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Isolation and characterization of organic solvent stable protease from alkaliphilic marine *Saccharopolyspora* species

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Protease enzyme from thermostable, organic solvent tolerant and less extensively studied marine *Sacccharoplyspora* species is reported in the present study. Genus of the isolated strain was identified as *Saccharopolyspora* based on 16S r-DNA sequencing as well as the morphological characteristics and biochemical properties. Optimum *p*H for cell growth and protease production was *p*H 10.0. Anionic (SDS) and non-ionic (Tweens) surfactants increased the cell membrane permeability, therefore enhancing the enzyme production. Proteolytic activity of the supernatant of the culture was stable in the presence of various organic solvents. Stability of the enzyme in the presence of organic solvents of the logarithm of the partition coefficient (log *P*) was equal to or more than 3.2. Enzyme retained 98, 97, 95 and 94% of its activity after the incubation in p-xylene, toluene, n-hexane and n-decane, respectively. Solvent-tolerant microorganism and the solvent-stable enzyme produced by this novel marine *Sacccharoplyspora* species can be used as biocatalyst for peptide synthesis in the presence of organic solvents.

[Keywords: Alkaliphilic, *Saccharopolyspora* species, Organic solvent stable, Protease, Salt tolerant, Peptide synthesis**]**

Introduction

Proteases find diverse applications in peptide synthesis, protein processing, food, pharmaceuticals and detergent industries[1,2]. It hydrolyses peptide bonds in aqueous environments and synthesizes them in non-aqueous environments. Proteases need to be stable in the presence of organic solvents for use as biocatalysts for peptide synthesis. However, enzymes in general are inactivated and give low rate of reactions under such conditions[3]. Several physical and chemical methods such as chemical modification, immobilization, entrapment, protein engineering and directed evolution have been employed for the stabilization of enzymes in the presence of organic solvents[4,5].

In recent years a new class of solvent tolerant microbes having unique ability to sustain under non-aqueous system has drawn considerable attention. Such organisms are attractive for applications in solvent bioremediation and biotransformation in non-aqueous media[6-8]. Studies concerning the screening of microorganisms, which produce organic solvent-stable proteases are very less. Hence, protease from solvent tolerant *Saccharopolyspora* sp. is novel. Actinomycetes were considered as major source for antibiotics[9,10], but very limited attention has been directed towards protease production[11-13].

Present study describes the isolation of solvent tolerant microorganisms from marine ecosystem. One of the isolates, identified to be *Saccharopolyspora* species grow well at high concentrations of solvents. It produced an extracellular protease, stable in the presence of various organic solvents.

Materials and Methods

Sediment samples were collected from Goa, Alibagh and Mumbai coastal region of India at the time of low tide. Heat pre-treatment at 40°C for 30 to 60 days was used for isolation of marine actinomycetes[14]. Marine soil samples were suspended in sterile water and thoroughly mixed on rotary shaker at 150 rpm for 20 min. Marine actinomycetes were

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isolated by using selective media such as glycerol yeast extract agar, glucose aspargine agar, starch casein agar and yeast extract malt extract agar. They were screened for protease production by using casein agar medium containing (w/v) 1% casein and 0.5% yeast extract prepared in artificial sea water (ASW). The artificial sea water (ASW) contains (g/l): NaCl, 23.37; Na_2SO_4, 3.91; $NaHCO_3$, 0.19; KCl, 0.66; KBr, 0.09; $MgCl_2$, 4.98; $CaCl_2$, 1.10; $SrCl_2$, 0.02 and H_3B0_3 0.02. Identification of strain was done by scanning electron microscopy (SEM), 16S r-DNA sequencing, biochemical and cultural characterization. The method used for preparation of culture for SEM was as described by Williams and Davies[15].

Glycerol yeast extract medium prepared in artificial sea water was used for development of inoculum. Seed culture was prepared in 100 mL of conical flasks containing 50 mL of medium by inoculating 2.0 mL of spore suspension containing 3.5 to 4.0 × 10^6 CFU/ml and cultivated under agitation at 200 rpm at 45°C for 4 days. Seed culture (50 mL) was inoculated in the 500 mL of fermentation medium containing (w/v) 2% maltose, 0.4% beef extract and 0.4% malt extract prepared in artificial sea water and fermentation was carried out 14 days under agitation of 200 rpm at 45°C. The *p*H of the medium was adjusted to 10.0. After removal of cells by centrifugation at 5000 × g for 15 min, cell free supernatant was subjected to purification and characterization.

The enzyme was precipitated by bringing the culture filtrate to 90% saturation with solid ammonium sulphate and kept at 4°C for overnight. Precipitate was centrifuged at 12000 × g for 30 min. Precipitate was dissolved in glycine-NaOH buffer of *p*H 10.0 and dialyzed for 48 h against the same buffer. Dialyzed sample was assayed for protease activity and protein content[16].

Enzyme activity was determined by the caseinolytic activity method of Mcdonald and Chen[17] and Nilegaonkar *et al*[18] with minor modification. Briefly, the enzyme (1 mL) was mixed with 4 mL of 1% casein (w/v) in 10 mM glycine NaOH buffer *p*H 9.5 and incubated at 37°C for 10 min and the reaction was stopped by adding 5 ml of 5% trichloroacetic acid. Mixture was centrifuged at 8000 *g* for 10 min and the chromogenic reaction mixture containing 1 mL of centrifuged solution and 5 mL of 0.4 mol/l Na_2CO_3 and 1 mL of Folin-phenol reagent was shaken carefully and incubated at 37°C for 10 min, then cooled to room temperature. Absorbance was measured at 660 nm. A blank was prepared in the same manner except that the reaction mixture contained 1 mL of enzyme solution. Tyrosine was used as the standard. One unit of protease activity was defined as the amount of enzyme which liberates 1 µg.

Effect of *p*H on production of enzyme was studied by adjusting the *p*H (3.0-12) of the basal medium before sterilization. Optimum temperature was studied by incubating basal medium at 25-75°C. Effect of NaCl on enzyme production was studied by adding different percentage of NaCl to the basal medium.

In order to study the effect of carbon sources the basal medium was substituted with different carbon sources [2% (w/v)] such as starch, glucose, maltose, xylose, sucrose, glycerol, fructose and galactose were tested for enzyme production. Organic nitrogen sources like peptone, casein, yeast extract, malt extract, beef extract, tryptone, histidine, L-asparaginase, DL-alanine were tested for enzyme production.

Effect of surfactants on protease production was studied by adding (0.04 mol/l) different surfactants such as tween-20, 40, 60, 80, triton X-100, CTAB, EDTA, SDS (0.1-0.5%) to the basal medium at *p*H 10.0 and at 37°C.

The strain was cultured in 500 mL Erlenmeyer flask containing 60 mL medium and 30 mL of organic solvent at 37°C with shaking at 140 rpm. All flasks were plugged with butyl-rubber stoppers to prevent evaporation of organic solvent. Growth of actinomycetes was monitored by measuring dry cell mass.

To check the effect of organic solvent on protease activity, 3 mL of enzyme was incubated with various organic solvent with shaking at 140 rpm for 1 h. Organic solvents stability of protease was determined by incubating 3 mL of enzyme with 1 mL of various organic solvent with shaking at 150 rpm for different time interval. Activity measured in absence of organic solvent was considered as 100%.

Results

Fifty two actinomycetes strains from marine sediments collected from west coast of India were isolated as a part of the study by pre-heat treatment at 40°C. Strain was selected for protease production as it appeared to be the best producer of extracellular protease in both liquid and solid media.

The species showed good growth at 37°C to 55°C at *p*H 10.0 in 7 days on glycerol yeast extract medium but optimum temperature for growth was found to be 55°C. Aerial and substrate mycelium were observed by light microscopy and both the mycelium fragmented into short rods (Fig. 1). Short spiral spore chain was also observed on aerial mycelium. 16S r-DNA sequencing (data not shown) and SEM confirmed that the isolated strain was found to be a member of *Saccharopolyspora* genus. Strain was deposited in Gene Bank with an accession number of HM440345. Morphological, biochemical and physiological properties of isolated strain are listed in Table 1.

Actinomycetes have slow growth rate. Enzyme production had started in early log phase but there was drastic increase in production of enzyme at late growth phase and early stationary phase and protease production was continued up to late stationary phase and achieved maximal production up to 51636 U/mL after that it declined (Fig. 2).

The effect of *p*H on enzyme production and growth of strain was investigated over broad *p*H range (*p*H 3.0-12.0). Results (Fig. 3) suggest that the enzyme production was depress in lower range of *p*H, while it was enhanced in the neutral to alkaline *p*H (7.0-12.0) Maximum production of enzyme 52184 u/L was obtained at *p*H 10.0. Strain even shows 83% relative production at *p*H 12.0. Similarly strain grows well at *p*H 8.0-11.0 with optimum growth was found at *p*H 10.0.

Optimum temperature for growth and enzyme production was investigated from 4°C-85°C and 55°C (Fig. 4) was found to be maximal for cell growth and protease production. Result showed the salt has pronounced effect on growth and enzyme production (Fig. 5). Strain shows maximum growth and enzyme production in presence of 11% NaCl. Strain grows well at 7-13% NaCl then cell growth was found to decrease drastically. Strain shows 12124 u/L of enzyme production at 17% salt and maximum yield of 65449 u/l was produced at 11% salt concentration.

Effect of carbon sources (2% w/v) was studied in basal medium where glucose was replaced with different carbon sources (Fig. 6). Among the investigated carbon sources xylose (80568 u/L) was the best for cell growth and enzyme production which enhanced the enzyme production. Galactose (62982 u/L), fructose (51064 u/L) and glucose (49685 u/L) was also increased the protease

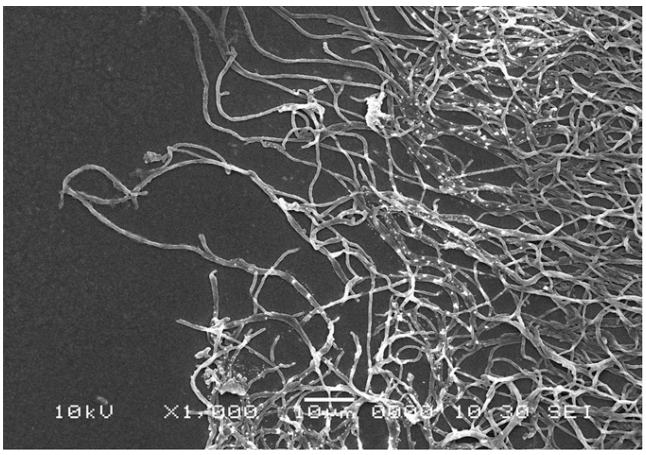

Fig. 1 – Scanning electron microscopy of strain at ×1,000 resolution grown on glycerol yeast extract agar plate at 55°C for 15 days

Table 1—The cultural, biochemical and physiological characters of strain

Tests	Tests
Gram stain	+
Spore chain	Short spiral
Spore surface	Smooth
Substrate mycelium	Short rod fragments
Colour of aerial mycelium	White
Colour of substrate mycelium	Brownish white
Hydrolysis of	
Starch	+
Casein	+
Gelatine	+
Utilisation of carbon sources	
Starch	+
D-Glucose	+
Maltose	+
D-Xylose	+
Sucrose	+
Glycerol	+
D-Fructose	+
D-Galactose	+
Utilization of nitrogen sources	
Peptone	+
Casein	+
Yeast extract	+
Malt extract	+
Beef extract	+
Tryptone	+
Histidine	+
L-Asparginase	+
DL-alanine	+
Growth Temperature (°C)	37-65, optimum-55
*p*H	7-12, optimum-10
NaCl tolerance % (w/v)	3.5-17

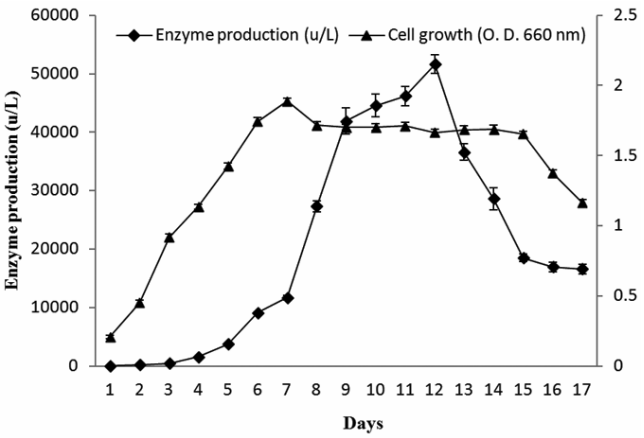

Fig. 2 – Growth kinetics of strain with reference to protease production

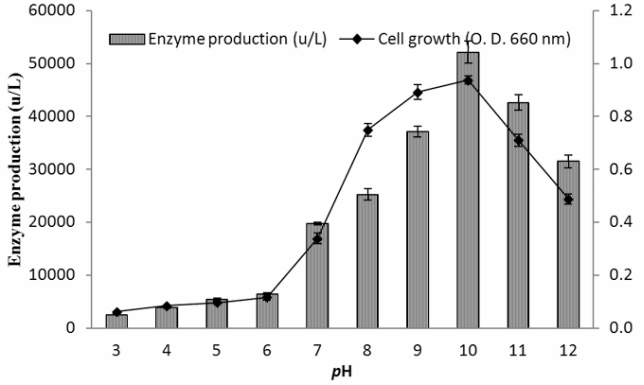

Fig. 3 – Effect of *p*H on protease production and growth of strain

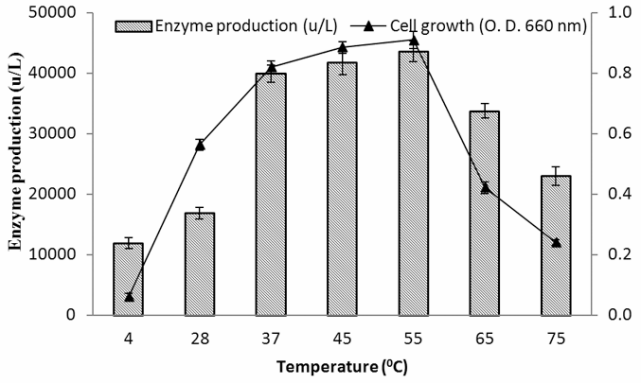

Fig. 4 – Effect of temperature on protease production and growth of strain

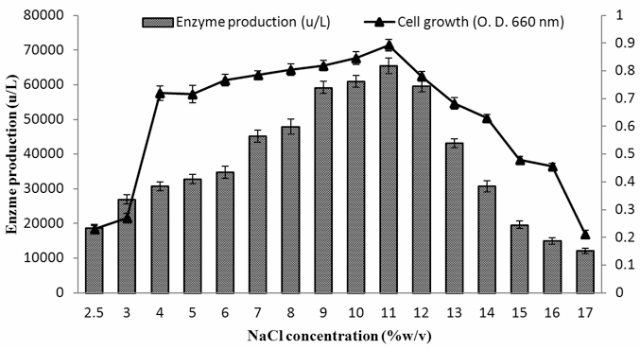

Fig. 5 – Effect of NaCl on protease production and growth of strain

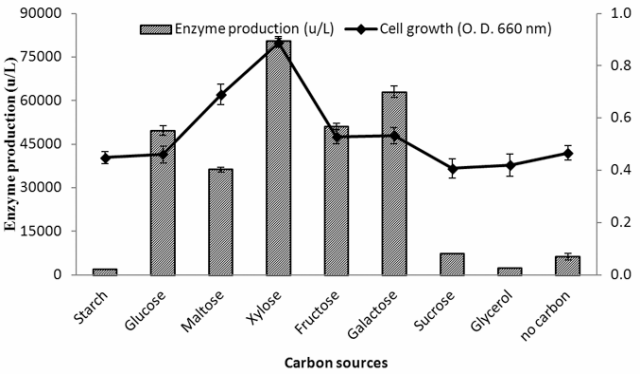

Fig. 6 – Effect of carbon sources on protease production and growth of strain

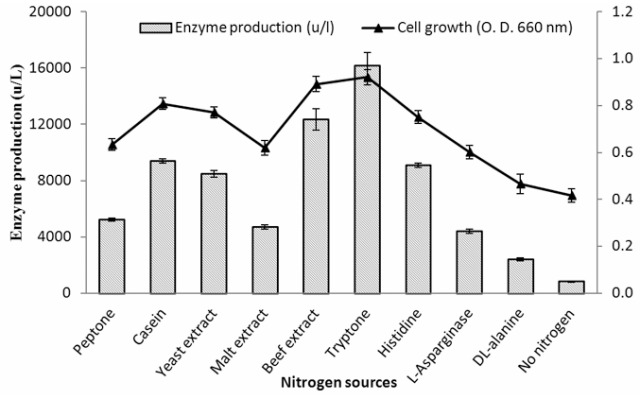

Fig. 7 – Effect of Nitrogen sources on protease production and growth of strain

production. While other sugars, such as sucrose and maltose supported protease production, starch and glycerol affected the production.

The effect of nitrogen sources on growth of strain and enzyme production was also studied by replacing beef extract with 1% of various nitrogen sources (Fig. 7). Among the various nitrogen sources tested tryptone was the best for protease production which has enhanced the production 16139 u/L, while other nitrogen sources have positively enhanced the production and growth of strain.

Enzyme production was increased in presence of all surfactant used (Fig. 8). The enzyme production was drastically increased in presence of SDS 0.3% (19694 u/L), whereas strain showed good growth in presence of various surfactants while SDS 0.1% inhibited the growth of strain.

Growth of strain in liquid medium containing organic solvents and without organic solvent was compared (Fig. 9). The parameter lop *P* is the logarithm of partition coefficient of the solvent between octanol and water. Parameter is a quantitative measure of the solvent polarity. Observation showed that organic solvent with log *P* value 5.6 such as n-decane had little effect on growth of strain. Strong inhibition on growth was observed in those solvent whose log *P* values were below zero, such as n-butanol (log *P* = 0.8), ethanol (log *P* = -0.24).

Enzymes are usually inactivated or denatured in the presence of organic solvent. The effect of organic solvents on protease activity was studied. Relative activity, which remained after 1h incubation in 25% organic solvent, is shown in Table. 2. Activity of enzyme without any solvent was taken as 100%. Remaining activity of protease was found to be 94, 95, 98, 97, and 92% in presence of n-decane, n-hexane, p-xylene, toluene and benzene respectively.

The effect of organic solvent on protease stability was shown in Fig. 10. In this study, various organic solvents with log *P* values low to high were selected and incubated with enzyme for 48 h. Stability study shows enzyme retained more than 80% activity after 48 h. incubation in n-decane (log *P* = 5.6), n-hexane (log *P* = 3.5), p-xylene (log *P* = 3.1), toluene (log *P* = 2.5) and benzene (log *P* = 2.0). Whereas in n-butanol (log *P* = 0.8), ethanol (log *P* = -0.24) and methanol (log *P* = -0.76) enzyme stability was found to be decreased.

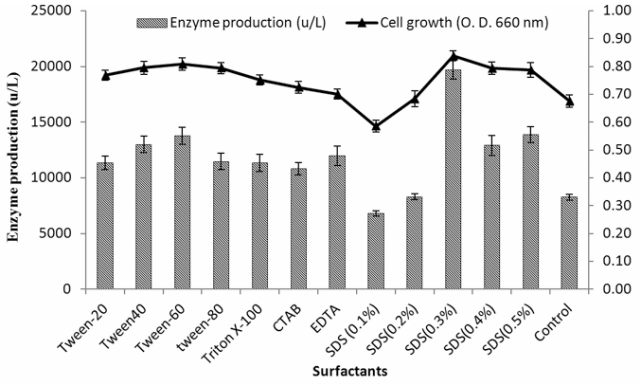

Fig. 8 – Effect of surfactant on growth and enzyme production

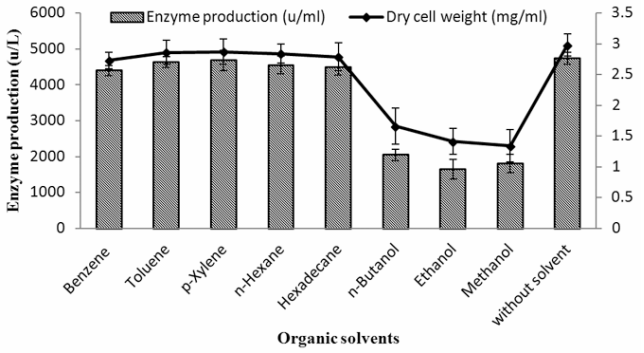

Fig. 9 – Effect of organic solvent on growth of strain and enzyme production

Discussion

In the present study, actinomycetes isolate from marine environment was identified as *Saccharopolyspora* sp. Results given in Table 1, suggest that Gram positive, white colour aerial mycelium and able to hydrolyse the starch, casein and gelatine. It can tolerate the 3.5-17% NaCl, optimum

Table 2—Effect of organic solvent on protease activity

Solvent	log *P*	% Relative activity
n-Decane	5.6	94.91
n-Hexane	3.5	95.97
p-Xylene	3.1	98.73
Toluene	2.5	97.94
Benzene	2.0	92.74
n-Butanol	0.8	79.24
Ethanol	-0.24	68.07
Methanol	-0.76	54.78

One millilitre of 25% organic solvent was incubated with 3 mL of protease at 37°C 140 rpm for 1 h. The remaining protease activity was measured. The activity of protease without organic solvent was taken as 100%.

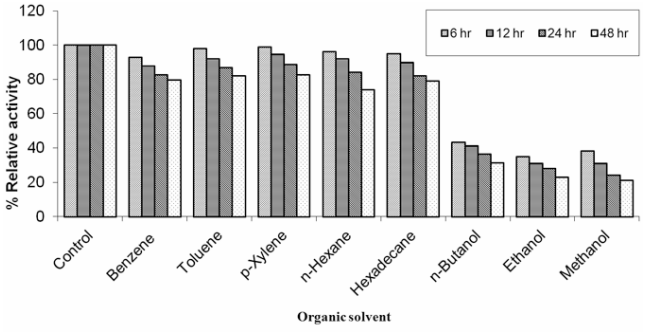

Fig. 10 – Effect of organic solvent on protease stability

temperature and *p*H. Present isolate was agreement with the report of Yuan *et al*[19] from soil, with short spiral spore chain on aerial mycelium and the author has reported characteristics of the genus *Saccharopolyspora*. 16S r-DNA sequencing (data not shown) and SEM[20] confirmed that the isolated strain was found to be a member of *Saccharopolyspora* genus. Growth curve indicates the kinetics of enzyme production and it was more of growth associated. Effective induction may not occur until the stationary phase has been reached and the readily available carbon source was depleted. This secretion pattern is different from those reported in previous studies in which the majority of proteases were not or only produced at a low rate in the exponential growth phase and normally reached the maximal production in the stationary phase[21-23]. Relatively high level of protease was produced by isolated strain compared to *Bacillus cereus* MCM B-326[18]. Based on these merits, strain could be an efficient and economical microorganism with potential applications in industrial production of enzymes.

Extracellular protease was detected over broad *p*H range (*p*H 7.0-12), with optimum protease production and growth of strain exhibited at *p*H 10.0. Cell growth and protease production was higher at alkaline *p*H as compared to in acidic and neutral *p*H. However at extreme acidity of *p*H below 6.0, the production and growth was found to be greatly reduced. The optimum *p*H for production of protease determined in this study is in agreement with the optimum *p*H for the protease *Nocardiopsis* sp. TOA-1[24]. Nascimento and Martins[25] reported an optimum *p*H of 8.0 from thermophilic *Bacillus* sp. strain SMIA-2. Meanwhile, Laxman *et al*[26] has reported optimum *p*H of 9.0 for protease from *Conidiobolus coronatus.* Temperature is critical parameter which needs to be controlled and this usually varied from organism to organism[27]. Studies by Frankena et al[28] showed a link between enzyme synthesis and energy metabolism in bacteria and this was controlled by the temperature and oxygen uptake. Optimum temperature for growth of strain and protease production was in agreement with production of protease by *Bacillus licheniformis*[29]. Meanwhile, the optimum temperature for the protease by *Bacillus*[25] sp. SMIA-2 was 60°C. Strain was found to be moderately halophilic in nature as maximum growth and enzyme production was obtained in presence of 11% (w/v) of NaCl and enzyme retain 65% of its activity in presence of 13% (w/v) of NaCl. There was no protease production in absence of NaCl. These result clearly indicate halophilic nature of the strain and enzyme, where salt appears to be prerequisite for protease production and growth of strain. Similar behaviour of other halophiles producing protease was reported by Li *et al*[30] from actinomycetes strain YIM 90007, which produces protease in presence of 10% (w/v) of NaCl and Kaur *et al*[31] reported that *Bacillus* sp. P-2 can tolerate 20% NaCl concentration and produced little amount of protease. Kumar *et al*[32] reported similar result for *Bacillus clausii* produces maximum enzyme at 10% (w/v) of NaCl.

Carbon sources greatly influenced the enzyme production[33]. Reported studies showed that glucose was a promising source for enzyme production it has been reported that lack of glucose in a medium resulted in dramatic decrease in the protease production. On the other hand glucose has reported to suppress protease production[34]. Present results are good in accordance with the previous studies and xylose was found the best carbon source for protease production. In present study 2% glucose repress the enzyme production compared to the xylose. Similar effect of glucose on enzyme production was also observed by Heineken *et al*[35]. Nitrogen sources affected enzyme secretion in case of recently reported actinomycetes[36]. Complex organic nitrogen sources such as yeast extract and peptone being rich in amino acids and short peptides displayed enzyme repression, when used at higher concentrations. This trend has earlier been shown in case of *Bacillus wrmus*[37]. From the results presented in Fig. 6, it was evident that growth and enzyme production was optimum at 1% (w/v) tryptone and the similar result was reported from *Streptomyces clavuligerus*[38]. However, at higher concentrations enzyme secretion was repressed. Thus, nitrogen sources stimulated protease synthesis up to certain threshold levels beyond which enzyme secretion was repressed. However, to the best of our knowledge, there is no report on the nitrogen repression of alkaline protease from actinomycetes.

Anionic and nonionic surfactants were found to have strong influence on enzyme secretion. This may be due to the solubilization of membrane protein that leads to an increase in cell membrane permeability, thereby enhancing the secretion of biomolecules[39]. Interestingly, tween-80 and SDS did not affect the specific enzyme activity, though enzyme titres

increased. This may be due to the result of release of other proteins from permeabilized cell membrane[39,40].

Organic solvent tolerant microorganisms are a novel and unique group of extremophilic microorganism that thrive in the presence of very high concentrations of organic solvents[41]. In general, organic solvents are extremely toxic to microorganism, as they disrupt the cell membrane, by altering with the structural and functional integrity of the cell[42,43]. Physiological investigation of microbes has revealed a correlation between solvent toxicity and its log *P* value. The greater the polarity of a solvent, the lower its log *P* value and the greater will be its toxicity[44]. Previous studies have reported some organic solvent-tolerant strains that can produce solvent-stable protease, most of them were from *Pseudomonas* genus[45,46] and few were *Bacillus cereus*[47]. In present investigation *Saccharopolyspora* sp. could be considered as a novel strain producing solvent-stable protease. To date, some organic solvent-stable proteases have been isolated and reported. The effect of organic solvent on protease differs from protease to protease. Similar stability pattern of isolate was observed as like *Bacillus pumilus*[48]. It also showed stability in presence of organic solvent with log *P* of 3.5 or higher. Similarly, Geok *et al*[45] also found that protease from *P. aeruginosa* strain K was also stable in presence of organic solvent with log *P* equal to or higher than 4.0. This may be due to replacement of some water molecule of the enzyme with solvent molecule which stabilizes the structure of the enzyme. To achieve a high yield of peptide synthesis, the stability of protease was very important because enzyme were denatured or inactivated in presence of organic solvent before completing the reaction. The present finding suggested that the *Saccharopolyspora* sp. itself and protease has shown stability in presence of various organic solvent over 24 h and enzyme retained almost 40% of its activity after 48 h incubation in various organic solvents. This organic solvent stable protease could be used as a biocatalyst for peptide synthesis in organic media.

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