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ORIGINAL ARTICLE

Solid-state protease production using anchovy waste meal by moderate halophile *Serratia proteamaculans* AP-CMST isolated from fish intestine

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Abstract The possibility of utilising anchovy waste meal as a substrate for protease production by the fish gut isolate *Serratia proteamaculans* AP-CMST was assessed through solid-state fermentation. A time course for protease production revealed 72 h to be the optimum duration for higher production (146.24 U/g). The most suitable pH, temperature and moisture level observed for higher protease production were pH 7 (123.5 U/g), 30°C (97.22 U/g) and 75% (126.7 U/g), respectively. Protease production by *S. proteamaculans* AP-CMST was high in medium with added xylose (198.21 U/g), peptone (118.42 U/g), Triton X-100 (152.56 U/g) and manganese sulphate (178.33 U/g) when compared to other tested medium components. The halotolerancy of *S. proteamaculans* AP-CMST for protease production was 4% sodium chloride (155.65 U/g). Enzyme recovery from fermented anchovy waste meal was greatest (130.52 U/g) when 10% ethyl acetate was used as the extractant, and the optimum time range for extraction was 90–120 min.

Keywords Proteolytic bacterium · *Serratia proteamaculans* AP-CMST · Anchovy waste meal · Solid-state fermentation

Introduction

Proteases belong to a class of enzymes known as hydrolases, which catalyse the hydrolysis of various bonds with the participation of a water molecule. Proteases execute a wide variety of functions and have various important biotechnological applications. The most important industrial use of proteases is in laundry detergents, where they help remove protein-based stains (e.g. blood and egg) from clothing. The second largest use of protease is in cheese making. Proteases are also used for bating (softening) leather, modifying food ingredients, as meat tenderisers, and in flavour development (Fukushima 1983; El-Shora and Metwally 2008). Proteases have also been studied for their role in blood clotting (Kaminishi et al. 1994) and inflammatory diseases (Okamoto et al. 2001).

Proteases can be produced either by submerged fermentation (SMF) or solid-state fermentation (SSF). Of these, the latter has been given preference because it is a simple, low-investment process that requires only low energy consumption and allows easy control of contamination due to the low moisture levels employed. SSF processes utilize mainly cheap nutrient sources and agricultural residues. Earlier studies using arrowroot starch (Ganesh Kumar and Parrack 2003), sardinella meal (Ellouz et al. 2001, 2003) and Bengal gram (Zambare et al. 2007) have demonstrated protease production using these low cost substrates. The present study was designed to optimise culture conditions under SSF for protease production by *Serratia proteamaculans* AP-CMST isolated from the gut of the estuarine fish *Etroplus suratensis* using low cost anchovy waste meal as substrate. The anchovy fish catch accounts for a major portion of the world's overall fish catch, the majority being used for fish meal production and only a comparatively small amount being used for human consumption. Anchovy meal is known for its nutrient richness, containing 63–66% protein and 9.14% lipid (Kratzer et al. 1994; Bimbo 1990; Turan et al. 2007), which may help promote excellent microbial growth.

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Materials and methods

Bacterium and enzyme activity

The bacterium AP-CMST used in this study was isolated from the gut of *Etroplus suratensis* collected from the Rajakkamangalam estuary in Kanyakumari District, Tamilnadu, India. AP-CMST produced a clear zone when streaked on skim milk agar after 24 h, and was identified as *Serratia proteamaculans* based on 16S rRNA gene sequence analysis (Rainey et al. 1994); the sequence was submitted to GenBank (accession no. FJ752236).

Solid-state fermentation and protease extraction

Anchovy waste was obtained from a local fish exporting industry and powdered well. *Serratia proteamaculans* AP-CMST was cultured using enrichment medium containing (w/v): beef extract (0.15%), peptone (0.5%), sodium chloride (1.0%) and glucose (0.5%), pH 7, at 32°C for 24 h. Then, 5% of enriched seed culture was inoculated into a 150-ml flask containing 10 ml mineral medium (w/v) (potassium dihydrogen orthophosphate 0.1%; sodium chloride 1%; magnesium sulphate 0.01%; ammonium nitrate 0.5%), along with 5 g solid anchovy waste meal. The moisture level in the medium was maintained at 70%. The culture was then incubated for 72 h at 32°C, and after incubation the fermented solid was added to 50 ml distilled water and placed in a shaker at 150 rpm for 1 h for protease extraction. It was then filtered through muslin cloth and the cells were harvested by centrifugation at 10,000 *g* for 15 min; the supernatant was used for further assay.

Protease assay

To 0.25 ml culture supernatant, 1.25 ml Tris buffer (100 mM; pH 7.2) and 0.5 ml 1% aqueous casein solution were added. The mixture was incubated for 30 min at 30°C. Then, 3 ml 5% tricholoroacetic acid (TCA) was added to this mixture, whereby it formed a precipitate. The mixture was further incubated at 4°C for 10 min, and then centrifuged at 5,000 *g* for 15 min. Thereafter, 0.5 ml supernatant was taken, to which 2.5 ml 0.5 M sodium carbonate was added, mixed well and incubated for 20 min. To this mixture, 0.5 ml folin phenol reagent was added and the absorbance was read at 660 nm using a UV-Vis Spectrophotometer (TECOMP 8500, Hong Kong). The amount of protease produced was measured with the help of a tyrosine standard graph (Takami et al. 1989). Based on the tyrosine released, protease activity was expressed in units per gram of dry solid anchovy substrate (U/g).

Optimisation of culture conditions for solid-state protease production

In the present study, solid-state protease production by *S. proteamaculans* AP-CMST was optimised by varying physical parameters (fermentation time, pH, temperature and initial moisture level), nutrient sources (carbon and nitrogen), surfactants, trace elements and sodium chloride.

The optimum time required for protease production in basic mineral medium was determined at every 24 h of fermentation up to 144 h. To assess the effect of temperature on protease production, the substrate inoculated with bacterial culture was incubated at various temperatures (10, 20, 30, 40, 50 and 60°C) at pH 7.0. The optimum pH for solid-state protease production was determined in production media at different pH values, for which preautoclaved medium was individually prepared at pH 4, 5, 6, 7, 8, 9 and 10 and inoculated with experimental bacterium at 32°C. To determine the effect of initial moisture content on protease production by SSF, the initial moisture content of the anchovy waste meal was adjusted to 30, 40, 50, 60, 70, 80, 90 and 100% with distilled water. These experiments were conducted using basic mineral medium.

The influence of nitrogen source (2% w/v) on protease production was investigated by replacing ammonium nitrate in mineral medium with skim milk powder, casein, beef extract, yeast extract, peptone, sodium nitrate, potassium nitrate, ammonium chloride, urea or ammonium sulphate. Different carbon sources, including glucose, sucrose, fructose, lactose, xylose, maltose, mannitol, raffinose and sorbitol were employed (1% w/v) to determine their influence on solid-state protease production. Suitability of various surfactants, viz. Tween 20, Tween 40, Tween 60, Tween 80, Triton X-100 and polyethylene glycol was studied by incorporating them into the mineral medium at the concentration of 0.5% (w/v). Different trace elements, such as calcium chloride, magnesium chloride, EDTA, barium chloride, copper sulphate, zinc sulphate, manganese sulphate and zinc chloride (all at 0.05% w/v) were assessed for their influence on protease production. Since the test bacterium was isolated from an estuarine environment, the effect of various concentrations (2, 4, 6, 8, 10 and 12% w/v) of sodium chloride was assessed for its effect on protease production. These latter five experiments were performed in pH-, temperature- and moisture-content-optimised basic mineral medium. All experiments were carried out in triplicate, and average values are presented. The data obtained in the present study were analysed using Microsoft Excel 2003 (http://www.microsoft.com).

Effect of different extractants on protease extraction from fermented solid medium

Enzyme recovery is a critical factor in SSF. For the effective recovery of protease, the fermented biomass was

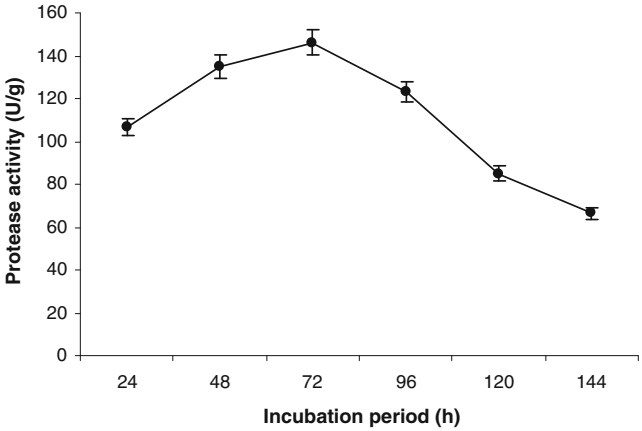

Fig. 1 Effect of incubation time on solid-state protease production by *Serratia proteamaculans* AP-CMST in basal mineral medium with 70% moisture level at pH 7.0 and 32°C

extracted with six different solvents, i.e. distilled water, Tris buffer (pH 7.2), ethyl acetate (10%), glycerol (10%), ethanol (10%) and acetone (10%). To determine the optimum time requirement for enzyme extraction in the tested solvents, the assay was carried out at 30-min intervals for up to 3 h.

Results and discussion

The influence of incubation time on protease production by *S. proteamaculans* AP-CMST was statistically significant ($P<0.0001$); maximum production (146.24 U/g) was obtained at 72 h (Fig. 1). With longer incubation times, protease production declined. This may be due to the fact that the cells may have reached the decline phase and display low protease synthesis.

Physical factors are important in any fermentation for optimisation of biochemical production. The important physical factors that determine the rate of bioprocessing are: pH, temperature, aeration and agitation. In the present study, the effect of temperature on protease production by SSF revealed that 30°C was optimal (97.22 U/g) and enzyme production declined at the higher temperatures tested (Table 1). This may be attributed to growth reduction and enzyme inactivation at higher temperatures. The influence of temperature on protease production was statistically significant ($P<0.0001$). There are reports describing the effect of temperature on SSF of protease that correlate with and support the present study. Soares et al. (2005) reported optimum protease production by *Bacillus subtilis* under SSF with soycake at 35°C. Joo and Chang (2005) reported that *Bacillus* sp. I – 312 under SSF with soy meal showed optimum protease production at 32°C.

The effect of initial pH on SSF of protease showed that the pH range 5–7 favoured higher protease production, and that production was relatively high at pH 7.0 (123.5 U/g) (Table 1). At higher pH levels (8–10), protease production decreased. The variation in protease production at different pH was statistically significant ($P<0.0001$). It is postulated that the metabolic activities of microbes very much respond to pH change. At higher pH, the metabolic action of a bacterium may be suppressed and thus enzyme production inhibited. Similar trends have also been observed in SSF of protease by some fungal species. Tunga et al. (1998) stated that SSF of protease by *Rhizopus oryzae* increased when the pH was raised to 7.0. Chutmanop et al. (2008) also noted an increase in solid-state protease production by *Aspergillus oryzae* at pH 7.0. A similar range of pH optimum (6–7) for protease production was also noticed in *Bacillus subtilis* (Soares et al. 2005) and *Bacillus* sp. I – 312 (Joo and Chang 2005).

The initial moisture level in the medium is a critical factor in SSF. In the present study, the optimal initial moisture level of the medium accelerated solid-state protease production by *S. proteomaculans* AP-CMST with anchovy waste meal. Optimal enzyme production was registered at the 75% moisture level (126.7 U/g). At a moisture level of 30%, minimum enzyme production was noted (Fig. 2). The variation in protease production at different moisture levels was statistically significant ($P<0.0001$). This is consistent with a report by Anandan et al. (2007) on SSF of wheat bran

Table 1 Effect of incubation temperature (at pH 7 and 70% moisture level) and pH (at 32°C and 70% moisture level) on solid-state protease production by *Serratia proteamaculans* AP-CMST in basal mineral medium. Each value is a mean of triplicate analysis

Temperature (°C)	Protease activity (U/g)	pH	Protease activity (U/g)
10	45.40±0.81	2	97.88±0.92
20	77.26±0.70	3	101.79±0.96
30	97.22±1.08	4	113.70±0.65
40	73.99±0.91	5	119.20±0.98
50	55.80±0. 59	6	121.50±0.54
60	35.50±0.58	7	123.50±0.62
		8	84.90±0.64
		9	75.90±0.90
		10	66.00±0.78

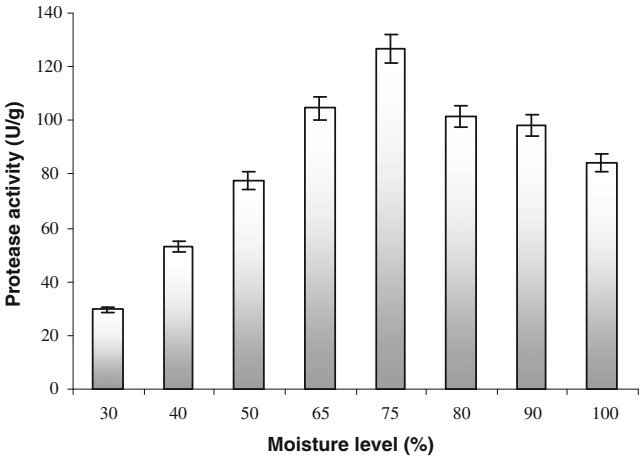

Fig. 2 Effect of initial moisture level on solid-state protease production by *S. proteamaculans* AP-CMST in basal mineral medium at pH 7.0 and 32°C

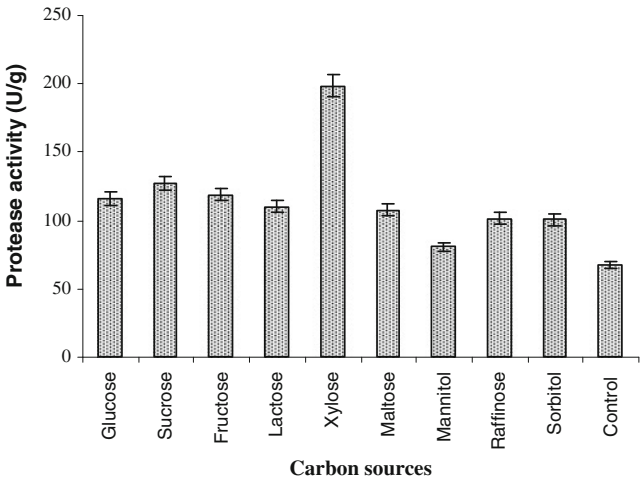

Fig. 4 Effect of supplementary carbon sources on solid-state protease production by *S. proteamaculans* AP-CMST

by *Aspergillus tamari*, wherein maximum protease was reported at the 65% moisture level. Mahanta et al. (2008) also reported maximum protease production in the same substrate with initial moisture levels of 50% and 66.7%. It was also reported that moisture levels higher than the optimum limit reduce protease production to a greater extent, and that this may be due to sealing of water in between particle spaces of the substrate with a consequent reduction in the oxygen available for microbial growth (Bogar et al. 2003).

The nitrogen source is critical in fermentation media; supplying a suitable nitrogen source favours higher level enzyme or metabolite production. In the present study, supplementary nitrogen sources accelerated protease production under SSF. Furthermore, this experiment showed that the complex organic nitrogen sources gave higher protease production than inorganic nitrogen sources, with production being highest in peptone (118.42 U/g) supplied medium. Other organic nitrogen sources supporting high protease production were: beef extract, yeast extract and casein (111.28 U/g, 107.76 U/g and 100.53 U/g, respectively). On the other hand, the lowest protease production was observed in ammonium chloride (12.8 U/g) supplied medium—only 10.8% when compared with the highest value observed in peptone (Fig. 3). The variation in protease production between nitrogen sources was statistically significant ($P<0.0001$). The reason that organic nitrogen sources were superior may be that inorganic nitrogen sources serve only as a nitrogen source, whereas organic nitrogen sources are multi-nutrient sources also containing free amino acids, carbohydrates, essential fatty acids, etc. These constituents serve as multi growth promoters that also contain some inducers for protease production. Our results on organic nitrogen source-induced protease production correlate well with the results of Prakasham et al. (2006), who reported that protease production by *Bacillus* sp. under SSF of green gram husk was influenced greatly by organic nitrogen sources, especially yeast extract. Higher protease production by *Aspergillus tamarii* was also reported in peptone- and skim-milk-supplied medium (Anandan et al. 2007).

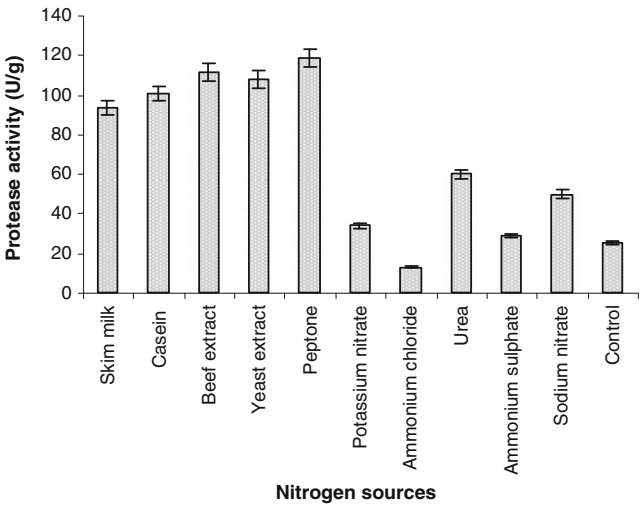

Fig. 3 Effect of supplementary nitrogen sources on solid-state protease production by *S. proteamaculans* AP-CMST.

The effect of various supplementary carbon sources on SSF of protease by *S. proteamaculans* AP-CMST revealed the existence of significant variation ($P<0.0001$) between them. The influence of xylose was greater (198.21 U/g) than the other carbon sources tested (Fig. 4.). Sucrose was the second best supplementary carbon source, producing 126.44 U/g; however, this was only 63.79% of the highest production observed with xylose. On the other hand, mannitol gave rise to the lowest protease production (80.67 U/g). It can be assumed that different carbon sources

Table 2 Effect of surfactants and metal ions on solid-state protease production by *S. proteamaculans* AP-CMST. Each value is a mean of triplicate analysis

Surfactant	Protease activity (U/g)	Metal ions	Protease activity (U/g)
Tween 20	81.42±0.74	Calcium chloride	143.08±0.48
Tween 40	105.11±0.97	Magnesium chloride	173.08±1.20
Tween 60	104.47±0.80	EDTA	140.14±0.74
Tween 80	122.33±0.99	Barium chloride	173.25±1.0
Triton X-100	152.56±1.14	Copper sulphate	168.46±1.05
Polyethylene glycol	149.29±0.46	Zinc sulphate	172.46±1.10
Control	148.15±0.70	Manganese sulphate	178.33±0.54
		Zinc chloride	173.41±0.95
		Control	133.2±0.66

have varied influence on the production of extracellular enzymes, especially protease, by different strains. Enhanced protease production in carbohydrate-enriched solid substrate has been documented by many other researchers. Prakasham et al. (2006) reported high protease production by *Bacillus* sp. under SSF in green gram husk medium supplied with xylose and maltose. Samarntarn et al. (1999) reported that protease production by *Aspergillus oryzae* U152 was high in the presence of supplementary carbohydrate carbon sources, especially lactose.

Despite an enhanced protease production under SSF by *S. proteamaculans* AP-CMST in medium with added surfactants, negative results over the control medium (148.15 U/g) were also noted in certain cases. Higher protease production was found in medium supplied with Triton X-100 (152.56 U/g), which was 3.0% higher than the control. The other surfactant that gave a positive effect was polyethylene glycol (149.26 U/g) (Table 2). This implied that the protease from this bacterium is stable towards the non-ionic surfactant, Triton X-100. This result is positively correlated with the Triton X-100- and SDS-stable protease production reported in *Bacillus clausii* I 52 (Joo et al. 2003) and *Bacillus* sp. (Nascimento and Martins 2006). Our results are also consistent with reports of polyethylene glycol-induced lipase production by *Yarrowia lipolytica* (Alberts et al. 2003) and surfactant-aided solid-state production of amylase (Goes and Sheppard 1999).

Metal ions and trace elements are often required in fermentation medium for enhanced protease production, depending on the source of enzyme. The present results show that manganese sulphate emerged as the most suitable trace element (178.33 U/g) for protease production. Here, the protease production noted in control medium was 133.2 U/g (Table 2). Protease production in medium supplemented with other tested trace elements did not vary much except in media with added metal chelator EDTA (140.14 U/g) and calcium chloride (143.08 U/g), which recorded 78.58% and 80.5% of the highest production observed in the presence of manganese sulphate, respectively. The overall results inferred the existence of statistically significant ($P<0.0001$) variation in protease production between the various metal ions used. Similar to this study, Patke and Dey (1998) reported higher proteolytic activity by *Streptomyces megasporus* in medium supplemented with cupric chloride, magnesium chloride and manganese chloride. This result also finds support from metal ion

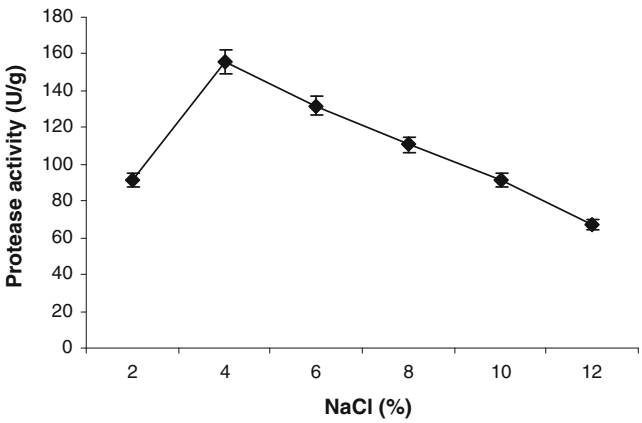

Fig. 5 Effect of different concentrations of sodium chloride on solid-state protease production by *S. proteamaculans* AP-CMST

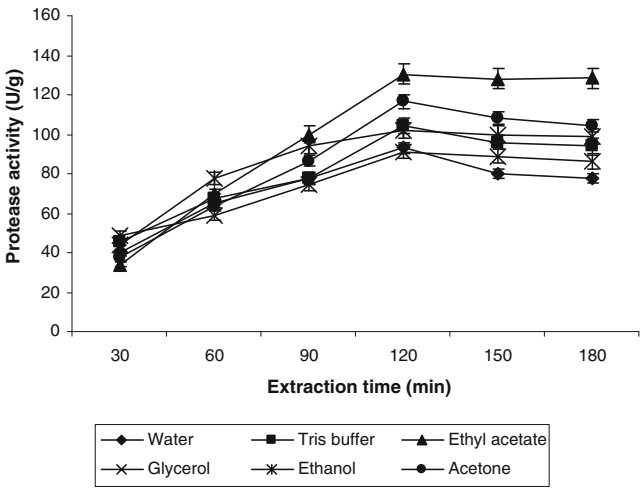

Fig. 6 Effect of various extractants on protease extraction from anchovy waste meal after 72 h of fermentation

mediated protease synthesis by *Pseudomonas aeruginosa* (Abd Rahman et al. 2005). Nascimento and Martins (2004) also reported the positive effect of manganese sulphate on protease activity by *Bacillus* sp.

Sodium chloride is an important nutrient factor for *S. proteamaculans* AP-CMST, because this bacterium was isolated from an estuarine habitat and absolutely requires Na^+ for its growth and physiological activities. The sodium chloride optimisation study indicated that 4% was the optimum concentration for SSF of protease (155.65 U/g). Above this concentration, production decreased gradually, and at 6% the enzyme produced was 131.68 U/g—retaining 84.6% productivity compared to the optimum level. Production was relatively low in 12% sodium chloride, at about 43.14% (67.16 U/g) of the higher production observed at the optimum concentration (Fig. 5). The variation in protease production between sodium chloride concentrations was statistically significant ($P<0.0001$). This study inferred that this bacterium is moderately halophilic because it is an estuarine fish gut isolate. In estuarine environments, the influence of salinity is greater due to its frequent fluctuation. The enhanced protease production in medium supplemented with 4% sodium chloride is the result of a requirement for salt in order to maintain the osmotic balance of this bacterium. This present results are in accord with the salinity induced protease production reported in *Bacillus* sp. (Patel et al. 2006) and *Bacillus subtilis* at 10% sodium chloride (Setyorini et al. 2006). This study also correlates with the results of Vidyasagar et al. (2007) on protease production by *Chromohalobacter* sp. at 4 M sodium chloride concentration.

Enzyme recovery is a critical process in SSF. The selection of a suitable solvent is an important factor and hence, in the present work, seven different solvents were tested for enzyme extraction. Two-way ANOVA indicated that the variation in protease extraction due to solvent was statistically more significant ($P<0.001$) than variation due to extraction time ($P<0.01$). Among the solvents used, ethyl acetate (10%) gave maximum recovery (130.52 U/g), and glycerol gave low enzyme recovery (91.12 U/g) (Fig. 6). Extraction by distilled water, Tris buffer and ethanol also resulted in low recovery when compared to ethyl acetate. In this experiment all these solvents had an optimum extraction time of 1.5–2 h, with maximum protease being extracted within this period. Ethyl acetate was used as a potent solvent for the extraction of microbial metabolites, especially antimicrobial products, from fermented broth (Sunish Kumar et al. 2005; Patel et al. 2009). In the case of protease extraction, such attempts have been quite limited. Tunga et al. (1998) reported that a mixture of ethanol (10%) and glycerol (3%) was efficient for the extraction of protease produced by *Rhizopus oryzae*. Agrawal et al. (2005) found that extraction of protease produced by *Beauveria felina* under SSF condition was high in 1% sodium chloride.

Conclusion

Proteases are industrially important enzymes with many applications, especially in the food industry. The enzyme from fish intestinal bacteria is an unexploited bioresource for enzyme production. The present study reports the production of protease by the *Etroplus suratensis* intestinal isolate *Serratia proteamaculans* AP-CMST under SSF of anchovy waste meal. Successful SSF of anchovy waste meal under optimised environmental (pH, temperature and moisture level) and nutrient (carbon, nitrogen, trace elements, surfactants, and sodium chloride) conditions yielded maximum protease production. This proteolytic bacterium could be used effectively for protease production from low cost marine protein resources and also as a microbial probiotic in the formulation of functional aqua feed with high efficiency.

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