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Optimal production of alkaline protease from solvent-tolerant alkalophilic *Pseudomonas aeruginosa* MTCC 7926

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A new organic solvent-tolerant bacterial strain UD-5 was isolated from industrial solvent effluent-sediment stream, collected from Ankleshwar (India) and documented for the production of solvent-tolerant alkaline protease. Based on phylogenetic analysis of the 16S rRNA gene sequence and phenotypic analysis, as well as biochemical analyses, this strain was identified as *Pseudomonas aeruginosa* MTCC 7926. It secreted an extracellular, thermostable; alkaline protease in a casamino acid-rich salt medium (*p*H 9) at 40°C, in static condition. The solvent-tolerance of bacterium in the presence of *n*-hexane, dodecane, cyclohexane and DMSO, xylene and tolune was analyzed. Effect of *p*H, temperature, agitation, carbon and nitrogen sources and various salts on the growth and protease production by strain UD-5 of solvent-tolerant *P. aeruginosa* MTCC 7926 from-manmade ecological habitat was studied. It provided an alternative source of alkaline protease with additional attributes of industrial applications compared to proteases reported earlier. Its ability to grow in organic solvents especially in water-miscible solvents (DMF and DMSO) proves its potential for applications in non-aqueous reactions in addition to usage in detergent, laundry additives and leather finishing.

Keywords *Pseudomonas aeruginosa,* alkaline protease, solvent tolerant, optimization

Introduction

Microbial proteases (peptidyl-peptide hydrolases, EC: 3.4.11-19), representing one among the three largest groups of industrial enzymes, account for 65% of total worldwide enzyme sales[1]. Among them, alkaline protease is of particular interest, which alone accounts for 40% of total world enzyme production[2] because of its usage in detergent, laundry additives, leather finishing, silk industry, feeds modification, food processing, brewing, pharmaceuticals, diagnostic reagents, peptide synthesis, silver recovery from X-ray/photographic film, preparation of organic fertilizer and waste treatment[3]. Their application in detergent formulations as a sole and key ingredient makes up 89% of total protease sales[3,4]. Further, its global sales are estimated to increase more than $1.0-1.2 billion in the coming decade[1]. Currently, microbial proteases also contributed to the development and synthesis of edible peptides from different proteins[4]. At present, alkaline proteases are produced by a wide range of microorganisms, including bacteria, molds and yeasts, its large proportion being derived from *Bacillus* strains.

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For a majority of innovative applications like (i) organic synthesis, (ii) chemical analysis, (iii) downstream processing of fine chemicals, (iv) therapeutic/diagnostic kits, and (v) study of protein structure *vis-a-vis* function, proteases need to be active and stable under harsh conditions, their performance being greatly influenced by several factors. Alkaline proteases with stable high activity in high alkaline range, high temperature and non-aqueous environment have novel biotechnological applications.

Protease-catalyzed reactions in organic solvents have been studied extensively for the synthesis of peptides and esters because these media can increase the solubility of either substrates or products, facilitate product recovery and suppress water-induced side reactions[5]. Although physical and chemical methods have been developed for enhancing enzyme stability in the organic solvents, proteases, naturally endowed with stable and active attributes, are any time superior and preferred[6]. It is anticipated that these solvent-tolerant bacteria secrete organic solvent tolerant enzymes. Based on this hypothesis, several earlier reports indicated high activities and stability of proteases in the presence of organic solvents for novel industrial

applications[6-8]. However, very few reports exist on the optimization of production of alkaline protease from *Pseudomonas* sp.

In this regard, *P. aeruginosa* Pst, *P. aeruginosa* PST-01, *P. aeruginosa* strain K, *P. aeruginosa* MN 7, *P. putida* Idaho and *Pseudomonas aeruginosa* PseA were grown on media containing high concentration of organic solvents for optimal production of proteases[6,7,9-13]. Such microorganisms would be useful for bioremediation of proteinaceous wastes (especially food, hospital and leather) contaminated by hydrophobic solvents, which is otherwise not possible due to microbiocidal nature of solvents. Hence, it is imperative to search for microorganism(s) endowed with unique and naturally stable protease in organic solvents and study optimization of physical and nutritional factors for its production.

In view of these requirements necessary for various industrial applications, the present work describes isolation of a solvent tolerant strain of *P. aeruginosa* from solvent contaminated wastes with an emphasis on the optimization of nutritional requirements for the effective production of organic solvent–stable, extracellular alkaline protease.

Materials and Methods

Microorganism

The microbial isolate, designated as UD-5, was isolated from the solvent polluted sediment, at a depth of 15 cm, located at Gujarat Industrial Development Corporation, Ankleshwar, (India). It was eventually identified as *P. aeruginosa* on the basis of its morphological, biochemical and cultural attributes (Table 1). It secretes an extracellular, solvent tolerant, thermostable and alkaline metallo-protease (35 kDa)[8]. The strain was maintained on nutrient agar slants at 4°C and sub-cultured every 4 wks.

Screening of Microorganisms

The isolates were obtained by suspending 20 g solvent-sediment in 100 mL sterile saline and transferring a 5 mL aliquot to 100 mL medium comprising of (gL^{-1}): glucose 10, peptone 5, yeast extract 5, KH_2PO_4 1, and $MgSO_4.7H_2O$ 0.05, adjusted to *p*H 9.0 with sterile 0.4% (w/v) Na_2CO_3. Each culture was incubated (30°C, 150 rpm, 72 h) and subsequently subjected thrice to batch culturing in order to obtain pure culture. Thus, 24 protease secreting isolates were screened out on skimmed milk agar medium (SMA), comprising of (gL^{-1}): skimmed

Table 1—Morphological, cultural and biochemical characteristics of the isolate UD -5

Morphological characteristics	
Shape	Rod
Gram stain	Gram negative
Motility	+
Cultural characteristics	
Growth at (temperature)	
20°C	+
37°C	+
40°C	++
42°C	+
Growth at (*p*H)	
8.0	+
9.0	++
10.5	+
NaCl (% tolerance)	
2%	+++
4%	++
6%	+
7%	-
Biochemical characteristics	
Oxidase activity	+
Catalase activity	+
Lysine dehydrogenase	+
Ornithine decarboxylase	+
Arginine dihydrolase activity	+
Nitrate reductase	+
Esculin hydrolysis	-
Hydrogen sulfide formation	-
Tween 20 hydrolysis	-
Tween 80 hydrolysis	-
Indole production	-
Methyl red test	-
Voges-Proskauer test	-
Citrate utilization	+
Hydrolysis of:	
Gelatin	+
Starch	-
Utilization of :	
Glucose	+
L-Arabinose	+
D-Galactose	-
Lactose	-
L-Rhamnose	-
Sucrose	-
Trehalose	-
D-Xylitol	-
D-Sorbitol	-
Inositol	-
Malonic acid	-
Propionic acid	+
Poly-β-hydroxybutyrate	+

+: Growth; - : No growth

milk 28, enzymatic casein hydrolysate 5, yeast extract 2.5, dextrose 1, agar 15 and *p*H 9.0. The strains showing maximum clarified zone around the colonies on SMA plates at 40°C after 24 h were selected for further investigation.

Screening of Organic Solvent-tolerant Strain

Microorganisms producing maximum clarified zone around colonial growth (i.e. high protease secretors) on SMA medium were analyzed for organic solvent-tolerance as per Ogino *et al*[7]. For this purpose, cell suspension (0.1 mL, 0.5 OD at 540 nm) of each isolate was individually spread on nutrient agar plate. Each plate was then separately overlaid with 7 mL of *n*-octane, *n*-decane, dodecane, *n*-hexane, chloroform, cyclohexane, benzene, toluene, xylene, and dimethyl-sulphoxide. The plates were stacked in a large stainless steel container with a tight cover, at 40°C for 24 h. After 24 h, colonies emerging from each isolate, formed on the surface of nutrient agar plates, flooded with the above-mentioned organic solvents were isolated. These isolates were further inoculated in Erlenmeyer flasks (500 mL capacity) containing 100 mL nutrient liquid medium and 30 mL each organic solvent. The flasks were plugged with rubber stopper so as to prevent evaporation of an organic solvent and each flask was incubated at 40°C for 72 h. The cell growth was monitored by measuring dry weight of the culture.

Identification of Strain

The newly isolated strain (UD-5) was eventually identified on the basis of its morphological, biochemical and cultural characteristics. The identification was confirmed by (i) Microbial Type Culture Collection (MTCC), Chandigarh using Biolog Microbial Identification System (Biolog automated Microstation system, USA), and (ii) taxonomic characterization on the basis of nucleotide sequence of 16 S rRNA gene.

DNA Extraction and PCR Analysis of Bacterial Strain UD-5

The total genomic DNA of strain UD-5 was isolated as per Sambrook *et al*[14]. The bacterial 16S rRNA gene was PCR amplified from the total chromosomal DNA using universal eubacteria specific primer 16 F27 (5′-CCA GAGTTTGATCMT GGCTCA-3′) and 16 R1525 × P (5′TTC TGC AGTCTAGAAGGAGGTGWTCCAGCC-3′). The reaction system (25 µL) consisted of 10X buffer 2.5 µL, 2 m*M* dNTP 2.5 µL, 10 pMol/L 16F27 1.25 µL, 10 pMol 17R1525XP 1.25 µL, 10U Taq DNA polymerase 0.2 µL, template DNA 2 µL and water 15.3 µL. The conditions for PCR amplification were (i) denaturation at 95°C for 1 min, (ii) annealing at 55°C for 1 min, (iii) extension of annealing at 55°C for 1 min, and (iv) final extension at 72°C for 10 min. PCR was run for 35 cycles using Applied Biosystem PCR cycler system. The PCR product was purified with 0.6 volumes of PEG-NaCl and incubated for 10 min at 37°C. The precipitate was collected by centrifugation for 20 min at 12,000 rpm. The pellet was washed twice with 70% ethanol, dried under vacuum, re-suspended in distilled water at concentration of >0.1 pmolμL^{-1} and the purified product was sequenced using BIGDYE terminator kit 3.1CABI Perkin Elmer, USA. The sequencing reactions were run on ABI-PRISM 31D automated sequencer (Model 3730; Applied Biosystem, USA).

The 16S rRNA sequences obtained above were aligned using BLAST analysis at NCBI server (http://www.ncbi.nlm.nih.gov/BLAST). For comparison, currently available sequences at NCBI were used and multiple sequence alignment performed by using Bioedit 7.0.

Phylogenetic Study

The evolutionary history was inferred using the Neighbor-Joining method[15]. The evolutionary distances were computed using the Kimura 2-parameter method[16] and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Phylogenetic analyses were conducted in MEGA4[17].

Enzyme Production

The culture was grown at 40±2°C without shaking (static) for 48 h in 100 mL medium containing (gL^{1}): casamino acids, 10; Na_2CO_3, 4; KH_2PO_4, 1; $MgSO_4.7H_2O$, 0.05 in a 500 mL capacity Erlenmeyer flask by inoculating 4 mL aliquot of a 24 h-old seed culture (0.5 OD at 540 nm)[8]. To monitor medium optimization, activity was estimated from the cell-free supernatant, recovered by centrifugation (12,000 rpm, 4°C and 10 min). Each experiment was carried out in triplicate.

Measurement of Cell Density

The culture broth was harvested by centrifugation (12,000 rpm, 4°C and 10 min) and the cells mass washed twice with cold distilled water. The washed cells were dried under vacuum at room temperature

until a constant weight was attained[15]. Values obtained were an average of three independent determinations.

Protease Activity Assay

Alkaline protease activity, using casein as a substrate, was assayed as per Nakanishi *et al*[18]. An aliquot of protease (1.0 mL) was added to 1.0 mL casein (0.6 % w/v in 0.2 *M* carbonate-bicarbonate buffer, *p*H 9.0) and the reaction mixture was incubated at 55°C for 10 min. The reaction was stopped by adding chilled 3.0 mL TCA mixture (0.11 *M* trichloracetic acid, 0.22 *M* sodium acetate and 0.33 *M* acetic acid). The inactivated reaction mixture was maintained at room temperature for 30 min, filtered through Whatman filter paper No. 1 and absorbance was measured at 275 nm with tyrosine as a standard. One unit of alkaline protease activity was defined as the amount of enzyme required to produce peptides equivalent to 1.0 µg of tyrosine in the filtrate per minute per mL at *p*H 9.0 and 55°C.

Protein Assay

Protein was estimated as per Lowry *et al*[19] using bovine serum albumin (BSA) as a standard. The culture-free spent liquor was mixed with 1.0 mL 6 N cold TCA and centrifuged (3000 rpm, 4°C for 10 min). The settled protein precipitate was washed thrice in ether and dissolved in 1.0 mL of 0.2 *M* carbonate-bicarbonate buffer (*p*H 9.0) and its suitably diluted aliquot was used for determination of protein concentration at 750 nm.

Optimization of Physical Parameters for Protease Production

The influence of *p*H on growth and protease production was examined by growing the strain UD-5 in a basal medium (gL^{-1}): casamino acids, 10; KH_2PO_4, 1; Mg $SO_4.7H_2O$, 0.05, at 40°C for 48 h and varying *p*H (7-11) was adjusted separately by pre-sterilized 1% Na_2CO_3. The influence of temperature on growth and protease secretion was examined by growing the strain in the same production medium, at *p*H 9, different temperatures (30-42°C) for 48 h. Similarly, the influence of agitation was examined in basal medium at *p*H 9, 40°C for 48 h under various speeds of rotation (50-150 rpm). For preparation of inoculum, a suspension was prepared by mixing pre-grown *P. aeruginosa* MTCC 7926 with saline (0.85% w/v) to achieve 0.5 OD at 540 nm. Effect of inoculum size was studied in the range 2-10% (v/v). In each experiment, cell density was determined spectrophotometrically at 540 nm and protease activity was determined under standard assay conditions. Each optimization parameter was investigated independently in triplicate.

Optimization of Ingredients of Production Medium for Protease Production

For this purpose, various nitrogen sources (viz. beef extract, bovine serum albumin, casamino acids, casein, feather meal, gelatin, peptone, soybean meal and yeast extract) were examined for optimum protease production by separately adding at 1% in the basal medium.

Similarly, various carbon sources (glucose, fructose, arabinose, citrate, and xylose) were examined for optimum protease production by separately adding 1% in the basal medium. One set was kept as a control without any carbon-source. The effect of medium supplementation with different concentrations of glucose (0-2%), casamino acids (0-2%), sodium carbonate (0-0.8%) and KH_2PO_4 (0-0.2%) was also studied. Each optimization was performed independently in triplicate.

Growth and Protease Production Profile

The growth and protease production were investigated in the optimized production medium containing ($g\ L^{-1}$): casamino acids, 10; $MgSO_4.7H_2O$, 0.05; KH_2PO_4, 1; Na_2CO_3, 2; *p*H 9.0 and 40°C under static condition.

Statistical Analysis

For statistical analysis, standard deviation of each experimental result was evaluated using SigmaStat software and Microsoft Excel.

Results

Isolation and Characterization of Solvent Tolerant Proteolytic Strain

Twenty four strains were isolated for protease secretion on SMA medium (*p*H 9, 40°C). Among these, UD-5 exhibited maximum clarified zone, its ratio of diameter of clarified zone: colony diameter was 8.2 on SMA (*p*H 9, 30°C) after 48 h. Among the 24 strains, 4 strains viz., UB-1, UB-2, UC-3 and UD-5 were found organic solvent-tolerant. Of these, based on their solvent tolerance and proteolytic activity, UD-5 was selected; it grew on nutrient agar medium overlaid with organic solvents at 40°C for 72 h. It also grew on nutrient agar medium overlaid with n-hexane, cyclohexane, n-octane, n-decane, do-decane, p-xylene, acetone, chloroform and DMSO, but not in the presence of benzene and toluene (Fig. 1).

Microbiological characteristics of UD-5 strain are summarized in Table 1. On the basis of these morphological and biochemical characteristics, UD-5 strain was identified as *P. aeruginosa* and further verified by Biolog System at Microbial Type Culture Collection, Chandigarh (MTCC accession number 7926). The partial 16S rRNA gene sequence (1390 bp, GeneBank accession number: GQ461355) was compared with sequences available from public databases. After analysis of 16S rRNA gene and subsequent BLAST analysis, the isolate UD-5 was finally confirmed as *P. aeruginosa.* The phylogenetic tree is depicted in Fig. 2.

Optimization of Physical Parameters

The effect of temperature on growth and protease production was examined at 30, 32, 35, 37, 40 and 42°C. While the optimum temperature for growth was 40°C, the strain could grow at 42°C, suggesting its thermo-tolerant nature. It secreted maximum alkaline protease (366 UmL^{-1}) at 40°C and thereafter protease secretion steeply declined (154 UmL^{-1}) with a loss of 58% (Fig. 3a).

The effect of *p*H on growth and protease production was studied by growing the strain at varying *p*H (7, 8, 9, 10 & 11). It grew maximally in the *p*H range of 8 and 10 indicating its alkalophilic nature. After incubation at 40°C for 48 h under static condition, optimum growth and protease production was observed between *p*H 8 and 9. However, alkaline protease production drastically declined beyond the *p*H 9 reaching to negligible levels at *p*H 11 (Fig. 3b).

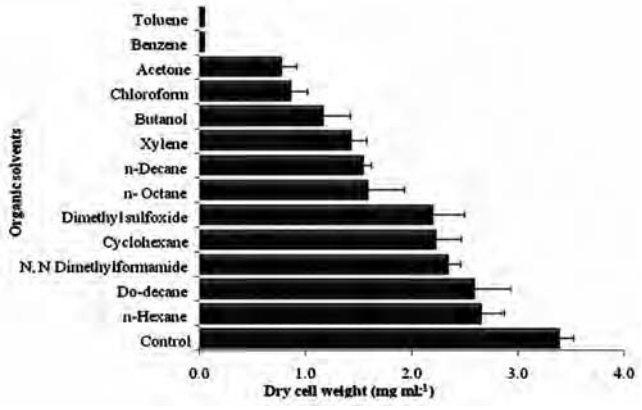

Fig. 1—Dry cell weight of *P. aeruginosa* MTCC 7926 cultured in 100 mL of the nutrient liquid medium (*p*H 9) and 30 mL of organic solvent at 40°C for 72 h.

In order to find the effect of agitation, *P. aeruginosa* inoculated in the medium containing (gL^{-1}): casamino acids, 10; KH_2PO_4, 1; $MgSO_4.7H_2O$, 0.05; Na_2CO_3, 20 (*p*H 9, and 40°C), was maintained static (zero rpm) and agitated at 50, 100, 150 and 200 rpm. While considerable growth was observed at 50 rpm, maximum alkaline protease production (368.9 UmL^{-1}) was detected under static condition (Fig. 3c). Growth and protease production of *P. aeruginosa* MTCC 7926 was maximum at 4 % v/v inoculum incubated for 48 h (Fig. 3d).

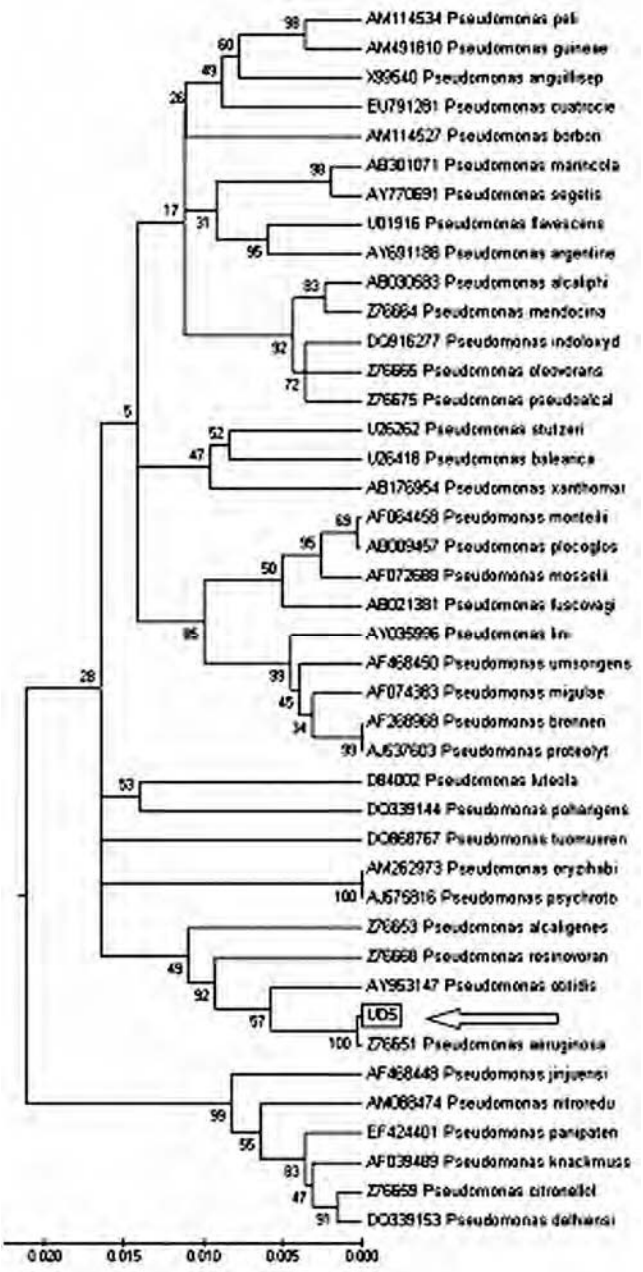

Fig. 2—Phylogenetic tree of strain UD-5. The optimal tree with the sum of branch length = 0.4765 is shown.

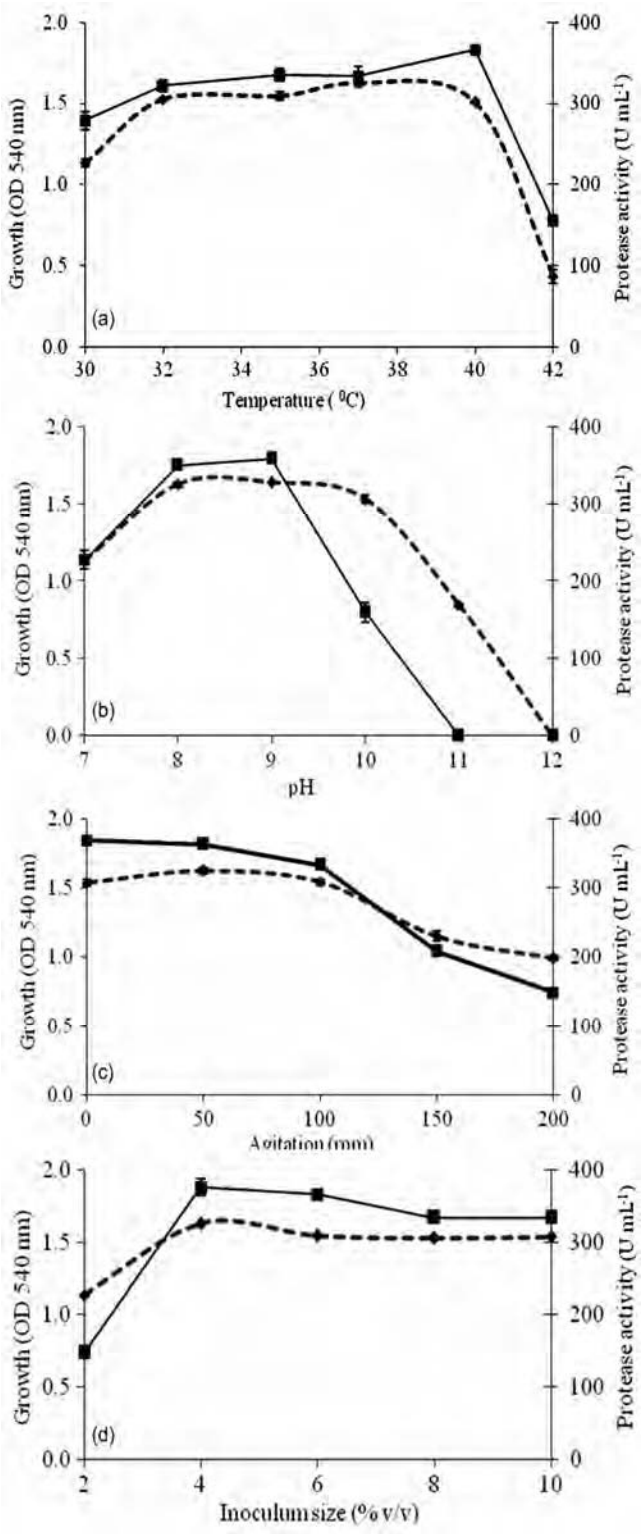

Fig. 3—Effect of physical parameters on the growth and alkaline protease production by *P. aeruginosa*. (a) temperature; (b) *p*H; (c) agitation; & (d) inoculum.

Optimization of Ingredients of Production Medium for Protease Production

Among the various nitrogen sources (in the absence of carbon source), casamino acids exhibited conspicuously higher protease (363.7±5.3 UmL^{-1}) production by *P. aeruginosa* at 40°C, *p*H 9 in 48 h (Table 2), followed by beef extract, peptone, yeast extract, soy meal and BSA (>300 UmL^{-1}). Poor production of protease was observed with casein (269.3 UmL^{-1}), gelatin (246.2±13.3 UmL^{-1}) and the least (42.0 UmL^{-1}) with feather meal. Hence, optimum concentration of casamino acids was examined at 0.0, 0.5, 1.0, 1.5 and 2.0% concentration. Protease production by *P. aeruginosa* was fairly steady between 0.5 and 1.5% casamino acids at 40°C and *p*H 9, attaining maximum growth and maximum protease production at 1.0% (Fig. 4a). Various carbon sources examined at 1% for protease production by *P. aeruginosa* under similar conditions results are summarized in Table 3. In the present instance, glucose suppressed not only bacterial growth, it also repressed protease production in the defining order from 0.5- 2% concentration *vis-à-vis* no glucose (Fig. 4b).

Effect of phosphate ions (PO_4^{-3}) was examined for enhanced growth and production of protease by *P. aeruginosa.* Protease activity, concomitant with growth, gradually increased to 362.2±7.1 UmL^{-1} at 0.1% phosphate *vis-a-vis* 284.1±8.2 UmL^{-1} in control (no extraneous supplementation of phosphate) (Fig. 4c). In the same way, role of Na_2CO_3 (0.0-0.8% concentration) required to maintain *p*H of the medium and protease production was examined. The growth, concomitant with production was optimum (358.8 UmL^{-1}) in the range of 0.2-0.4% Na_2CO_3; thereafter, growth declined gradually, while protease activity declined fast reaching almost negligible at 0.8% Na_2CO_3 (Fig. 4d).

Growth and Protease Production Profile

A time course study performed to determine bacterial growth and protease production of *P. aeruginosa* in optimized medium is shown in Fig. 5.

Discussion

The demand for microbial proteases with high activity and stability, in high alkaline range, high temperature and non-aqueous environment for novel industrial applications continues to stimulate the

Table 2—Effect of nitrogen sources (1%) on protease production by *P. aeruginosa*

Nitrogen supplementation (1%)	Protease activity (U/mL)	Specific activity (U/mg)
Casamino acids	363.7±5.3	1147±56
Beef extract	341.2±9.3	798.3±67
Peptone	309.6±6.7	916.0±81
Yeast extract	308.2±6.3	876.3±54
Soy meal4	302.8±5.0	841.2±40
Bovine serum albumin	300.2±8.7	721.0±50
Casein	269.3±7.4	676.6±87
Gelatin	246.2±13.3	456.4±70
Feather meal	42.6±1.0	173.4±40

*G*rown in the basal medium at 40°C and *p*H 9.0; ±, Standard deviation; each nitrogen source was 1% added in the basal medium having composition (g/L): KH_2PO_4, 1; $MgSO_4.7H_2O$, 0.05.

Table 3—Effect of carbon sources on protease production by *P. aeruginosa* at 40°C and *p*H 9

Carbon source	Enzyme activity (U/mL)	Specific activity (U/mg)
Control	360.0±04.9	932.1±45
Sorbitol	297.6±17.6	776.6±82
Glucose	251.4±09.0	724.1±31
Fructose	164.5±14.8	381.3±73
Xylose	155.2±14.5	540.8±44
Arabinose	103.2±08.4	347.1±75
Citrate	96.2±17.3	322.3±41
Succinate	70.1±07.2	284.7±32

±, Standard deviation; The concentration of each carbon source was 1% in the basal medium having composition (g/L): casamino acids, 10; KH_2PO_4, 1; $MgSO_4.7H_2O$, 0.05; control was without any carbon source.

search for new enzyme sources. Several alkalophiles reported earlier are isolated from Soda Lake and marine environment, while the present investigation has revealed the presence of alkalophilic bacteria from man-made, solvent-contaminated habitat for the first time.

Generally, organic solvents are toxic to living organisms because of their adverse effects on biological membranes. Several researchers correlated solvent toxicity with the hydrophobic character of the solvent, expressed by the logarithm of its partition coefficient between octanol and water, denoted by log *P* value[7,20]. The solvents with log *P* values below 4 are considered extremely toxic as their degree of partitioning into the aqueous layer is higher[21]. Therefore, presence of solvents may lead to reduction in the growth rate. However, cells which are adapted to solvents can achieve the maximum growth rate in the presence of solvents. To confirm that reduction in growth was not caused by oxygen limitation, the strains UD-5 was also cultivated in the absence of organic solvent in an Erlenmeyer flask with a rubber stopper. Accordingly, compared to the optimum growth in control (without solvent), growth was (i) maximum in the presence of n-hexane (log *P* = 3.6), do-decane (log *P* = 5.6), and cyclohexane (log *P* = 3.2), (ii) moderate in the presence of DMSO (*log P* = -1.4), and DMF (*log P* = -1), *n*-octane (log *P* = 4.5) and *n*-decane (*log P* = 5.6) (iii) less in acetone (log P = - 0.23), chloroform (*log P* =2.0), butanol (*log P*= 0.8), and xylene (*log P* 3.1) and (iv) no growth in the presence of *p*-toluene (*log P* = - 2.46) and benzene (*log P* = 3.1). It is indeed intriguing that UD-5 tolerated do-decane more efficiently as compared to *n*-octane and *n*-decane.

These results are in accordance with those of Ogino *et al*[7], except that solvent tolerance of UD-5 was comparatively better than those of *P. putida* IH-2000, *P. aeruginosa* ST-001, *P. putida* Idaho and *P. aeruginosa* PseA, and *Pseudomonas* sp. TOR[12,13,22-24].

Several reports had already indicated incubation temperature as a critical parameter for regulating synthesis and secretion of alkaline protease by microbes[25,26], by affecting (i) energy metabolism and oxygen uptake[27], (ii) translational synthesis of protein[28] and (iii) the physical properties of cell membrane[29]. Accordingly, the effect of temperature

on growth and protease production by *P. aeruginosa* MTCC 7926 was examined. The optimum temperature for its growth was 40°C; the strain could grow at 42°C but not beyond 50°C, emphasizing its mesophilic nature. It secreted maximum alkaline protease (366 UmL^{-1}) at 40°C and protease secretion steeply declined (154 UmL^{-1}) thereafter with 58% loss of activity. Bayoudh *et al*[30] reported alkaline protease production by *P. aeruginosa* MN1 at 30°C. *P. aeruginosa* MTCC 7926 was comparatively better than *P. aeruginosa* strain K which was able to grow at 37°C (range 37-45°C) and showed 24% loss of protease activity upon incubation at 40°C for 48 h[29].

The *p*H of medium has profound effect on alkaline protease production. Interactions between the metabolic reactions and genetic regulatory mechanism(s), product and by-product formation in the bioprocess for alkaline protease production and transport of various nutrient components across the cell membranes are dependent on *p*H[31]. *P. aeruginosa* MTCC 7926 grew maximally in the *p*H range of 8-10 indicating its alkalophilic nature. The maximum protease production was observed between *p*H 8 and 9, suggesting the optimal range. However, alkaline protease production drastically declined beyond the *p*H 9, reducing to negligible at *p*H 11. *P. aeruginosa* san-ai strain was capable of secreting protease at *p*H 10[32], while neutral *p*H maximized the protease production in *P. aeruginosa* strain K[29]. Proteases secreted by *Bacillus* sp. showed activity over a wide range of *p*H (7-11)[3,33].

P. aeruginosa MTCC 7926 secreted maximum alkaline protease production (368.9 UmL^{-1}) under static condition and it remained fairly constant up to 50 rpm indicating that neither mass transfer nor permeability of casamino acids was a problem. Nearly 50% reduction in protease production detected beyond 100 rpm was likely due to shearing forces. High agitation speed caused a reduction in protease production which might be due to (i) cell lysis, (ii) excessive cell permeability related to abrasion by shear forces, and (iii) oxygen limitation in dense

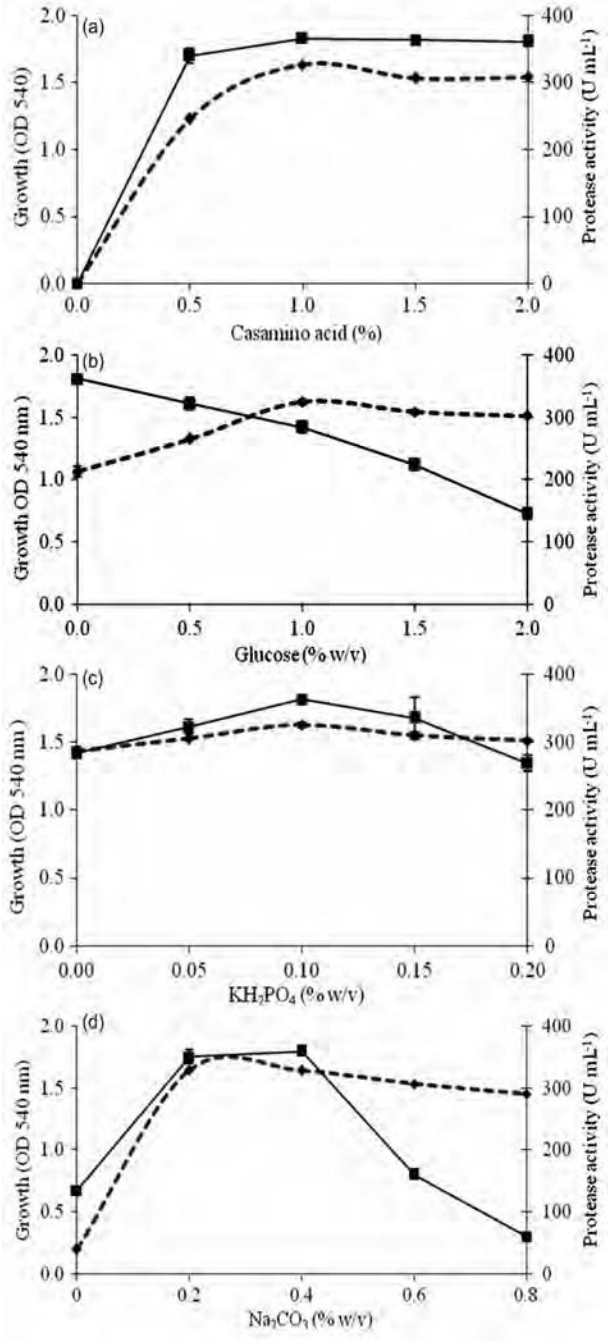

Fig. 4—Effect of ingredients of production medium on the growth and optimum protease production by *P. aeruginosa.* (a) cosamino acids; (b) glucose; (c) phosphate ions; & (d) Na_2CO_3.

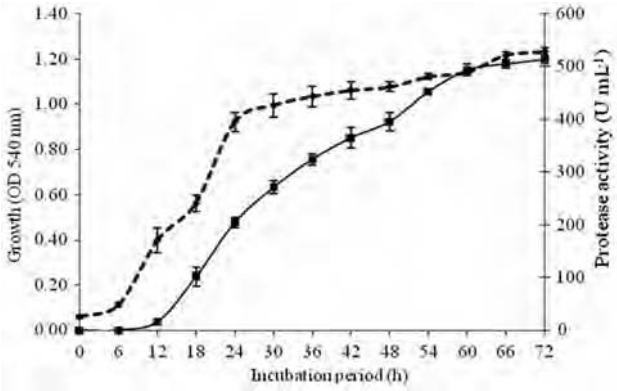

Fig. 5—Profile of growth (- - ◆ - -) and protease production (——■——) of *P. aeruginosa* as a function of time in the basal medium (gL^{-1}): casamino acids, 10; $MgSO_4.7H_2O$, 0.05; KH_2PO_4, 1; Na_2CO_3, 2; *p*H 9 at 40°C under static condition.

pellets as a result of the formation of extracellular polysaccharides[34]. In fact, Frankena *et al*[27] reported an increase in protease production when oxygen was limited. Similar response of agitation on the growth and protease production was reported with *P. aeruginosa* strain K too[29].

It is essential to establish an effective inoculum development programme regardless of the scale of fermentation. Such a programme not only aids consistency on small scale, but is invaluable in scaling up the fermentation for providing homogenous and contamination free inoculum. The inoculum size had impact on cell growth, minimization of contamination and enzyme synthesis, depending on the characteristics of the strains[35]. Growth and protease production of *P. aeruginosa* MTCC 7926 was maximum at 4% v/v inoculum incubated for 48 h. Higher inoculum size might be responsible for lack of oxygen and early nutrient depletion. An inoculation of 2-5% culture was an optimum value for *Bacillus* type of strains as reported in the literature which supported our findings. Other reports include, 5% (v/v) for *Bacillus licheniformis* ATCC 21415[36] and 10% (v/v) for *Bacillus* sp[37].

Nitrogen is a vital component of nucleic acids, proteins and secondary metabolites. Casamino acids was the most effective inducer of protease synthesis by *P. aeruginosa* MTCC 7926, compared to other complex nitrogen sources studied. Although casamino acids is costly compared to yeast extract and soy meal (2nd and 3rd best inducers), it is still preferable due to its inducibility for highest specific activity (Table 2). It is well settled that proteins induce protease secretion. However, in the present instance, not only this established fact is contra-indicated, but most simple substrate also induced optimal secretion is a bit puzzling. The *raison de etre* for induction of protease by its end metabolite apparently appears without rationale or certainly non-understood rationale.

Among various carbon sources examined at 1% for protease production by *P. aeruginosa* under comparable conditions, (i) absence of carbon source induced 360.0±4.9 UmL^{-1} secretion, (ii) sorbitol showed 297.6±17.6 UmL^{-1} protease, (iii) glucose induced 251.4±9.0 UmL^{-1}, (iv) other sugars showed protease secretion in the reducing order, fructose (164.5 UmL^{-1}), xylose (155.2 UmL^{-1}) and sucrose (138.0 UmL^{-1}) and (v) the least inducers were arabinose (103 UmL^{-1}), citrate (96 UmL^{-1}) and succinate (70 UmL^{-1}) (Table 3). Rahman *et al*[9] reported improved protease production upon supplementation with sorbitol to the production medium. Alkaline protease secretion was repressed with increased concentration of glucose. A similar observation about repression of protease production by glucose was reported earlier[9,38]. This has once again validated that the protein inducible protease secretion is repressed by most easily utilizable carbon source and higher growth of biomass does not necessarily translate into higher production of protease.

Phosphate ions (PO_4^{-3}) enhanced growth and protease production by *P. aeruginosa* MTCC 7926. In most studies, potassium phosphate has been used as a source of phosphate and buffering component of the medium[39,40]. Concomitant with growth, protease activity gradually increased to 362.2±7.1 UmL^{-1} at 0.1% phosphate *vis-a-vis* 284.1±8.2 UmL^{-1} in control (no extraneous supplementation of phosphate). These results are in agreement with an earlier report on the need of phosphate supplementation for alkaline protease production by *Bacillus firmis*[40].

The growth, concomitant with enzyme production was optimum (358.8 UmL^{-1}) in the range of 0.2-0.4% Na_2CO_3; thereafter, growth declined gradually, while protease activity declined fast reaching almost negligible levels at 0.8% Na_2CO_3 An optimal protease production was observed at a concentration of 0.8% (w/v) sodium carbonate[41]. Role of Na_2CO_3 was to maintain *p*H of the medium and ensure protease production.

A time course study shows that (i) logarithmic growth phase commenced at 6 h and continued thereafter until 24 h, (ii) maximum growth in biomass was attained after 48 h, (iii) protease production initiated just before 18 h continued until 54 h and (iv) protease activity remained steady in the medium even after 72 h indicating its resistance to inactivation and stability. This is commercially a desirable aspect for maximum recovery during processing.

Conclusion

Several researchers have taken keen interest in the hydrolysis of proteins and synthesis of peptides, using protease. However, very few efforts have been made to screen solvent-tolerant microbes, aiming to produce solvent-tolerant proteases which have their own commercial implications for rapid termination of reaction in desired direction. In this

context, the present study focused on the isolation of solvent-tolerant bacterium *P. aeruginosa* MTCC 7926 which is an alternative source of protease with additional attributes compared to proteases reported earlier. It secreted optimum protease in a medium consisted of (gL^{-1}): casamino acids, 10; KH_2PO_4, 1; $MgSO_4.7H_2O$, 0.05 and Na_2CO_3, 4, at *p*H 9, 40°C in 48 h without agitation.

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