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Scale up production of Protease using *Pseudomonas aeruginosa* MCM B-327 and its Detergent Compatibility

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Abstract

The maximum protease activity was obtained from *P. aeruginosa* MCM B-327 with soybean meal 1%, tryptone 1%, initial medium pH 7, agitation rate 250 rpm, aeration rate 0.75 vvm and fermentation temperature 30 °C, under submerged fermentation conditions (SmF). The protease productivity at 10 and 120L fermenters was found to be 16,021 and 9,975 $UL^{-1}h^{-1}$ respectively. Kinetics of cell growth revealed that specific cell growth rate was 0.025 h^{-1}. Protease was active and stable at different pH, temperatures, in anionic, cationic and non-ionic detergent additives, as well as in commercial detergents. The protease exhibited blood stains removing performance indicating its potential in detergent industry. The dried ammonium sulphate precipitated protease was stable at room temperature for a period of one year. The Protease has shown properties suitable for its application in detergents. The results contribute to basic knowledge and application of protease from *P.aeruginosa* to detergent industry. The studies will help to optimize the production of this protease for biotechnological applications.

Keywords: Protease, *Pseudomonas aeruginosa*, aeration, agitation, kinetics

Introduction

Proteases are hydrolytic enzymes catalyzing hydrolysis of proteins to amino acids. Proteases account for about 65% of the total industrial enzyme market (Oskouie *et al.* 2008). These proteases have wide-ranging applications in detergent, pharmaceutical, food, chemicals, degelatinization of photo films and leather industries (Gupta et al. 2002, Zambare *et al.* 2011a, 2011b). Most commonly used proteases, especially in detergent and leather industries, are alkaline proteases (especially in detergent and leather industries). Performance of protease in the detergent is influenced by factors such as pH, ionic strength, wash temperature and time, detergent composition, and mechanical handling. Both performance and stability of the enzyme are influenced by detergent, surfactants and bleach systems (Olsen, 1999). Protease enzymes boost the efficacy of surfactant and dispersant systems by degrading proteinaceous soils into smaller, more dispersible fragments, thereby directly facilitating the soil removal process. In addition, they decrease the redeposition of proteins, especially hydrophobic proteins such as those found in blood, resulting in improved overall whiteness (Showell 1999). These proteases were produced from plants, animals and microbes. Microbial proteases are mainly produced from bacteria, fungi and yeasts under either submerged fermentation or solid state fermentation (Rao et al. 1998). Among bacteria, the genus *Bacillus* is the most dominant reported for protease production (Maria et al. 2001, Nilegaonkar et al. 2007) followed by *Aspergillus* fungus (Samarntarn and Tanticharoen, 1999).

From an industrial point of view, extracellular production of enzymes is very important. The secretion of microbial enzymes in fermenter vessel depends on several factors, such as the composition of the culture medium, carbon and nitrogen source, mineral salts, trace elements, type of strain, and fermentation conditions such as pH, temperature, dissolved oxygen concentration, agitation etc. (Wang and Shih, 1999; Ocampo and Ezquerra, 2002). In aerobic fermentation, oxygen has an influence on enzyme secretion and this may be because of metabolic activities in the organism (Raimbault, 1998). The volumetric oxygen transfer to the organism is varied from species to species and is mainly dependent on agitation speed and aeration rate in the bioreactors (Shin and Kim, 1996). The agitation and aeration plays a significant role in the overall productivity of the fermentation process (Alves et al. 2010). However, very little attention has been paid to the role of aeration rate and agitation speed in the production of extracellular protease. Dhandapani et al. (2012) reported impact of aeration and agitation on protease secreted by *Aspergillus tamari* in fermenter. Scale up production of alkaline protease using *Conidiobolus coronatus* demonstrated in 150 L fermenter (Laxman et al 2005). Sivprakasam et al. (2007) reported scale up production of protease by *Pseudomonas aeruginosa* in bench scale fermenter. Saran et al.

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(2007) carried out protease production form *Bacillus* sp. in 30 L bioreactor. Kinetics data for the protease production using *Pseudomonas aeruginosa* were reported (Sivprakasam et al. 2008, Mahadevan et al. 2010).

We have reported a novel protease with non-collagenolytic and non-keratinolytic properties from *P. aeruginosa* MCM B-327, which differentiate it from other types of proteases like pseudolysin, staphylolysin, alkaline protease and lysine-specific endopeptidase, with applications in dehairing and degelatinization (Zambare *et al.* 2011a, 2011b, 2013). These results attracted us to study the scale up. Therefore, the aim of the present research work was to investigate the appropriate aeration and agitation rates for alkaline protease production in a stirred tank bioreactor from *P. aeruginosa* MCM B-327. The appropriate relationships among different agitation and aeration rates and protease production were also established for further scale up study. Furthermore, crude enzyme characterization and shelf life were studied for detergent compatibilities.

Materials and Methods

Chemicals

All chemicals and reagents used were of analytical grade. All substrates, like Nutrient broth, soybean meal and tryptone were purchased from HiMedia (Mumbai, India). Ammonium sulphate was purchased from Sd-fine Chemicals (Mumbai, India). Commercial detergents were purchased from the local market of Pune (India).

Microorganism and inoculum development

Pseudomonas aeruginosa producing protease was isolated from vermicompost pit soil, Pune (India). The stock cultures were maintained on Nutrient agar (NA) slants at 4ºC and as a glycerol stock at -20°C. *Pseudomonas aeruginosa* was initially grown on NA medium in a Petri dish and then transferred to the seed culture medium by inoculating a single colony with the help of nichrome wire-loop. For 10L and 120 L fermenters, the seed cultures of 700 mL and 10L were developed in nutrient broth at 30°C on a rotary shaker at 150 rpm for 18-21h.

Fermentations

The optimization of production of protease was carried out using production medium ST (1% soybean meal, 1% tryptone and pH 7.0) in 10L stirred-tank glass fermenter (Model 4 CR, B.E. Marubishi Co. Ltd. Tokyo, Japan) with technical specifications described in Table 1. The culture medium was inoculated with 10% (vv^{-1}) (approximately 10^9 cells mL^{-1}) of the seed culture. The influence of aeration rate and agitation speed in 10L fermenter were examined within the ranges from 0 to 250 rpm (with 0.75 vvm aeration) and 0.5 to 1.0 vvm (with 250 rpm agitation), respectively. Protease activity, cell growth, pH and protein content of the broth were determined after every 6 h. The batches were harvested immediately as the enzyme activity droped down from maximum value. The cell free enzyme was obtained by centrifugation (10,000 rpm). It was partially purified by ammonium sulphate precipitation at 60% saturation. The % yield of ammonium sulphate precipitated enzyme, on the basis of substrate utilized and productivity ($UL^{-1}h^{-1}$) of enzyme were calculated. The time course of protease production in the production medium in 10L fermenter was carried out (in production medium) with optimum agitation and aeration rates at 30°C.

Pilot scale production of protease

Pilot scale experiments were carried out in a 120 L SS fermenter (Biochem Engineering Pvt. Ltd. Pune, India). Technical details of the fermenter are given in Table 1. In geometrically similar vessels kinematic similarity was achieved by keeping identical values of impeller Reynolds No., Froude No., impeller tip speed and superficial velocity, to control the fluid motions and appropriate mixing of medium components for growth of organism and product formation in the fermenter. It is very difficult to maintain all these parameters constant while scaling-up of fermentation; therefore in present investigation, constant impeller tip speed and superficial air velocity were employed as a principal scale-up factor. Following equations could be employed for scale up in geometrically similar vessels with equal impeller Reynolds No. (N_{re}) (n=2, Eq. 1), equal Froude number (N_{Fr}) (n=0.5, Eq. 1), equal impeller tip speed (n=1, Eq. 1) and equal superficial air velocity (V_s) (Eq.2) (Treybal 1981).

$$\frac{N_1}{N_2} = \left(\frac{d_{i2}}{d_{i1}}\right)^n \quad (1)$$

$$V_s = (H_L / H_t) D(VVM) \quad (2)$$

Where, N_1-impeller tip speed for 10 L, N_2-impeller tip speed for 120 L, d_{i1}- impeller diameter of 10 L, d_{i2}-impeller diameter of 120 L, D-fermenter diameter, VVM-volumetric air flow rate/volume of media, H_L/H_t-Aspect ratio, H_L-liquid depth, H_t-fermenter height. The estimated values of aeration and agitation for pilot scale fermenter are given the Table 2, 3 and 4. Soybean and tryptone (ST) medium having pH 7 was inoculated with 10% (v/v) inoculum incubated at 30°C, with 66.1 rpm, 0.28 vvm. Protease production in the broth was determined after every 6 h. Enzyme was precipitated as described above.

Table 1: Technical details of bench and pilot scale fermenters

Sr. No.	Dimensions	Bench scale	Pilot scale
1	Total volume (L)	10	120
2	Working volume (L)	7	100
3	Impeller numbers	2	2
4	Impeller diameter d_i (cm)	7.4	
5	Tank height H_t, (cm)	37	16.8
6	Tank diameter D_t (cm)	18.5	42
7	H_t/D_t	2	2
8	d_i/D_t	0.4	0.4
9	Liquid height, H_L (cm)	26	70
10	Aspect ratio, H_L/H_t	0.70	0.833

Table 2 :Impeller tip speed for bench and pilot scale fermenters

For 10 L	
Agitation, rpm	Impeller tip speed, m/min
0	0
100	23.23
150	34.85
250	58.09
For 120 L	
66.1	34.85

Analytical method

Protease activity was measured using caseinolytic assay (Zambare *et al.* 2011a). The culture supernatant (1 mL) was

incubated in 4 mL of 0.625% casein at 35°C for 30 min. The reaction was stopped by the addition of 5 mL of trichloroacetic acid (5%) and the casein hydrolysis product was measured by modified Folin–Ciocalteu method, against inactive enzyme. A standard graph was generated using standard tyrosine of 10–50 µg mL^{-1}. One unit (U) of protease activity was defined as the amount of enzyme, which liberated 1 µg tyrosine per min at 35°C. Protein concentration was measured by the method of Biuret (Jayaraman *et al.* 2003) using BSA as the standard. The specific activities (Umg^{-1} protein) and productivities ($UL^{-1}h^{-1}$) were calculated accordingly. Hemoglobin hydrolysis assay was determined using the method described by Sarath *et al.* (1996).

Table 3: Superficial velocities for bench and pilot scale fermenters

For 10 L		
Aeration, vvm	Volumetric air flow rate, L/min	Superficial air velocity, cm/s
0.5	3.5	0.108
0.75	5.25	0.162
1	7	0.216
For 120 L		
0.28	28	0.162

Table 4: Estimated values of aeration and agitation for bench and pilot scale fermenters

For constant impeller Reynolds No.			
Fermenter, L	Agitation, rpm	Impeller diameter, cm	N_{re}
10	150	7.4	25636.70
120	29.1	16.8	25634.15
For constant Froude No.			
Fermenter, L	Agitation, rpm	Impeller diameter, cm	N_{Fr}
10	250	7.4	0.13
120	99.55	16.8	0.13
For constant impeller tip speed			
Fermenter, L	Agitation, rpm	Impeller diameter, cm	Impeller tip speed, cm/s
10	150	7.4	34.85
120	66.1	16.8	34.85

Five mL haemoglobin substrate (2%) was incubated with 1 ml of enzyme solution at 35°C for 20 min. The reaction was stopped by addition of 10 mL 5% trichloroacetic acid and kept for 10 min at the same temperature. The assay mixture was centrifuged and the supernatant was read at 280nm against inactive enzyme as blank. One haemoglobin hydrolysis unit (HHU) was defined as the amount of enzyme, which increases the absorbance at 280nm under assay conditions.

Protease properties and detergent compatibility

The ammonium sulphate precipitated enzyme of *P. aeruginosa* MCM B-327 was used for protease characterization. The effect of pH on enzyme activity and stability (30 min and 3h) was studied under different pH values in the range of 5–12 (pH 5-6, 50 mM acetate buffer; pH 7–8, 50 mM phosphate buffer; pH 9–10, 50 mMTris–HCl buffer; pH 11–12, glycine–NaOH buffer at 35°C). The effect of temperature on enzyme activity and stability (30 min and 3h) was studied in the range of 25–80°C with 50 mM phosphate buffer (pH 8) conditions.

The effects of detergent additives [1%, sodium dodecyl sulphate (SDS), sodium tripolyphosphate, sodium tetraborate, Tween 80 and Triton X100] and commercial detergents [1%, Nirma® (Nirma Chemicals, India), Surf excel® and Rin Shakti® (Hindustan Liver Ltd, India), Tide® and Ariel® (Procter and Gamble, India)] on the enzyme activity were studied at 35°C for 30 min. The residual activity was assayed against enzyme control after 30 min at 35°C (Kumar and Bhalla, 2004). Hemoglobin hydrolysis was determined using the method described above.

Blood spot and washing performance of protease

A cotton cloth piece (3"x 3" size) was stained with human blood and was fixed by (with) ironing it. The blood spots were treated with water, 1% commercial detergents (Nirma® and Surf excel®), crude enzyme (100U or 20 HHU) and 1% commercial detergents with crude enzyme (100U) for 30 min at room temperature. Afterwards the cotton cloths were washed with tap water, air dried and compared for the washing performance by visual observation (Vijayalakshmi *et al.,* 2011).

Storage stability

The precipitated enzyme was used for stability studies. The dried powder of ammonium sulphate precipitated enzyme was stored at three different temperatures, viz. room temperature (28 ± 2°C), 4°C and -20°C. Samples from these stocks were withdrawn to test its storage stability.

Statistical analysis

Each experiment was carried out independently and the analysis presented here is the mean of triplicates and the standard deviation (SD) was calculated by using Microsoft Excel Program.

Results

Protease production at 10 and 120L fermenters was carried out in ST medium using *P. aeruginosa* having generation time of 153.50 min and 21h age of inoculums.

Optimization of Protease production at 10L fermenter

Effect of aeration

The critical effect of aeration rate on protease production and growth of *P. aeruginosa* is shown in Fig. 1. A high level of protease activity (735.73 U ml^{-1}) at 48 h and growth of *P. aeruginosa* (5.3 x 10^9 CFU ml^{-1}) at 6 h were observed at the moderate aeration rate (0.75 vvm) (Fig. 1A,B). Increase in aeration resulted in decrease in enzyme activity above and below 0.75 vvm. The maximum specific activity of protease was 1016.73U mg $protein^{-1}$ with 0.75 vvm and it was decreased to 709.52 and 559.22U mg $protein^{-1}$ for 0.5 and 1 vvm respectively. The maximum cell density of *P. aeruginosa* was observed with 0.75vvm aeration (Fig. 1B). The pH of medium increased from 7.0 to 8.6. As the pH reached a value of 8.6 (Fig. 1D) the protein content of the cell broth decreased (Fig. 1C) which may be because of hydrolysis of proteins by the protease secreted from the culture. Table 2 shows specific activity of protease as 1017 U mg^{-1} with 0.75 vvm supply of air and its decrease to 710 Umg^{-1} and 559 Umg^{-1} for 0.5 vvm and 1 vvm respectively.

Effect of agitation

The submerged fermentations were studied under different agitation speeds (0 to 250 rpm). At 250 rpm, the maximum protease production (769 U ml^{-1}) at 48 h and growth of *P. aeruginosa* (5.3 x 10^9 CFU ml^{-1}) at 6 h were observed

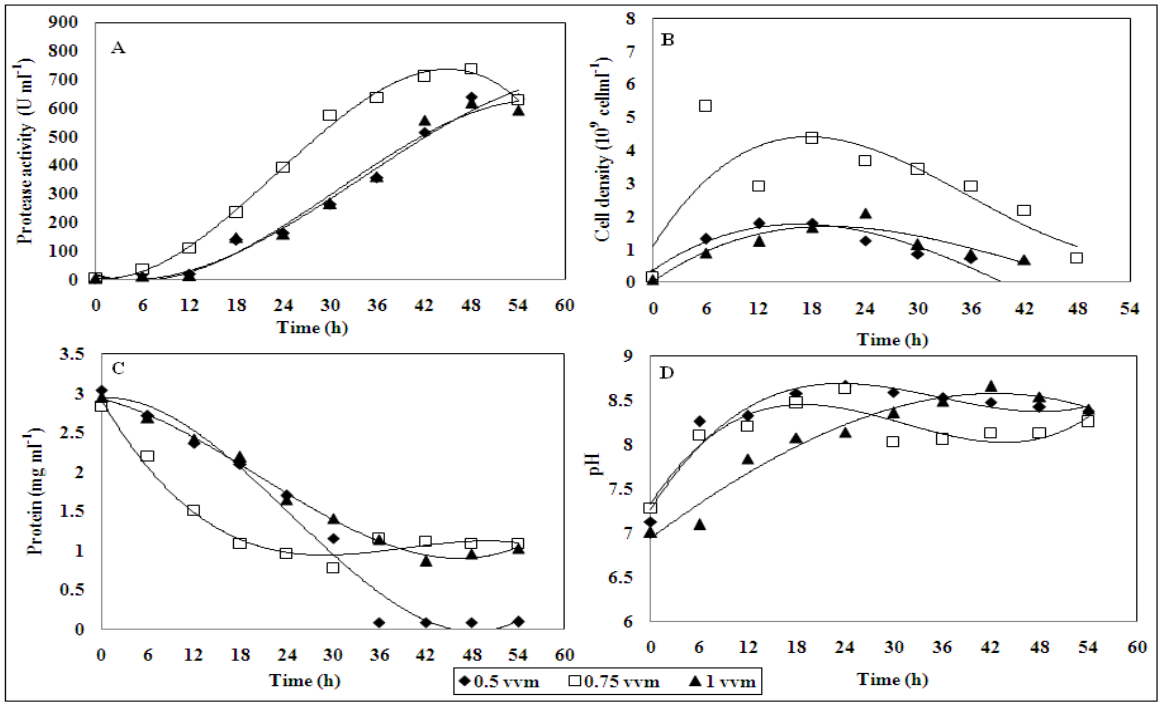

Figure 1: Optimization of aeration flow rate on protease production from *P. aeruginosa* (A), cell density (B), protein (C), pH (D) in 10L fermenter, ST medium (pH 7), 150 rpm aeration at 30°C. For protease activity, error bars represent the mean of triplicate analysis ± standard deviation.

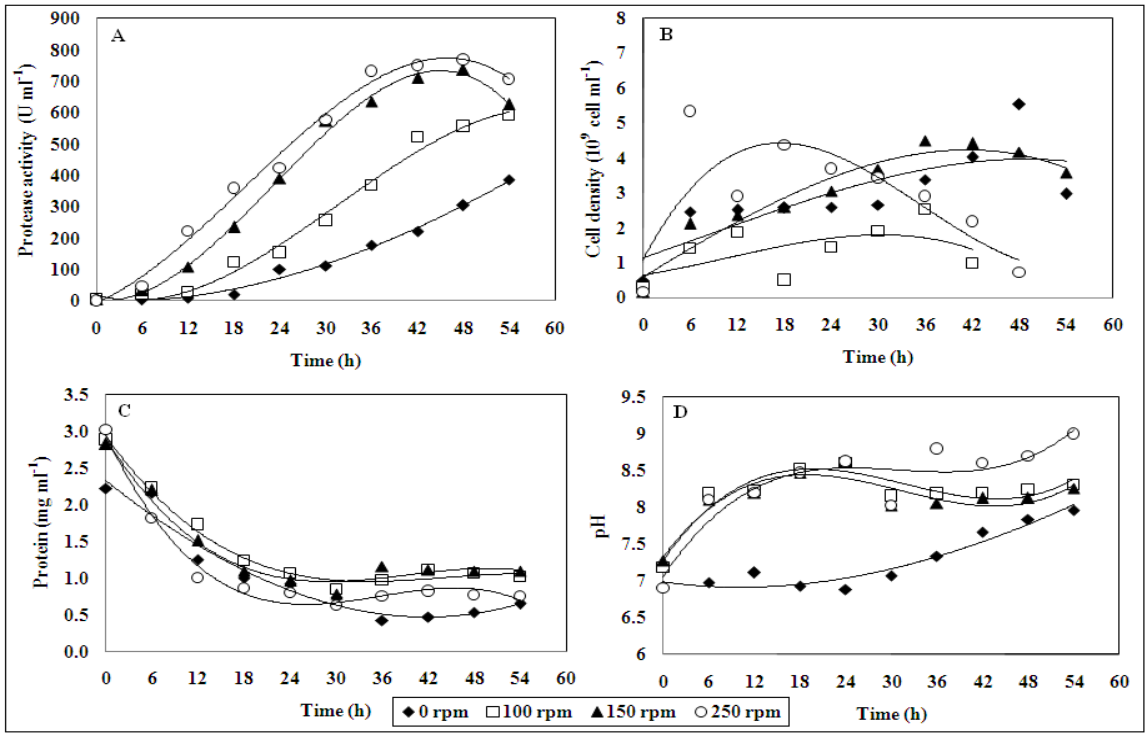

Figure 2: Optimization of agitation speed on protease production from *P. aeruginosa* (A), cell density (B), protein (C), pH (D) in 10L fermenter, ST medium (pH 7), 0.75 vvm aeration at 30°C. For protease activity, error bars represent the mean of triplicate analysis ± standard deviation.

(Fig. 2A,B). Fig. 2B shows higher growth at a higher agitation rate of 250 rpm and then the growth decreased with increased protease production. At zero agitation rate, the log phase was prolonged and afterwards it entered into decline phase. The pH of the medium increased from 7.0 to 8.6. The pH change pattern was the same for all agitation rates except for 0 rpm. In the absence of agitation the increase in pH was slow. This may be due to the poor cell growth resulting into slow protein hydrolysis (Fig. 2C). Decrease in rotein content was observed with different agitation rates (Fig. 2D).

Table 5 shows that the specific activity 1383 U mg^{-1} protein was maximum at 150 rpm with yield 43.5% and productivity 15,328 U $L^{-1}h^{-1}$. Thus the optimum agitation rate, 150 rpm was used for further scale up in 120 L fermenter.

Table 5 Effect of agitation and aeration on productivity of protease from *P. aeruginosa* MCM B-327 in 10 L fermenter

Parameters	Harvesting Time (h)	Yield (%)	Specific activity U/mg protein	Productivity U $L^{-1}h^{-1}$
Aeration (vvm)				
0.5 vvm	48	67	710	13,323
0.75 vvm	48	77	1017	16,021
1 vvm	54	54	559	11,447
Agitation (rpm)				
0	72	15	457	8,635
100	54	41.5	686	10,951
150	48	43.5	1383	15,328
250	48	77	1017	16,021

Time course of protease production in 10L fermenter was carried out under optimum conditions. Maximum protease activity was 779.02 UmL^{-1} at 48 h (Fig. 3). The initial pH was 6.8-7.0, which increased to 8.2-8.6.

The cell growth pattern showed lag phase up to 6h, log phase from 6 to 36h, stationary phase from 36 to 42h and decline phase from 42 to 60h. The maximum protease production was observed in decline phase of cell growth. The protein content decreased from 6 to 30 h i.e. in log phase of cell growth. This decrease in protein was because of fast hydrolysis of the substrate. The protein content again increased up to 36h and remained constant. This decrease and increase in protein content after 30h was because of decrease of substrate concentration and increase of protease enzyme in the broth. The change in pH of the fermented broth may be because of the release of amino acids, peptides or ammonia.

Protease scale up

The pilot scale production of protease at 120L fermenter was done in ST medium. The maximum protease activity was 599 UmL^{-1} at the end of 60h (Fig. 4). The yield, specific activity and productivity of protease in 120L fermenter was 50%, 463.95 U mg^{-1} protein and 9975 $UL^{-1}h^{-1}$ respectively.

Protease production and cell growth kinetics at different aeration and agitation rates

Protease formation kinetics was carried out at different aeration rates (0.5, 0.75 and 1.0 vvm) and agitation 150 rpm. Maximum protease formation rates of 25.55 (42 h), 30.75(30 h) and 33.88 U/ml/h (48 h) were observed at 0.5, 0.75 and 1.0 vvm respectively.

Rate of protease formation varied in the range 0.46 to 25.55, 2.26 to 30.57 and 0.45 to 33.88 U/ml/h at aeration 0.5, 0.75 and 1.0 vvm respectively (Fig 5 a). Negative protease formation rates were observed (-5.98 U/ml/h, -14.719 U/ml/h and - 4.81 U/ml/h) for 0.5 , 0.75 and 1.0 vvm respectively at 54 h.

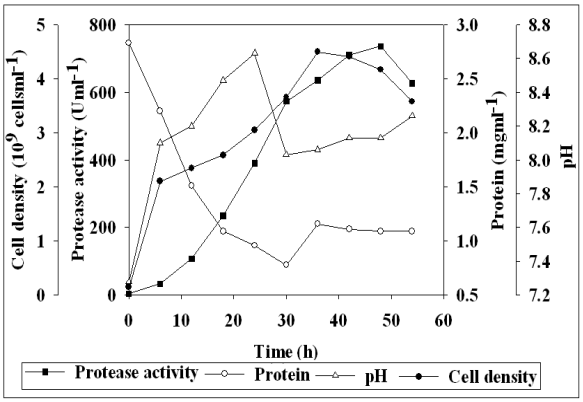

Figure 3: Time course of protease production in 10L fermenter (ST medium, pH 7, 0.75 vvm aeration, 150 rpm at 30°C). For protease activity, error bars represent the mean of triplicate analysis ± standard deviation.

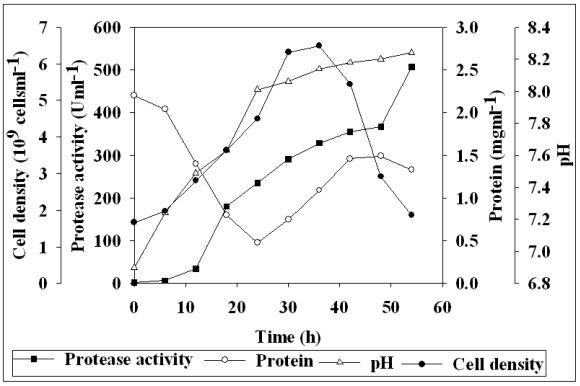

Figure 4: Protease productions at pilot scale, 120L fermenter (ST medium, pH 7, 0.28 vvm aeration, 66.1 rpm at 30°C). For protease activity, error bars represent the mean of triplicate analysis ± standard deviation.

Negative rate of protease formation may be due to initiation of the death phase of growth cycle. Cell growth rate varied in the range of 0.03 to1.7 x 10^8, 2.4 to 8.6 x 10^8 and 0.5 to 1.3 x10^8, 2.4 to 8.6 x 10^8 cells/ml/h at 0, 0.5, 0.75 and 1.0 vvm respectively. Net cell growth rate was zero during 12-36 h of fermentation. Afterwards cells entered into the death phase of growth cycle (Fig 5 b).

The protease formation kinetics were carried out at different agitation rates (0, 100, 150 and 250 rpm) and aeration 0.75 vvm. Maximum rates of protease formation of 16.31 (24 h), 26.32(42 h), 30.57 (30 h) and 33.06 U/ml/h (36 h) were observed at 0, 100,150 and 250 rpm respectively. Rate of protease formation varied in the range 0 to 16.31, 0.92 to 26.33, 2.26 to 30.57 and 1.81 to 33.06 U/ml/h for 0, 100, 150 and 250 rpm respectively (Fig 5 c). Negative rate of protease formation was observed after 72h (-4. 63 U/ml/h), 48 h (-14.72 U/ml/h) and 48h (-9.28 U/ml/h) for 0, 150 and 250 rpm respectively but was not observed till 54h at 100 rpm.

Cell growth rate varied in the range of 0.36 to 3.3 x 10^8 , 0.76 to 1.9 x 10^8, 0.4 to1.8 x 10^8, 2.4 to 8.6 x 10^8 cells/ml/h at 0, 100, 150 and 250 rpm respectively (Fig 5 d).

Protease production and cell growth kinetics in 120 L fermenter

Scale up protease production was carried out in 120 L SS fermenter at aeration 0.28 vvm and agitation 66.1 rpm. Protease formation rate varied in the range 0.9-18.79 U/ml/h (Fig 5 e). Negative rate of protease formation was observed at 48 h (-3.62 U/ml/h) (Fig 5 f). Cell growth rate varied in the range of 0.2 to 3 x 10^8 at aeration 0.28 vvm and agitation 66.1 rpm. Negative rate of protease formation is the crucial point where cells enter into the death phase of growth cycle.

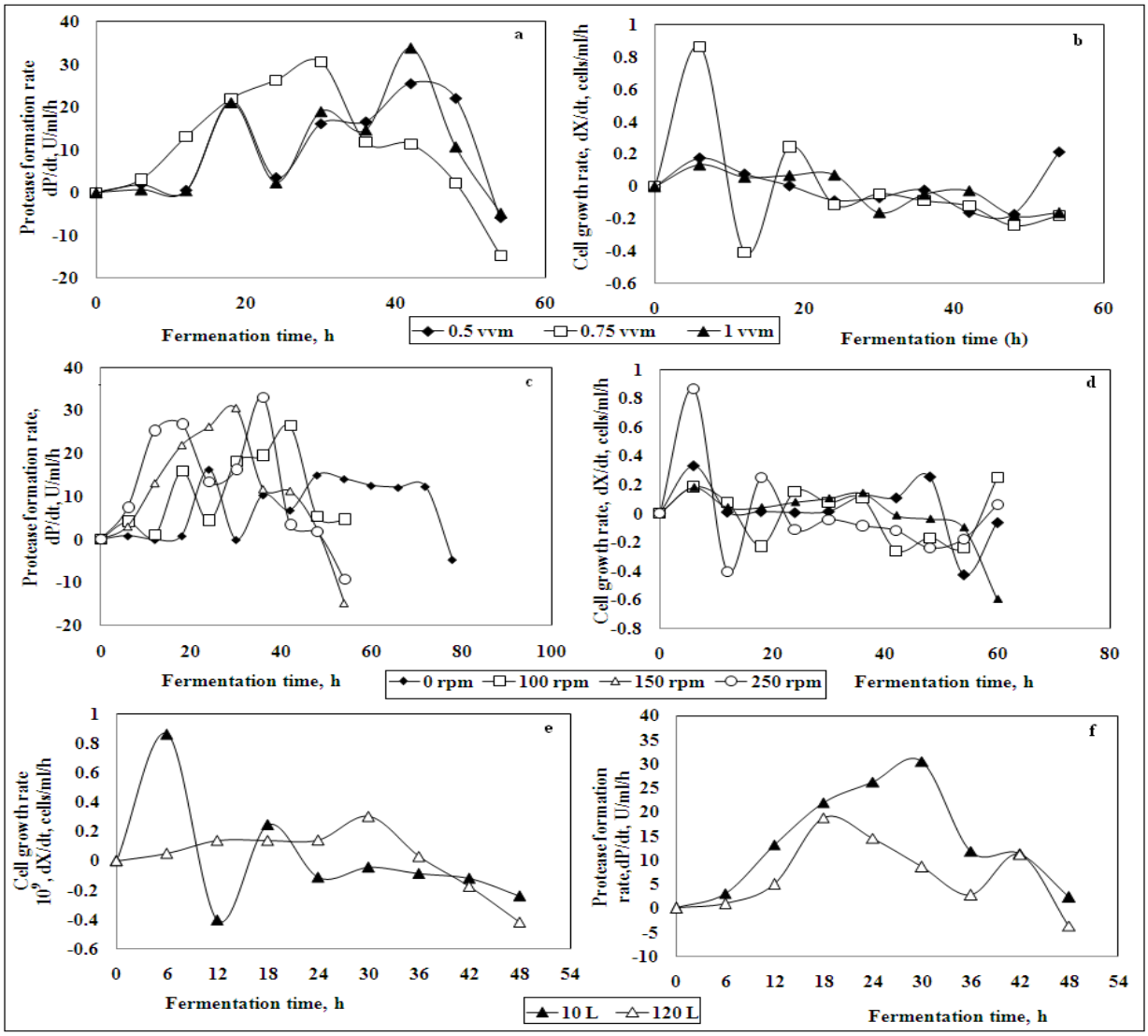

Figure 5: Kinetics of protease formation rate and cell growth rate at different aeration (a, b), agitation (c, d) and on scale up in 120 L fermenter (e, f)

Properties of Protease

Protease from *P. aeruginosa* MCM B-327 was active in pH range 5-12 with optimum pH of 8.0 and stable in pH range of 6-10. The enzyme retained 85 and 70% of its activity at 35°C, for pH 9.0 and 10.0, respectively, up to 3h (Fig. 6). Likewise, protease from *B. licheniformis* MZK03 remained stable over a wide pH range (6 to 11) with an optimum pH of 8.5 (Sayem *et al.* 2006).

The protease was active in temperature range 25-80°C and optimum at 35°C and was stable up to 45°C followed by a rapid loss of activity after 55°C. The enzyme retained more than 75% and 50% activity at 55°C for 30 min and 3 h respectively. However, the enzyme was completely inactivated at 80°C (Fig. 7).

The enzyme was stable in all detergent ingredients under study except SDS (20% inhibition). Surfactants like Tween 80 and Triton X100 had no inhibitory effect on protease activity (Table 6). Therefore, marginal loss of protease activity in some of the laundry detergents may be attributed to inhibitory effect of component of these detergents. In contrast, some of the components of the detergent, for example Tween 80 and sodium tripolyphosphate have a slight stimulatory effect on activity of *P. aeruginosa* protease.

Using the standard reaction mixture, the proteolytic activity was monitored in 50 mm Tris-HCl buffer, pH 8 at different

temperatures eg.from 25–80°C for 30 min and 3h. Error bars represent the mean of triplicate analysis ± standard deviation.

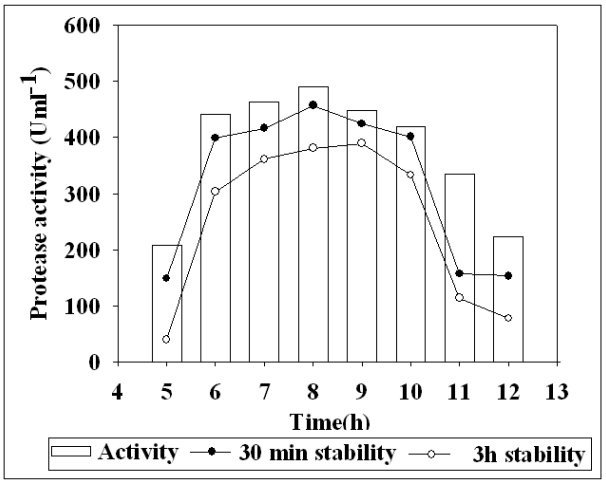

Figure 6: Effect of pH on protease activity and stability. Using the standard reaction mixture, the proteolytic activity was monitored at 35°C in 50 mm buffers of different pH 5-12) for 30 min and 3h. Error bars represent the mean of triplicate analysis ± standard deviation.

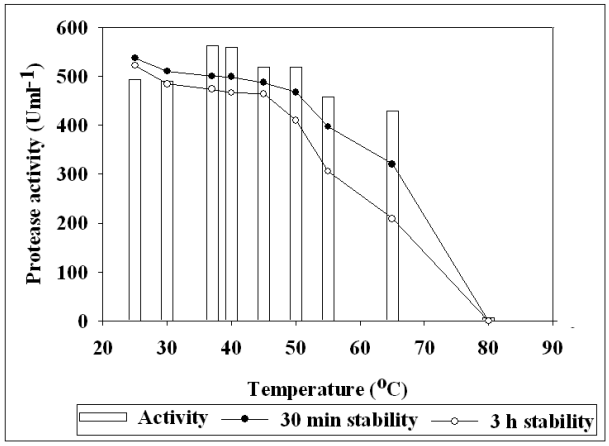

Figure 7: Effect of temperature on protease activity and stability.

SDS-Sodium dodecyl sulphate, the proteolytic activity was monitored at 35°C in 50 mm Tris-HCl buffers pH 7 for 30 min incubation.

The enzyme showed 69-92% activity after 30 min in commercial detergents (Table 6). The protease from *P. aeruginosa* showed similarly stability in commercial detergents as Tide®< Ariel®< Nirma®< Rin Shakti®< Surf excel®.

Protease from *P. aeruginosa* showed efficient removal of bloodstain from cotton fabric with Surf excel® and Nirma® separately. It required 30 min to remove the bloodstain from cotton cloth with only detergents at room temperature. However, protease assisted detergent removed blood stain within 10 min. The removal of bloodstain from cloth with different washing agents is shown in Fig. 8.

Storage stability

In our study, the enzyme (dried ammonium sulphate precipitate) was 100% stable up to 12 months when stored at room temperature (28 ± 2°C), 4°C and -20°C (data not shown).

Table 6: Effect of detergent additives and commercial detergents on protease activity

Additives (1%)	Residual protease activity (%)
Detergent additives	
None	100
SDS	80
Sodium tripolyphosphate	103
Sodium tetraborate	98
Tween 80	102
Triton X100	97
Commercial detergents	
Tide®	69
Ariel®	72
Nirma®	74
Rin Shakti®	85
Surf excel®	92

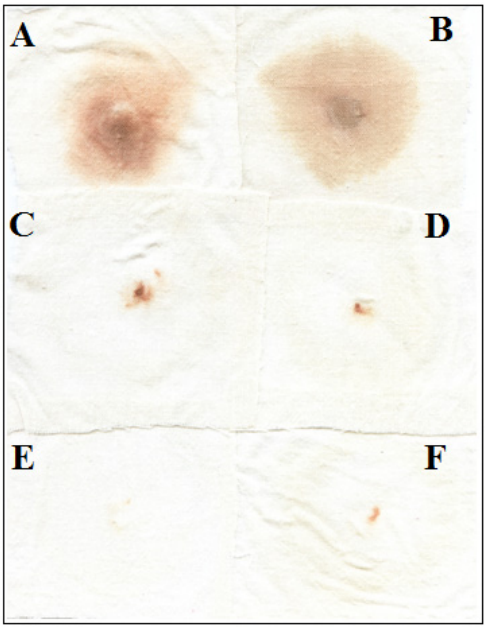

Figure 8: Blood stain washing performance of *P. aeruginos*a protease with and without detergents. A-control blood spot washed with tap water, B- blood spot washed with crude protease, C- blood spot washed with Surf Excel®, D- blood spot washed with Nirma®, E- blood spot washed with Surf Excel® with crude protease, F- blood spot washed with Nirma® with crude protease.

Discussion

For the fermentation process, bacterial inoculums developed in different ways had influence on enzyme production. Anstrup (1974) described a two-step inoculum development program for the production of protease by *B. subtilis*. Inoculum for seed fermenter was grown for 1 to 2 days on a solid or liquid medium and then transferred to a seed vessel where the organism was allowed to grow for a further 10 generations before transfer to the production stage. Humphrey, (1998) reported that, on scale up, culture age increases and it could undergo as many as 25-50 generations, from the frozen inoculum stage. Thus, based on generation time and age of inoculums, 8-9 generations *P. aeruginosa* were used in fermentation studies.

Several investigators have reported similar trends during protease production in batch fermentations (Nadeem *et al.* 2009; Irfan *et al.* 2010). The maximum protease yield of 77% and productivity 16,021 $UL^{-1}h^{-1}$ was obtained with 0.75 vvm. Jonsson (1967) reported that the maximal productivity of protease from *Alternaria tenuissima* was 0.057 $UL^{-1}h^{-1}$ at aeration of 0.5 LPM. Similarly, *Bacillus firmus* exhibited maximum enzyme yields at an aeration rate of 7 LPM and an agitation rate of 360 rpm (Moon and Parulekar, 1991). However, these results indicated that by lowering the aeration rate and agitation speed there was a reduction in the protease yields. A report showed increased protease production by *Bacillus* sp. at agitation speed of 600 rpm and aeration rate of 0.5 vvm (Fujiwara and Yamamoto, 1987). The protease activity declined after 48 h. The reason for decrease of activity might be deactivation or self-digestion of the enzyme (Samarntarn and Tanticharoen, 1999). Laxman et al. (2005) carried out fermentation with aeration of 0.5 vvm and agitation of 400-500 rpm. Hameed et al. (1999) also reported that the culture pH was 6 on inoculation, dropping to 5.3 after 12 h before rising steadily to ~8 at the end of fermentation. The optimal conditions reported by them for protease production by *Bacillus subtilis* K2 in 20 L fermenter were uncontrolled pH and agitation rates between 300 and 500 rpm, with activity reaching maximum in 60 h. From the literature, it is seen that less time is required for protease production by *P. aeruginosa*. Pastor et al. (2001) observed highest protease yield from *Bacillus subtilis* 3411 with the agitation speed of 750 rpm and aeration rate of 1 LPM. Samarntarn and Tanticharoen (1999) worked on alkaline protease production in 5L fermenter using genetically modified *Aspergillus oryzae* at aeration 0.5vvm and agitation of 5000 rpm. Some studies reported that agitation speed of 200 rpm resulted in increased protease production by *Bacillus halodurans* (Ibrahim and Al-Salamah, 2009), *Halobacterium* sp. (VijayAnand *et al.* 2010), and *Teredinobacter turniae* (Elibola and Moreira, 2005). In conclusion, cell growth and alkaline protease production by *P. aeruginosa* MCM B-327 in stirred tank bioreactor was dependent on both aeration and agitation rates. At lower aeration and agitation rates, oxygen mass transfer was found to be limited, resulting in decreased cell biomass and protease productivity.

The addition of *Amaranth* seed meal in mechanically stirred fermenter at a concentration of 20 g/L resulted in 400% increase in protease production from *Bacillus subtilis* 3411 with agitation 750 rpm and aeration 1 LPM (Maria *et al.* 2001). Effect of agitation and aeration on protease production from *B. licheniformic* NCIM-2042 was studied by Potumarthi *et al*. (2007) and showed maximum specific protease production of 102 U mg^{-1} biomass at 72h. The present study showed improved specific protease production with decrease in fermentation time. Decreased fermentation time for maximum specific protease production may have significant impact on product cost.

An optimum pH of 8 was reported for protease from *Pseudomonas thermaerum* and it remained stable in pH range 5-11 at 60^{o}C (Gaur *et al.* 2010). Joo *et al.* (2004) reported the optimum temperature was around 45–50^{o}C, and stability was exhibited up to 50^{o}C of *Bacillus* sp.

Sivaprakasam et al. (2008) showed influence of aeration (0.5- 1.5 vvm) and agitation (50-250 rpm), on volumetric mass transfer coefficient using *Pseudomonas aeruginosa* in 2 L biocalorimeter. Specific cell growth rate was 0.025 h^{-1} observed at optimum condition, i.e. aeration 0.75 vvm and agitation 150 rpm. Mahadevan et al. (2010) demonstrated maximum specific growth rate of 0.63/h using *Pseudomonas aeruginosa* and 2 L biocalorimeter containing 1 L working volume.

Saran et al. (2007) showed scale up production of protease using *Bacillus* sp. (SBP-66) in 30 L bioreactor at aeration 5 vvm and agitation 700 rpm; resulting in maximum protease activity of 3978 U/ml. Laxman et al. (2005) investigated production of protease form *Conidiobolus coronatus* in 14 and 150 L fermenters. Optimum protease activity of 70-90 IU/ml and 40-50 IU/ml was obtained with 14 and 150 L fermenters respectively. Protease activity was lowered on increasing the size of fermenter. The observation is confirmed in this investigation.

Commercial laundry detergent formulations include bleaching agents, surfactants (anionic), and water softening agents, which acts as enzyme stabilizers in the detergent (Stoner *et al.* 2004). Najafi *et al.* (2005) showed 50% of maximal activity in 1% (w/v) SDS and 3 % (v/v) Tween 80, while , the present protease showed 20% inhibition in SDS and 102% activity in presence of tween 80. Nilegaonkar *et al.* (2007) have reported almost 100% inhibition by 1% SDS, 89% by sodium tripolyphosphate, 38% by sodium tetraborate and 21% by Tween 80 in case of protease of *Bacillus cereus*. These results of this study suggest that the enzyme is compatible with all the important ingredients of commercial laundry detergents.

There are a few reports on detergent/ bleach stable enzymes (Gupta *et al.* 2002; Nascimento and Martin, 2006). Kanekar *et al.* (2002) reported 100% and 70% protease activity in commercial detergent from *B. alcalophilus* and *A. ramosus* respectively. Venugopal and Saramma, (2006) have reported VM10 protease from *Vibrio fluvialis*, which retained 42% enzyme activity after 1 h of incubation with Ariel® and Henko®, 51% with Rin®, 47% with Sunlight®, 61% with Surf®, and 47% with Tide®, indicating maximum stability with Surf®. Nascimento and Martin (2006) studied thermophilic *Bacillus* sp. and showed 80% and 65% of protease activity after 30 min incubation at 60ºC in (the) presence of the detergent brands Tide® and Cheer®, respectively. Mala and Srividya (2010) also studied a protease from *Bacillus* sp. Y with excellent stability and compatibility with three locally available detergents (Kite, Tide and Aerial) for 3 h, retaining 70-80% activity at room temperature (30°C) and 10-20% activity at 50°C. Thus, the *P. aeruginosa* protease also has a potential role in detergent application.

To our surprise, enzyme alone was not found to be much effective for blood stain removal. The detergent ingredients were required for effective blood stain removal from cotton fabric. From the results, Surf excel® was found to be the best suitable commercial detergent for effective washing performances. Use of detergent stable protease from *A. ramosus* and *B. alcalophilus* in removing bloodstains from cotton fabric after 10 sec was also reported (Kanekar *et al.* 2002). Najafi *et al.* (2005) showed removal of bloodstain from cloth after 50 min without addition of any detergent. To remove the bloodstain from cotton fabrics, the present study enzyme showed better compatibility with Surf excel®. However, crude gut extract from *Spilosoma obliqua*, containing protease, amylase and lipase, could be used as a detergent additive, as the enzymes were found to be highly effective in the removal of bloodstains from cotton fabric in presence and absence of detergents (Anwar and Saleemuddin, 2000). The protease enzyme of present study was very effective compared to the enzymes reported earlier (Vijayalakshmi *et al.,* 2011) with respect to the short times and less enzyme units required for complete removal of stains.

Laxman et al. (2006) used additives such as sodium chloride, glycerol, sorbitol and polyethylene glycol (PEG) as enzyme stabilizers at two different temperature ranges, viz. 5–10 and 25–30°C. Glycerol was found to confer considerable stability to the enzyme even at room temperature. But, ammonium sulphate concentrated enzyme (0.9 saturated) was found to be stable for 2 years even at room temperature. The advantage of above formulation is that the salt itself acts as a preservative and there is no need of additional preservative. High stability is generally considered as an economic advantage because of reduced enzyme turnover. In addition, stable enzymes permit the use of high process temperatures, which may have beneficial effects on reaction rates, reactant solubility and the risk of microbial contamination (Eijsink et al. 2004).

The Pseudomonas aeruginosa MCM B-327 is a good source of protease with commercial importance.

Conclusions

Optimization of protease production was carried out in 10 L fermenter at aeration 0.75 vvm and agitation 150 rpm with specific cell growth rate of 0.025 h^{-1}. Kinetics revealed that maximum rate of protease production was 33.88 U/ml/h with cell growth rate 2.4 to 8.6 x 10^8 cells/ml/h at aeration 1 vvm and agitation 250 rpm. The production was scaled up to 120 L fermenter. The ammonium sulphate precipitated enzyme is stable over a broad range of temperatures and pH and in presence of detergent ingredients; this suggests the possible application of protease from P.aeruginosa in detergent industry.

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