0.01^{a}	11.42 ± 0.20^{a}	0.06 ± 0.03^{a}	0.08 ± 0.01^{a}
4	0.74 ± 0.08^{b}	27.44 ± 0.18^{b}	0.36 ± 0.49^{b}	1.28 ± 0.03^{b}
6	1.69 ± 0.14^{c}	55.27 ± 0.14^{c}	0.37 ± 0.02^{b}	1.76 ± 0.03^{c}
8	10.22 ± 0.43^{d}	72.73 ± 0.19^{d}	0.67 ± 0.03^{c}	1.88 ± 0.02^{d}
10	16.12 ± 0.17^{e}	86.57 ± 0.18^{e}	0.70 ± 0.05^{d}	1.93 ± 0.05^{e}
12	23.19 ± 0.03^{f}	112.11 ± 0.40^{f}	0.94 ± 0.04^{e}	2.16 ± 0.05^{f}
24	25.65 ± 0.10^{g}	112.50 ± 0.51^{f}	1.18 ± 0.01^{f}	2.16 ± 0.02^{f}
36	24.70 ± 1.07^{g}	112.28 ± 0.30^{f}	$1.36 \pm 0.03f^{g}$	2.25 ± 0.02^{g}
48	35.96 ± 0.02^{h}	112.43 ± 0.31^{f}	1.52 ± 0.05^{g}	2.36 ± 0.05^{h}
60	47.35 ± 0.69^{i}	112.19 ± 0.22^{f}	1.82 ± 0.14^{h}	1.95 ± 0.03^{e}

Table 6.4 Comparison of free and immobilised cells

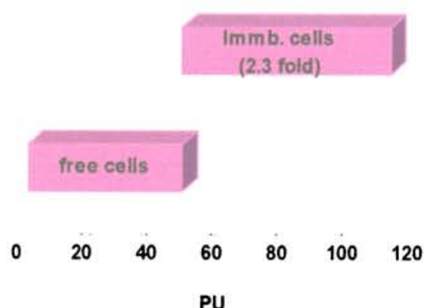


Fig.6.4 b Enhanced protease production by immobilised cells

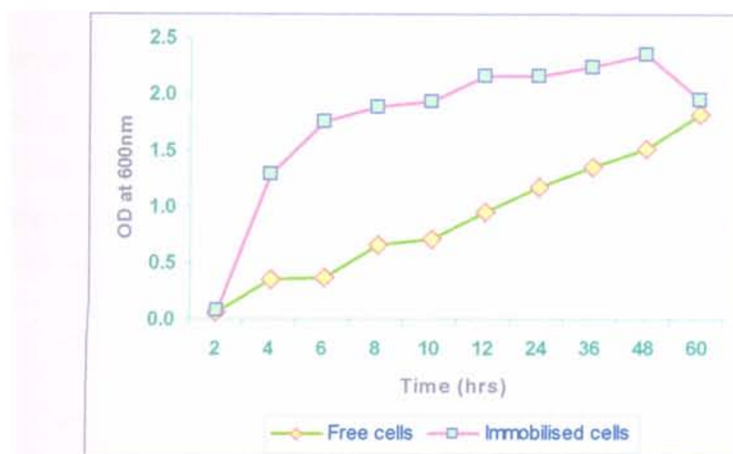
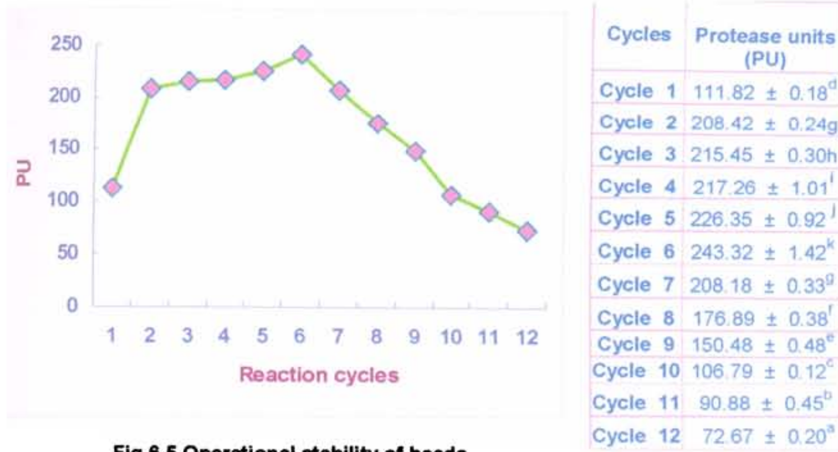


Fig.6.4c Cell densities of the media inoculated with free and immobilised cells

6.3.5 Estimation of operational stability of beads

Fig.6.5 and Table 6.5 show the protease production by immobilised cells for 12 reaction cycles. Alkaline protease production by immobilised *Vibrio* sp.cells increased with repeated reaction cycles, and reached a maximum after 6 cycles. The half-life was estimated as 9.5 reaction cycles.

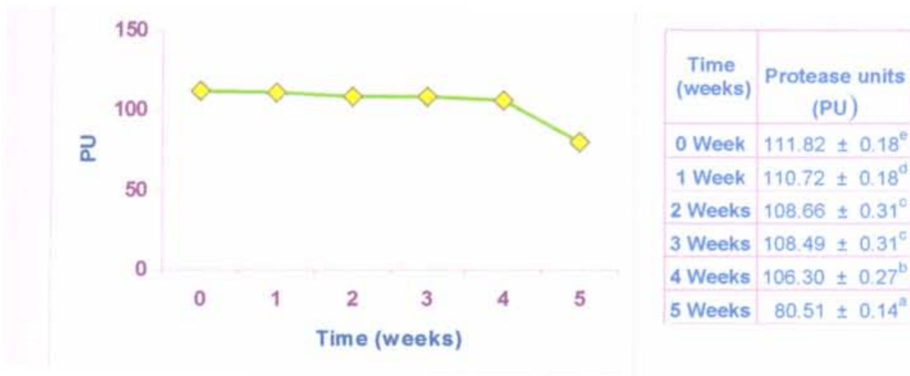


Cycles	Protease units (PU)
Cycle 1	111.82 ± 0.18^{d}
Cycle 2	208.42 ± 0.24g
Cycle 3	215.45 ± 0.30h
Cycle 4	217.26 ± 1.01^{i}
Cycle 5	226.35 ± 0.92^{j}
Cycle 6	243.32 ± 1.42^{k}
Cycle 7	208.18 ± 0.33^{g}
Cycle 8	176.89 ± 0.38^{f}
Cycle 9	150.48 ± 0.48^{e}
Cycle 10	106.79 ± 0.12^{c}
Cycle 11	90.88 ± 0.45^{b}
Cycle 12	72.67 ± 0.20^{a}

Fig.6.5 Operational stability of beads

6.3.6 Estimation of shelf life of beads

The ability of immobilised cells for protease production reduced on longer periods of storage, as illustrated in Fig.6.6 It was observed that the beads were viable and stable until 4 weeks of storage and beyond that, the quality of the beads became deteriorated.



Time (weeks)	Protease units (PU)
0 Week	111.82 ± 0.18^{e}
1 Week	110.72 ± 0.18^{d}
2 Weeks	108.66 ± 0.31^{c}
3 Weeks	108.49 ± 0.31^{c}
4 Weeks	106.30 ± 0.27^{b}
5 Weeks	80.51 ± 0.14^{a}

Fig.6.6 Shelf life of beads

6.4 Discussion

Cell immobilisation is one of the common techniques for increasing the over-all cell concentration and productivity. The separation of products from immobilised cells is easier compared with suspended cell systems. Immobilisation of cells may allow continuous operation of cultivation processes at high dilution rates. Last but not least, immoblisation is a strategy for protecting cells from shear forces. Many different techniques for immobilising cells have been proposed using polysaccharides for gel entrapment or encapsulation and the use of alginate gel beads stands out as the most promising and versatile method yet (Brodelius and Vandamme, 1987; Beshay *et al.*, 2002; Abd-El-Haleem *et al.*, 2003). Alginic acid and its derivatives are commercially available in a wide range of types, having different viscosities and gelling properties. Calcium alginate forms gels rapidly in very mild conditions and provides suitable media for the immobilisation by entrapment of whole microbial cells. Only a single type of sodium alginate was used in this work and it was found suitable for successful immobilisation of *Vibrio* sp. cells. The free cell density under different conditions of immobilisation corresponds to the cells diffused from the beads. These cells multiply in the medium and contribute to the extracellular alkaline protease production.

6.4.1 Effect of alginate concentration on alkaline protease production and cell leakage

The gel network differs depending on the conditions in which the gel beads are obtained (Nava *et al.*, 1996). In this study, various concentrations of sodium alginate solutions were used for the preparation of the biocatalyst beads in order to study its effect on protease production by *Vibrio* sp. V26 cells. The production of alkaline protease and free cell density improved with

increasing alginate concentration and reached its maximum at 2.5%. An increase beyond this concentration was accompanied by a decrease in the enzyme yield. This may be attributed to the impairment of diffusional properties of the gel because of its strong gel consistency, leading to lower enzyme yields. Other reasons could be the reduced rate of substrate mass transfer and the lower porosity of the gel beads, as reported by Shinmyo *et al.* (1982) and Fumi *et al.* (1992).

The results obtained in the present study are in agreement with other investigations (Dobreva *et al.*, 1996). Martinsen *et al.* (1992) studied the dependence of diffusion coefficients on alginate concentration in homogeneous as well as heterogeneous beads of *L.digitata*. They had observed that diffusion coefficient decreased with increasing alginate concentration for both homogeneous and heterogeneous beads. For alginate concentration higher than 1.9%, the diffusivities proved to be considerably higher in homogeneous beads than in heterogeneous ones, because of the fact that an increase in polymer concentration will favour a steeper gradient in the alginate network (Skjak-Braek *et al.*, 1989).

Effective biocatalysis in systems of immobilised enzymes or cells evidently requires that the transport of substrates and product within the alginate matrix should be as free as possible. Diffusion within the gel depends upon porosity, however, since gel matrix is negatively charged, the influence of electrostatic forces between the matrix and the ionic substrates must also be considered (Buchholz, 1979). For solutes with low molecular weights, Tanaka *et al.* (1984) found no reduction in diffusion coefficients in calcium alginate gel beads compared with the free diffusion in water. For larger solutes such as albumin, γ - globulins, and fibrinogen, however, the diffusion in the gel was retarded to an extent, depending upon the concentration of

sodium alginate and calcium chloride. Moreover, these proteins could diffuse *out of*, but not *into* the beads. It was, therefore, suggested that the structure of the calcium alginate gel formed in the presence of large protein molecules was different from that of the gels formed in their absence.

6.4.2 Effect of initial cell loading on alkaline protease production and cell leakage

The effect of cell loading of the beads on protease production and free cell density could be explained by the barrier formed by the gel to enzyme diffusion. Tanaka *et al.* (1984) investigated the diffusion of enzymes into and from calcium alginate beads in well-stirred solutions. In the present study, it was observed that the amount of cells that go into the beads have profound influence on protease production as there was lower productivity at lower amounts of cells. The optimum cell loading was found to be 10% (w/v); higher or lower inoculum levels resulted in reduced enzyme yield. Conversely an initial cell loading of 15%w/v gave the maximum cell density. Retention by the gel according to protein size could explain the easy diffusion of the *M.xanthus* proteases through the gel beads (Vuillermard *et al.*, 1988).

Diffusivity measurements of glucose in cell free and cell occupied Ca-alginate membranes have shown that the productivity of the cells might be limited by the presence of the alginate matrix (Hannoun and Stephanopoulos, 1986). Alginate gels allow fairly rapid diffusion of small molecules. But the cells obtain nutrients solely by diffusion, whereas in suspended cultures nutrients are carried by convective flow too. Diffusion is, however, significantly slower than convective transport in a well-stirred reactor. Furthermore, rapid growth near the gel surface leading to cell leakage and cell death in the inner core occurred. Furui and Yamashita (1985) determined effective diffusion

coefficients of solutes in immobilised cells prepared with polyacrylamide and k-carrageenan gels.

Internal mass transfer limitations of the nutrients are responsible in many cases for the uneven distribution of living cells within the immobilisation matrix after exposure to a growth medium (Radovich, 1985). In gel beads containing viable cells, a reaction front occurs, which leads to the formation of a high-density layer near the bead surface. The depth of the cell layer is dependent upon the fermentation conditions and the physical parameters such as bead diameter, substrate and product diffusion coefficients (Arnaud and Lacroix, 1991)

6.4.3 Effect of bead inoculum on alkaline protease production and cell leakage

The dependence of enzyme yield on bead inoculum was studied and it was observed that, with immobilised *Vibrio* sp. V 26, protease production was maximum with the 5% bead inoculum and further increase has lowered the net enzyme production. Similar results were obtained for the production of gluconic acid by *Aspergillus niger* immobilised in Ca-alginate beads (Rao and Panda, 1994). The decreased enzyme yield at higher bead inoculum could be attributed to the fact that, when the number of beads increases, the nutrient bead ratio decreases. Because of the competition between the cells, the nutrient concentration available in flasks with larger inocula could be inadequate for optimal growth and consequently for enzyme production. A bead inoculum of 5-10% favoured cell density and decreased thereafter.

With equal starting quantities of *Myxococcus xanthus* in the medium, higher proteolytic activities were obtained in the media with higher gel bead content (Vuillermard *et al.*, 1988). This may result from an increase in

diffusion surface with a greater number of gel beads. A modification of cell metabolism is also possible.

6.4.4 Alkaline protease production and cell density of free and immobilised cells

In the present study, the main advantage of cell immobilization was the improvement of protease production. It was observed that the protease production increased upto 2.3 fold in fermentation using immobilised cells. Vulliemard *et al.* (1988) observed that the proteolytic activity increased 4 times that of the free culture in the medium containing immobilised *M.xanthus* cells. The increase in enzyme yield could be due to the fact that immobilisation leads to higher volumetric activities within the same time of fermentation. Higher proteolytic activity and low free cell concentrations outside carrageenan beads containing *Myxococcus xanthus* was reported by Younes *et al.* (1984; 1987).

In contrast to the results obtained in the present study, Vulliemard *et al.* (1988) observed that cell immobilisation decreased protease production. Protease production in the medium with immobilised *S.marcescens* stayed lower than in the medium with free cells. A possible explanation is that proteolytic activity was mainly retained within the beads.

6.4.5 Estimation of operational stability of beads

The present investigation has shown that extracellular alkaline protease production by immobilised cells of *Vibrio* sp. increased gradually with repeated batch cycles. Increase of protease productivity of the immobilised cells during the early cycles may be caused by the growth of the cells in the gel. The cells gradually grew on the surface of the gel with

increasing cycles. The surface of the beads was suitable for the growth of cells, because the supply of oxygen and nutrients was better.

Gel entrapment is a fairly gentle cell immobilisation method, which gives good resistance to mechanical stress and provides a high cell concentration in a small space. However, larger beads limit diffusion of oxygen and nutrients. Alginate does not provide a surface for anchorage-dependent cells so only cells suitable for suspension-type growth can be trapped in alginate. This is one factor, which determines the suitability of different cells for alginate entrapment. The increased alkaline protease production by *Vibrio* sp. under immobilised conditions proves the appropriateness of the present investigation. When immobilised cells are transferred in a growth medium, limitations on the diffusion of substrates and products result in the formation of a high-cell density layer extending from the bead surface to the radial depth where lack of substrate or accumulation of inhibitory product and local physicochemical conditions prevent growth (Champagne *et al.*, 1994; Doleyres *et al.*, 2002 and Lacroix *et al.*, 2003). Cell release from gel beads in the liquid medium occurs spontaneously because of active cell growth in the high-biomass-density peripheral layer.

The present findings are in accordance with those obtained previously for the production of protease by immobilised *Serratia marcescens* and *Myxococcus xanthus* in calcium alginate beads (Vulliemard *et al.*, 1988). It was found that protease production by immobilised *Serratia marcescens* increased with repeated growth cycles, and reached a maximum after 5 cycles. Similar observations were reported by Beshay (2003) as well.

Protease production by immobilised cells was studied for 12 reaction cycles and alkaline protease production by immobilised *Vibrio* sp.V26 cells increased with repeated reaction cycles reaching a maximum after 6 cycles.

The half-life was estimated as 9.5 reaction cycles. In addition, mechanical stability of the biocatalysts was high enough, since no fragments of alginate beads were found in the culture broth.

6.4.6 Estimation of shelf life of beads

One of the advantages of immobilisation is the reuse of the beads and storage of beads until use in the next batch. It was found that the beads are viable and stable until 4 weeks of storage and beyond that the quality of the beads deteriorated. Similarly, Venugopal *et al.* (1989) studied the ability of the beads to solubilise fish meat during storage at 4^0C. It was observed that the beads retained proteolytic activity upto 30 days of storage.

The results of the present study prove that calcium alginate entrapment is a promising method of immobilisation of *Vibrio* sp. V26 cells for alkaline protease production. The major and specific advantages of this technique such as enhancement of production, long-term stability, the reusability and possibility of regeneration of beads have been fairly accomplished.

7

Application of alkaline protease on deproteinisation of prawn shell waste for chitin recovery

The shellfish processing industry in India generates 8.5 million tons of shell waste per year (FAO, 1998). In terms of weight, about 35-45% of shrimp is discarded as waste when processed into headless, shell-on products. Peeling process, which involves the removal of the shell from the tail of prawn, increases the total waste production upto 40-45%. On a global basis, the shrimp processing industry produces over 7,00,000 million tons of shell waste. Environmental implication of traditional disposal methods of such waste, coupled with the strengthening of environmental regulations in many countries, has created an interest in alternative methods of disposal/utilisation of this waste.

Crab shells contain only 12.9 - 23.5% protein based on the part of the shell used while shrimp wastes have 42% protein (Naczk and Shahidi, 1990).

Ash content is 29.2% - 36.8%. Content of crude chitin varies from 30% from crab backs to 40% for the other parts of the shell. Minerals in shellfish waste usually consist of 90% or more calcium carbonate, the rest being calcium phosphate. The high amount of calcium is a problem for recovery and disposal. Hansen and Illnes (1994) estimated the proximate composition of crustacean waste as, chitin 14-30%, protein 15-40%, minerals 35-53% and lipids 0-10%.

Since the head and shell comprise approximately 75% of the weight of a shrimp, proper utilisation of the waste will result in shrimp being of even greater value (Chen and Chen, 1991). Shell waste may serve as a potential source of raw material for the production of much value-added products including chitin/chitosan (Ashford *et al.*, 1977; Muzzarrelli, 1977; Johnson and Peniston, 1982), carotenoid pigments (Lambersten and Braekkam, 1971; Chen and Meyers, 1983; Manu-Tawaiah and Haard, 1987), and flavourants (Shahidi *et al.*, 1989; Pan, 1989). Many of these processes concentrated on the production of chitin/chitosan.

Chitin and its derivatives hold great economic value because of their versatile biological activities and agrochemical applications (Cosio *et al.*, 1982; Flach *et al.*, 1992; Wang *et al.*, 1995; Hirano, 1996). Chitin and its deacetylated form, chitosan, have found increasing number of uses in various fields in recent years, from the flocculation of solid particles in waste water to transparent films (Knorr, 1984; Skjak-Braek *et al.*, 1989). The world wide availability of chitin from shellfish is estimated to be over 39,000 tons, annually. Approximately 75% of the total weight of shellfish is considered as waste and from 20-58% of the dry weight of the waste is chitin depending upon the processing method (Kreag *et al.*, 1973).

Brzeski (1987) found that crustacean waste contains approximately 10 - 60 percent chitin on a dry weight basis, depending on the processing method. Chitin is the most abundant renewable natural polymer, after cellulose (Deshpande, 1986). It is a polymer of β (1-4) – N-acetyl-D glucosamine. Chitin occurs in three polymorphic forms, which differ in the arrangement of molecular chains within the crystal cell. α chitin is the tightly compacted, most crystalline polymeric form where the chains are arranged in an anti-parallel fashion; β chitin is the form where the chains are parallel and γ chitin is the form where the chains are one 'up' to every one 'down' (Muzzarelli, 1977).

Chitin in crustacean cuticle exists as a mucopolysaccharide bound to proteins (Hackman, 1954;Rudall, 1963; Hunt, 1970; Muzzarelli, 1977; Austin *et al.*, 1981; Brine, 1982). Horst (1989) examined the synthesis of chitin in the post larval stages of *Penaeus vannamei*. Analysis of the cuticle revealed 73% (by weight) amino acids and 27% N-acetyl glucosamine. Major amino acids in the cuticle are Asx (aspartic acid and asparagines), Glx (glutamic acid and glutamine), Thr, Ala, His and Leu.

The constituents of shell waste make them worthy of further processing and utilisation. Shell waste is mainly subjected to chemical processing for chitin/chitosan production. Current procedures for preparing chitin from shellfish waste consist of two main steps i.e. $CaCO_3$ separation or demineralisation with dilute acids or chelating agents (Tsugita, 1991), followed by protein separation or deproteinisation with dilute alkali or proteolytic enzymes. Alternatively, the deproteinisation process may precede the demineralisation step (Sandford, 1989). Subsequent to the demineralisation and deproteinisation steps, the product may be decolourised with acetone and/or hydrogen peroxide. However, the use of these chemicals

may cause a partial deacetylation of chitin and hydrolysis of the polymer resulting in final inconsistent physiological properties (Brine and Austin, 1981; Shimahara and Takiguchi, 1988; Gagne, 1993; Wang *et al.*, 1997). These chemical treatments also create waste disposal problems, because neutralisation and detoxification of the discharged waste water may be necessary. Furthermore, the value of the deproteinisation liquid is diminished due to the presence of sodium hydroxide (Gagne, 1993).

To overcome the shortcomings of the chemical treatments, studies have been conducted using microorganisms (Shimahara and Takiguchi, 1988) or proteolytic enzymes (Takeda and Abe, 1962; Takeda and Katsuura, 1964; Broussignac, 1968; Gagne, 1993; Wang and Chang, 1997; Wang and Chio, 1998). Bioconversion of shell waste is probably the most cost effective and environment-friendly procedure for waste utilisation. In order to obtain a less degraded chitin, some alternative methods for deproteinisation stage have been proposed. Proteolytic enzymes such as tuna trypsin (Takeda and Abe, 1962), Rhozyme-62 (Bough *et al.*, 1978) and cod trypsin (Simpson and Haard, 1985) have been used to deproteinise crustacean shell. Several work have been carried out on the chitin bioconversion itself (Carroad and Tom, 1978; Revah-Moiseev and Carroad, 1981; Cosio *et al.*, 1982). Revah-Moiseev and Carroad (1981) described the conversion of the enzymatic hydrolysate of shellfish waste chitin to single cell protein employing the yeast, *Pichia kudriavzenni.* Lactic acid fermentation of scampi (*Penaeus monodon*) shell waste for chitin recovery was described by Hall and de Silva (1994). Proteolytic strains like *Pseudomonas maltophila*, *Bacillus subtilis*, *Streptococcus faecium*, *Pediococcus pentosaseus* and *Aspergillus oryzae* were used for microbial extraction of chitin from prawn shell waste. Over 80% deproteinisation of the demineralised prawn shell waste was achieved by this bacterial consortia (Bustos and Healy, 1994). Healy *et al.*, (1994) investigated

the viability of lactic acid fermentation on demineralisation of prawn shell waste for deproteinisation under both aerobic and anaerobic conditions. In recent years, ample successes in conversion of proteinaceous waste into useful biomass by proteases have also been demonstrated (Venugopal *et al.*, 1989; Dalev and Simeonova, 1992; Atalo and Gash, 1993; Dalev, 1994, Yang *et al.*, 2000). Protease produced by *Bacillus spp.*, including *B. subtilis* and *B. firmus* are by far the most important group of enzymes being exploited.

Despite promising results on a laboratory scale, large scale application of such methods is unlikely because of the high price of the purified enzymes (Cano-Lopez *et al.*, 1987). The present study is intended to elucidate the potential of the crude extract of alkaline protease from *Vibrio* sp. V26 for the deproteinisation of prawn shell waste for chitin recovery, with as little deacetylation as possible.

7.1 *Materials and methods*

7.1.1 Pre-treatment of prawn shell waste

Prawn shell waste was collected from peeling sheds in Cochin. They were oven dried at 80^{0}C overnight. The dried shells were then powdered and stored in tightly sealed packets and kept under refrigeration (4^{0}C). The different stages in the pre-treatment of prawn shell waste are shown in Plate 8.

7.1.2 Demineralisation of prawn shell waste

The demineralisation of the waste was done using 1.75N acetic acid at room temperature for 12h. The ratio of waste to solvent was 1:15 (w : v). The demineralised material was recovered by filtration and was thoroughly rinsed with deionised water, and dried at 65^{0}C in an oven. A photograph of prawn shell waste after demineralisation is given in Plate 9.

Different stages of pre-treatment of prawn shell waste

Plate 8

Demineralised shell waste

Deproteinised shell waste

Prawn shell waste after demineralisation and deproteinisation

Plate 9

7.1.3 Preparation of crude enzyme

Alkaline protease was obtained by the biphasic fermentation of wheat bran by *Vibrio* sp. V26 as described in section 4.2.2 and the crude enzyme was extracted as per the procedure specified in section 3.2.3.

7.1.4 Deproteinisation of prawn shell waste

The demineralised materials were then deproteinised with the crude enzyme extract of alkaline protease. The ratio of demineralised waste to enzyme was 1 : 20 (w : v), and 4g of waste was used. The deproteinisation was conducted in Erlenmeyer flasks incubated for 72 h at 4^0C in an incubator-shaker with constant agitation at 150 rpm. A photograph of prawn shell waste after deproteinisation is given in Plate 9.

7.1.5 Protein estimation

The deproteinised material was filtered through Whatman No.4 filter paper and the protein in the residue recovered after filtration was measured by digesting the dry residue with 2N NaOH (1: 25, w : v) at 70^0C in a water bath for 1hr, and the protein content of the resulting liquid was measured by the method of Lowry *et al.* (1951) with Bovine Serum Albumin (BSA) as standard. The concentration of protein during purification studies was calculated from the standard curve.

7.1.6 Measurement of percentage of deproteinisation

The percentage of deproteinisation of the prawn shell waste was calculated as follows :

$$\%\text{ of deproteinisation} = \frac{\text{Total protein} - \text{Residual protein}}{\text{Total protein}} \times 100$$

7.1.7 Optimisation of process parameters for deproteinisation of prawn shell waste

The scheme followed was to optimise each parameter, and the optimal grade was adopted for subsequent experiments. The various process parameters studied were as follows:

7.1.7.1 Effect of incubation period on the deproteinisation of prawn shell waste

Constant weights of demineralised prawn shell waste were incubated with equal volumes of the crude alkaline protease for different time periods of 24h, 48 h, 72 h, 96 h and 120 h. The optimal incubation period giving maximum deproteinisation was employed for all the remaining experiments.

7.1.7.2 Effect of demineralised waste to enzyme ratio on the deproteinisation of prawn shell waste

The reaction was conducted using demineralised waste and alkaline protease at various ratios as 1: 10, 1: 15, 1 : 20, 1 : 25 and 1 : 30, in order to study the importance of demineralised waste to enzyme ratio on the deproteinisation of prawn shell waste.

7.1.7.3 Effect of temperature on the deproteinisation of prawn shell waste

The reaction mixture was incubated at different temperatures of 25^{0}C, 32^{0}C, 40^{0}C, 55^{0}C and 60^{0}C and the optimum temperature for maximum deproteinisation was determined.

7.1.7.4 Effect of shaking on the deproteinisation of prawn shell waste

To study the influence of shaking on the extent of deproteinisation, the reaction mixture was agitated at various speeds like 0, 50,100, 150 and 200 rpm, during incubation.

All the experiments were carried out in triplicates in order to corroborate the validity of the results obtained.

7.2 Statistical analysis

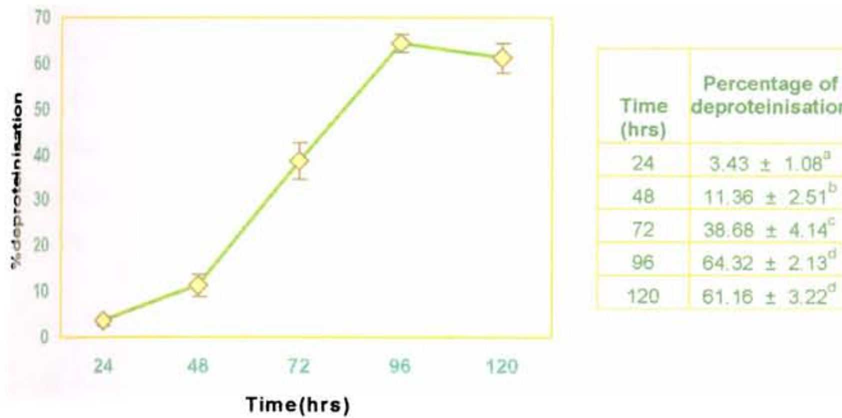
Data generated from the above experiments were analysed by one-way analysis of variance (ANOVA). Mean of the results was compared using SPSS 10.0 for windows at a significance level of $p<0.05$.

7.3 Results

The results of the optimisation studies of the process parameters for deproteinisation of prawn shell waste are shown as graphs and tables. The ANOVA results are also given in the tables as superscript alphabets. Values with the same superscripts do not vary significantly.

7.3.1 Effect of incubation period on the deproteinisation of prawn shell waste

Maximum deproteinisation was obtained after 96 h of incubation. The degree of deproteinisation showed a progressive increase from 24 h reaching a maximum after 96 h and further incubation did not contribute any increase towards deproteinisation. Fig. 7.1 describes the percentage of deproteinisation at different periods of incubation. ANOVA showed that incubation period has definite effect on protease production as the values obtained significantly differed from one another.



Time (hrs)	Percentage of deproteinisation
24	3.43 ± 1.08[a]
48	11.36 ± 2.51[b]
72	38.68 ± 4.14[c]
96	64.32 ± 2.13[d]
120	61.16 ± 3.22[d]

Fig.7.1 Effect of incubation period on deproteinisation

7.3.2 Effect of demineralised waste to enzyme ratio on the deproteinisation of prawn shell waste

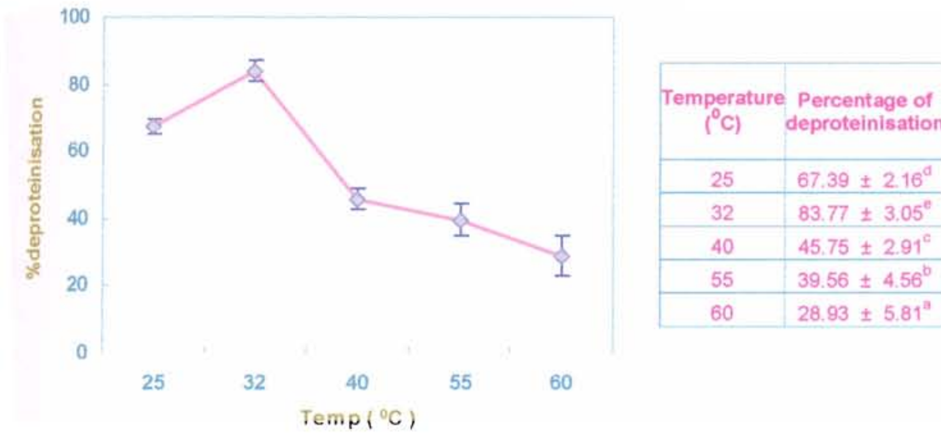
It was found that greater volumes of enzyme increased the deproteinisation until a demineralised waste to enzyme ratio of 1 : 20 beyond which there was hardly any increase in the yield (Fig.7.2).

Waste to enzyme ratio (w : v)	Percentage of deproteinisation
1 : 10	38.66 ± 4.03[a]
1 : 15	47.41 ± 2.01[b]
1 : 20	65.61 ± 2.93[c]
1 : 25	62.57 ± 2.88[c]
1 : 30	63.11 ± 2.54[c]

Fig.7.2 Effect of waste to enzyme ratio on deproteinisation

7.3.3 Effect of temperature on the deproteinisation of prawn shell waste

Deproteinisation occurred at all temperatures selected for the study but it was found that comparatively lower temperatures favoured deproteinisation, the optimum being 32°C. The percentage of deproteinisation declined sharply when the temperature of incubation was higher (Fig.7.3, proving the direct influence of temperature on the degree of deproteinisation.

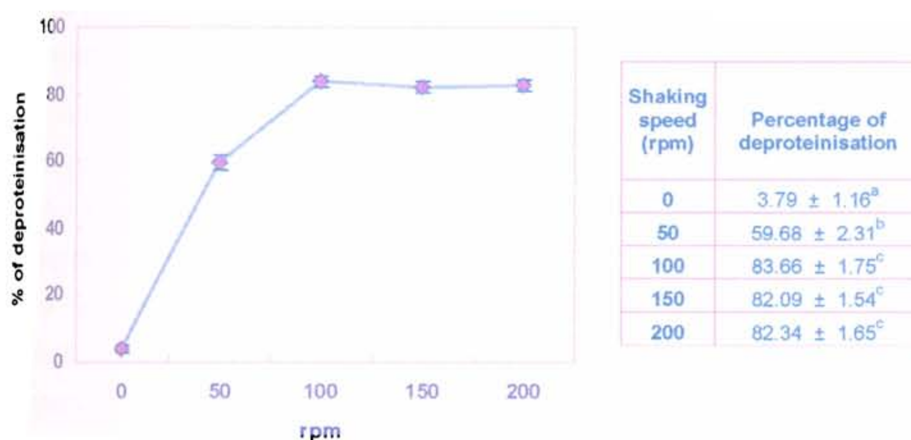


Temperature (°C)	Percentage of deproteinisation
25	67.39 ± 2.16^{d}
32	83.77 ± 3.05^{e}
40	45.75 ± 2.91^{c}
55	39.56 ± 4.56^{b}
60	28.93 ± 5.81^{a}

Fig.7.3 Effect of temperature on deproteinisation

7.3.4 Effect of shaking on the deproteinisation of prawn shell waste

There was a significant increase in deproteinisation when the reaction mixture was agitated than when it was kept stationary. The percentage of deproteinisation was maximal at the range of 100 to 200 rpm (Fig.7.4)



Shaking speed (rpm)	Percentage of deproteinisation
0	3.79 ± 1.16[a]
50	59.68 ± 2.31[b]
100	83.66 ± 1.75[c]
150	82.09 ± 1.54[c]
200	82.34 ± 1.65[c]

Fig.7.4 Effect of shaking speed on deproteinisation

7.4 Discussion

The use of proteolytic enzymes is a simple and inexpensive alternative to chemical methods currently employed in the preparation of chitin. It could be especially useful for the preservation of the natural state of this polymer. Optimisation studies were carried out to determine the reaction conditions in which the enzymatic deproteinisation was as complete as possible, without using excess enzymes.

7.4.1 Effect of incubation period on the deproteinisation of prawn shell waste

The demineralised shell waste has to be incubated with the crude enzyme long enough to react and deproteinise. A lower incubation time may limit the extent of deproteinisation and higher incubation periods would not increase the output beyond the maximum. The efficiency of the technology lies in locating the minimum time required for maximum deproteinisation. In the present study, maximum deproteinisation was obtained after 96 h of incubation. The degree of deproteinisation showed a progressive increase

from 24 h reaching a maximum after 96 h and further incubation did not contribute any increase towards deproteinisation.

7.4.2 Effect of demineralised waste to enzyme ratio on the deproteinisation of prawn shell waste

For effective utilisation of the deproteinisation process, it is necessary to find out the saturation limit of the enzyme with the substrate. In the current study, it was found that greater volumes of enzyme increased the deproteinisation until a demineralised waste to enzyme ratio of 1: 20 beyond which there was hardly any increase in the yield. The study proves that a volume of 20ml of the crude enzyme extract is required to deproteinise 1g of demineralised waste utilising the maximum potential of the hydrolytic activity of the enzyme.

In a similar study, Gagne and Simpson (1993) observed the influence of enzyme to waste ratio on deproteinisation of shrimp wastes using crude powder of chymotrypsin and papain using response surface analysis and found that at sub-optimum levels of enzyme, the response obtained is proportional to the amount of enzyme used. At enzyme levels closer to the optimal level, the response is no longer proportional to the amount of enzyme added, as this is approaching the saturation limit of the enzyme with the substrate. An enzyme to waste ratio of 7: 1000 and 10:1000 (w: w) was found to be optimum for obtaining highest levels of deproteinisation using chymotrypsin and papain respectively.

7.4.3 Effect of temperature on the deproteinisation of prawn shell waste

As in any other enzymatic reaction, temperature is an important factor which can influence the rate of the reaction. The reaction mixture of demineralised waste and crude enzyme should be incubated at a favourable temperature for successful deproteinisation. In the present case, deproteinisation occurred at all temperatures selected for study but it was

found that comparatively lower temperatures favoured deproteinisation, the optimum being 32°C. The percentage of deproteinisation declined sharply when the temperature of incubation was higher. This is in contradiction to the high thermostability of the enzyme. The protease under study was proved to be active over a temperature range of 30°C to 80°C, the optimum being 75°C. The stability studies of this enzyme revealed that it was absolutely stable upto 50°C (Venugopal, 2004). The nature of the substrate must be the reason for lower product yields at higher temperatures, prawn shell waste being a tough substrate for the enzyme to act upon. Broussignac (1968) demonstrated production of chitin involving a deproteinisation step using tuna proteinase at an optimum temperature of 37.5°C, papain at 37.5°C and a bacterial proteinase at 60°C.

7.4.4 Effect of shaking on the deproteinisation of prawn shell waste

Agitation of the reaction mixture is a critical factor in the deproteinisation step of chitin recovery because this is the means by which the enzyme molecules are homogenously mixed with the substrate. The reaction mixture was shaken at various rotating speeds to optimise the favourable speed for maximum deproteinisation. It was observed that there was a significant increase in deproteinisation when the reaction mixture was agitated than when it was kept stationary. The percentage of deproteinisation was maximal at the range of 100 to 200 rpm. On agitation, more molecules of the substrate might have exposed to enzyme action which would explain the above observation.

Deproteinisation of prawn shell waste using crude protease is a simple but viable alternative to the harsh, time-consuming and undesirable product yielding chemical treatments, for the recovery of chitin.

8

Summary

This thesis presents a detailed account of a cost – effective approach towards enhanced production of alkaline protease at profitable levels using different fermentation designs employing cheap agro-industrial residues. It involves the optimisation of process parameters for the production of a thermostable alkaline protease by *Vibrio* sp. V26 under solid state, submerged and biphasic fermentations, production of the enzyme using cell immobilisation technology and the application of the crude enzyme on the deproteinisation of crustacean waste.

The **important findings of the study are :-**

- *The bacterial strain, Vibrio sp. V26 has been successfully exploited* under SSF, SmF and biphasic fermentation systems
- The protease producing property of *Vibrio* sp. V26 was found to be non plasmid coded

- The fermentation process parameters for maximum production of alkaline protease by *Vibrio* sp. V26 under SSF, SmF and biphasic fermentations were optimised which demonstrated the effect of process parameters on the gross yield of enzyme as well as their independent nature in influencing the organism's ability to synthesise the enzyme
- Wheat bran with an average particle size < 425µm was found to be an excellent substrate for alkaline protease production by *Vibrio* sp. V26 under both SSF and SmF
- The optimum conditions for maximum enzyme production under SSF were :
  - 96 h of incubation
  - 80% (w/v) initial moisture content
  - 20% (v/w) inoculum size
  - Temperature 30^{0}C
  - Substrate weight to flask volume ratio, 1: 50
  - 1% (w/w) NaCl
  - *Maltose as supplementary carbon source*
  - Corn steep liquor as supplementary nitrogen source
- Extraction parameters of enzyme recovery from fermented mass in SSF were also optimised :
  - Carbonate-bicarbonate buffer was found to be the suitable extractant for maximum enzyme recovery from the fermented mass
  - A volume of 30 ml of buffer for 5g fermented biomass was optimum for enzyme extraction

    - Repeated extractions did not contribute much to enzyme recovery and a single wash was enough to serve the purpose

- Optimum conditions for maximum enzyme production under SmF were
    - 48 h of incubation
    - 80% (v/w) inoculum size
    - Temperature 30°C
    - 10% w/v solid substrate submerged in the medium
    - 0.2% (w/w) NaCl
    - Maltose as supplementary carbon source (10%,w/w)
    - Corn steep liquor as supplementary nitrogen source
- The optimum conditions for protease production under biphasic system were
    - Incubation period of 96 h
    - 50 ml of solid medium overlaid with 12.5 ml of liquid
    - Static conditions of incubation
    - 2% of agar in the solid phase medium
- Though the same microorganism *Vibrio* sp. V26 had been adopted for all the three fermentations, the product yield and time showed significant differences from one another whereas nutritional and temperature preferences did not demonstrate much variation
- In SmF, the protease yield was enhanced upto 4.34 fold (272.88 PU/g) compared to SSF

- In the biphasic system, there was a great increase in protease production, upto 19.35 fold (1216.73 PU/g) compared to SSF and 4.46 fold compared to SmF
- *Vibrio* sp. V26 cells were successfully entrapped and immobilised in Ca alginate gel beads for the production of alkaline protease
- Enzyme production by immobilised cells were found superior to that of free cells with an increase upto 2.3 fold
- The gel beads with entrapped cells were found to have very good operational stability with a half life of 9.5 reaction cycles and a shelf life of 4 weeks
- The optimum conditions of immobilisation for maximum enzyme production were found to be
  - 2.5% (w/v) alginate concentration
  - 10% (w/v) initial cell loading
  - 5% (v/v) bead inoculum
- Crude enzyme extract of alkaline protease produced by *Vibrio* sp. V26 was effectively used for the deproteinisation of prawn shell waste for chitin recovery
- A maximum of 83% of deproteinisation was achieved under optimal conditions, the optimum conditions being,
  - 96 h incubation,
  - demineralised waste to enzyme ratio of 1:20,
  - temperature 30^{0}C
  - agitation speed of the reaction mixture at 100 – 200 rpm.

The present investigation suggests an economic move towards improved production of alkaline protease at gainful altitudes employing different fermentation designs utilising inexpensive agro-industrial residues. Moreover, the use of agro-industrial and other solid waste substrates for fermentation helps to provide a substitute in conserving the already dwindling global energy resources. Another alternative for accomplishing economically feasible production is by the use of immobilisation technique. This method avoids the wasteful expense of continually growing microorganisms. The high protease producing potential of the organism under study ascertains their exploitation in the utilisation and management of wastes. However, strain improvement studies for the production of high yielding variants using mutagens or by gene transfer are required before recommending them to industries.

Industries, all over the world, have made several attempts to exploit the microbial diversity of this planet. For sustainable development, it is essential to discover, develop and defend this natural prosperity. The industrial development of any country is critically dependent on the intellectual and financial investment in this area. The need of the hour is to harness the beneficial uses of microbes for maximum utilisation of natural resources and technological yields. Owing to the multitude of applications in a variety of industrial sectors, there has always been an increasing demand for novel producers and resources of alkaline proteases as well as for innovative methods of production at a commercial altitude. This investigation forms a humble endeavour towards this perspective and bequeaths hope and inspiration for inventions to follow.

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* Not referred in original

Appendix

Type of fermentation	Yield (PU/g)
SSF	62.88 ± 0.408
SmF	272.88 ± 1.17
Biphasic	1216.73 ± 3.44

Table 5.1 Comparison of the maximum protease yield under solid state, submerged and biphasic fermentations

Time	PU/g		
	SSF	SmF	Biphasic
24	4.34 ± 0.061	192.49 ± 7.13	704.40 ± 7.41
48	9.17 ± 0.108	231.59 ± 1.46	816.86 ± 6.61
72	19.37 ± 0.141	214.35 ± 5.27	1057.59 ± 7.80
96	28.38 ± 0.263	199.41 ± 7.70	1220.38 ± 7.02
120	20.79 ± 0.186	186.46 ± 11.00	823.07 ± 8.45

Table 5.2 Comparison of the time impended for maximum protease yield under solid state, submerged and biphasic fermentations

Substrates	PU/g	
	SSF	SmF
W bran	21.35 ± 0.86	231.49 ± 1.43
R bran	0.35 ± 0.03	198.28 ± 12.21
Rawa	13.20 ± 0.06	49.39 ± 0.35
M bran	12.34 ± 0.10	54.57 ± 1.04
Barley	7.35 ± 0.25	32.49 ± 1.33
Ragi	1.96 ± 0.38	218.37 ± 5.94
Bread	1.63 ± 0.09	52.97 ± 1.31
Cassava	5.09 ± 0.20	45.87 ± 1.03
B gram	5.39 ± 0.40	103.75 ± 4.55
Soya	6.10 ± 0.47	52.59 ± 0.53

Table 5.3 Comparison of choice of substrates by *Vibrio* sp. V 26 for maximum protease production under solid state and submerged fermentations

Particlesize (µm)	PU/g	
	SSF	SmF
< 425	40.690 ± 0.270	251.9 ± 1.08
425-600	28.660 ± 0.180	187.97 ± 0.61
600-1000	24.380 ± 0.410	178.03 ± 1.07
1000-1425	21.430 ± 0.420	137.87 ± 1.50

Table 5.4 Comparison of choice of particle size of the substrate by *Vibrio* sp. V26 for maximum protease production under solid state and submerged fermentations

Carbon sources	PU/g					
	SSF			SmF		
Glucose	11.79	±	0.120	49.36	±	0.18
Sucrose	41.54	±	0.090	260.13	±	0.64
Maltose	45.01	±	0.110	268.34	±	0.89
Lactose	40.45	±	0.140	259.53	±	1.33
Glycerol	37.20	±	0.130	254.14	±	1.09
Starch	38.57	±	0.052	257.64	±	0.50
Mannose	43.38	±	0.140	262.31	±	0.77
Molasses	44.59	±	0.140	264.65	±	0.67

Table 5.5 Comparison of choice of supplementary carbon sources by *Vibrio* sp. V26 for maximum protease production under solid state and submerged fermentations

Nitrogen sources	PU/g					
	SSF			SmF		
B.extract	50.41	±	0.057	268.11	±	0.06
Casein	50.38	±	0.109	268.36	±	0.12
Gelatin	49.07	±	0.186	267.19	±	2.21
Peptone	47.52	±	0.266	263.79	±	0.24
Tryptone	46.34	±	0.148	262.56	±	1.96
Y.extract	47.87	±	0.544	265.48	±	0.41
C stp. liqr	54.98	±	0.191	272.7	±	0.79
NaNO3	12.42	±	0.275	121.57	±	0.56
NH4Cl	12.28	±	0.403	121.72	±	0.66
(NH4)2SO4	12.34	±	0.202	121.36	±	0.51
NH4NO3	12.53	±	0.635	121.59	±	0.51
Urea	12.29	±	0.252	121.65	±	0.79

Table 5.6 Comparison of choice of supplementary nitrogen sources by *Vibrio* sp. V26 for maximum protease production under solid state and submerged fermentations

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Temperature (°C)	PU/g	
	SSF	SmF
20	28.272 ± 0.249	189.77 ± 3.18
25	28.566 ± 0.101	193.31 ± 3.31
30	40.659 ± 0.269	232.54 ± 1.05
37	25.724 ± 0.080	229.21 ± 1.44
45	2.970 ± 0.079	70.84 ± 0.81

Table 5.7 Comparison of optimum temperature required by *Vibrio* sp. V26 for maximum protease production under solid state and submerged fermentations