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Solid State Protease Production by *Bacillus thuringiensis* AP - CMST using Trash Fish Meal as Substrate

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A proteolytic *Bacillus thuringiensis* AP-CMST was isolated from the gut of estuarine fish *Etroplus suratensis* and the factors affecting protease production in solid-state fermentation was assessed using trash fish - *Odonus niger* meal and the production was utmost in pH 7 and temperature 40°C. The effect of carbon and nitrogen sources on the protease production indicated that it was maximum in maltose and beef extract supplied medium. The other suitable nutrients observed for better protease production were Tween 60 and magnesium sulphate. The inorganic phosphates have little influence on protease production, though it was maximum in diammonium hydrogen orthophosphate added medium. The effect of various concentrations of NaCl indicated that the maximum enzyme production was noticed in 4% of NaCl.

Key words: Protease, Solid-State Fermentation, Trash Fish Meal, Beef Extract.

Extracellular proteases catalyze the hydrolysis of large proteins to smaller molecules. Proteases are used for various industrial applications, such as laundry detergents, leather preparation and food industry. Proteases are used best component for the development of biopharmaceutical products such as contact lens cleaner and they are also used to clean wide variety of polymeric protein substances[1]. Proteases are among the most important industrial enzymes accounting for 60% by mass of the total worldwide enzyme production[2]. Plants, animals and microbes are the popular sources of industrial protease. Among the proteases, microbial proteases are the most significant, because they are renewable sources and can be produced in large quantities, compared with animal and plant proteases. Microbial proteases also represent one of the largest classes of industrial enzymes, accounting for 40% by value of the total worldwide sales of enzymes[3].

Proteases can be produced either by submerged (SmF) or solid-state fermentation (SSF) methods. Among these two methods solid-state fermentation is pioneer technique to utilize the low cost and organic waste materials for the production of valuable biochemicals[4]. Moreover, the microbial protease production is varied by media composition such as carbon and nitrogen sources, metal ions, salts, surfactants etc. The other environmental factors such as incubation period, pH, temperature and aeration are also influenced the microbial protease production. Considering this fact the present study was designed to investigate the factors influencing protease production by fish intestinal *Bacillus thuringiensis* AP-CMST using fish processing industry wastes. *B. thuringiensis* is especially well known for biological insecticide production, but the enzymatic activity especially protease activity was not studied much, excepting few, an alkaline[5, 6] as well as neutral[7] proteases

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production by *B. thuringiensis*. Rojas-Avelizapa *et al*.[8] also reported the protease production by *B. thuringiensis* cultured in shrimp waste media. Fish processing wastes have a huge unexploited potential for value adding and every year tons of these material are simply dumped into the sea. Recent years these wastes considered as an inexpensive substrates for microbial fermentation. Esakkiraj *et al*., used different forms of tuna-processing waste such as raw fish meat, defatted fish meat, alkali hydrolysate, and acid hydrolysate as nitrogen source for protease production by *Bacillus* cereus in shake flask experiments and obtained maximum protease in defatted fish meat compared to that of commercial peptone[9]. Considering the importance, the present study was designed to optimize protease production by fish intestinal isolate *Bacillus* thuringiensis under SSF using processing wastes.

MATERIALS AND METHODS

Microorganism and protease activity

The strain AP-CMST used in this study was isolated from the gut of Etroplus suratensis collected from Rajakkamankalam estuary of Kanyakumari District, Tamilnadu, India by following the method described by Arbaciauskiene *et al*.,[10]. AP-CMST was observed as a strong protease producer in skim milk agar plates and it was identified as *Bacillus* thuringiensis by using 16s rRNA gene sequencing and analysis (GenBank accession no. HM623612).

Inoculum preparation and solid-state fermentation (SSF)

Fish processing wastes materials like viscera, head, tail etc. were collected individually for red snapper, tuna, red grouper and ray fish from fish processing centers of Kanyakumari District, Tamilnadu, India. The waste materials of individual fishes were made into powder form by following the procedure of Souissi *et al*.,[11]. Along with these wastes, low cost fish meal from balistid fish *Odonus niger* was also screened. The maximum protease producing substrate was taken for further study. Then 5 g of dry substrate was taken into a 250 ml Erlenmeyer flask and to this a salt solution (5 ml) containing K2HPO4 - 0.1 %; NaCl - 1 %; MgSO4.7H2O - 0.01 % and NH4NO3 - 0.5 %, and distilled water was added to adjust the required moisture level. The contents of the flask were thoroughly mixed, autoclaved, cooled to room temperature and inoculated with 24 h old fresh culture grown on medium containing (w/v) beef extract-0.15%, peptone-0.5%, NaCl- 1% and glucose- 0.5% and pH-7. Fermentation was carried out at 32°C for 24 h with initial moisture level of 85% and inoculum size of 2 ml, and this level were followed in all experiments. After incubation, 50 ml of distilled water was added into fermented solid medium and placed into a shaker at 150 rpm for 1 h. Then it was filtered through cotton cloth and was harvested by centrifugation at 10,000 rpm for 15 minutes. The supernatant was used for protease assay.

Protease assay

For measuring protease activity, 0.25 ml of culture supernatant was mixed with 0.5 ml of 1% aqueous casein solution and 1.25 ml Tris buffer (100mM, pH 7.2) and incubated for 30 min at room temperature. The reaction was terminated by adding 3ml of 5% TCA and it form precipitate. Further it was placed in freezer at 4°C for 10 min and was centrifuged at 5000 rpm for 15 min. From this 0.5 ml supernatant was taken and was added with 2.5 ml of Na2CO3 (0.5 M), mixed and incubated for 20 min at room temperature. To this mixture 0.5 ml of folin - phenol reagent was added and the absorbance was read at 660 nm using UV-Vis spectro-photometer. The amount of protease produced was measured with the help of tyrosine standard graph. Based on the tyrosine released, the protease activity was expressed in units per gram dry substrate (U/g)[12]. All the experiments were done in three sets and average values were reported.

Effect of nutritional and environmental factors on solid-state protease production

In the present study the solid-state protease production by *B. thuringiensis* AP-CMST was performed with varying physical parameters (pH and temperature), nutrient sources (carbon and nitrogen), surfactants, trace elements, phosphates and sodium chloride.

The effect of initial pH on solid-state production of protease was determined. For this the mineral media was individually adjusted with different pH. The tested pH were 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12. The effect of temperature on solid-state fermentation of protease production was determined by incubating the solid medium with

different temperatures such as 10, 20, 30, 40, 50, 60, 70 and 80°C.

The influence of carbon sources (0.5% w/v) on protease production was investigated by using 8 different carbon sources including glucose, sucrose, maltose, xylose, sorbitol, fructose, lactose and galactose. For the selection of suitable nitrogen source for protease production, 8 different nitrogen sources were screened at 1% (w/v) concentration in the mineral medium as follows; beef extract, yeast extract, peptone, skim milk powder, tryptone, urea, ammonium chloride, ammonium sulphate. The medium devoid of any of these carbon and nitrogen sources was used as control.

Different inorganic phosphate sources including diammonium hydrogen orthophosphate, disodium hydrogen phosphate, trisodium ortho phosphate, calcium hydrogen phosphate, potassium dihydrogen phosphate, bismuth phosphate, sodium dihydrogen orthophosphate were assessed (0.1% w/v) for their influence on protease production and media without phosphate source was taken as control. The different surfactants like Tween 20, Tween 40, Tween 60, Triton x 100, sodium dodecyl sulphate (SDS), polyethylene glycol (PEG) were screened (0.2 % (w/v) for their role on protease production and the media without surfactant was taken as control

As the bacterium is isolated from estuarine fish, and hence the effect of various concentrations (w/v) of NaCl (1, 2, 3, 4, 5, 6, 7, 8, 9 and 10%) were also tested for its efficiency to produce protease. Nine different metal ions such as magnesium sulphate, magnesium chloride, barium chloride, calcium chloride, copper sulphate, zinc sulphate, zinc chloride, mercuric chloride and EDTA were also individually screened at 0.05% (w/v) concentration for their effect of protease production and the media with out metal ion is taken as control.

RESULTS AND DISCUSSION

The present study on solid-state fermentation of fishery by products and low cost fish meal showed that trash fish *O. niger* meal had been readily utilized by *B. thuringiensis* AP-CMST for higher protease (17.0 U/g) production. From this result, it could be concluded that protein rich wastes from fishery by products can be efficiently utilized for protease production. The wastes of ray fish (12.74 U/g) and tuna (9.23 U/g) were also supported the protease production by B. thuringiensis AP-CMST. Red grouper wastes (8.5 U/g) and red snapper (5.6 U/g) produced lower protease than others. Fish wastes are considered as unexploited potential for value adding and in our area, it was also estimated that 1200-1500 tones of *O. niger* is being wasted every year in the coastal belt of Kanyakumari District, South-East coast of India[13]. Comparing to agroindustrial wastes fishery wastes can be readily utilized or spoiled by the bacteria because they are rich in nitrogen and this fishery wastes was also proved as a good substrate for also instead for microbial growth[14]. Recent studies also proved that fishery wastes can be used for industrial production of protease. Souissi *et al*.[15] used cuttle fish waste water to produce protease by *B. subtilis*. Low cost fish meal from Sardinella meal was used for protease production by *B. subtilis* and *Pseudomonas aeruginosa* MN7[16, 17].

Temperature is a vital physical factor that controls the protease production and also the

Table 1. Effect of carbon and nitrogen sources on protease production

Carbon sources	Protease (U/g)	Nitrogen sources	Protease (U/g)
Control	20.53	Control	20.34
Glucose	27.36	Beef extract	32.2
Sucrose	29.16	Yeast extract	26.4
Maltose	61.36	Peptone	24.65
Xylose	59.9	Skim milk powder	28.87
Sorbitol	23.42	Tryptone	17.6
Fructose	31.6	Urea	14.8
Lactose	30.2	Ammonium chloride	10.6
Galactose	34.49	Ammonium sulphate	9.9

temperature affects directly the protease production in solid-state fermentation and this was reported in many species. The present study on the effect of temperature on SSF of protease production showed that the temperature range of 30-40°C was the optimum for protease production (Fig. 1) (16.5 U/g). The higher tested temperatures may affect the growth rate of the bacterium and

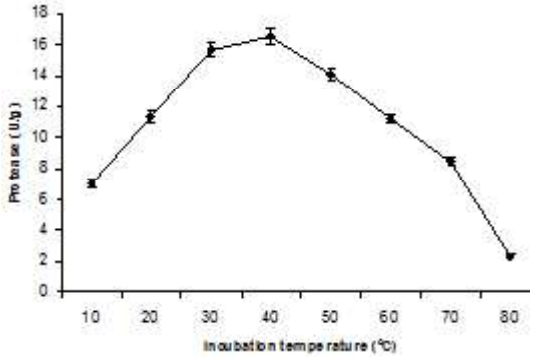

Fig. 1. Effect of incubation temperature on protease production

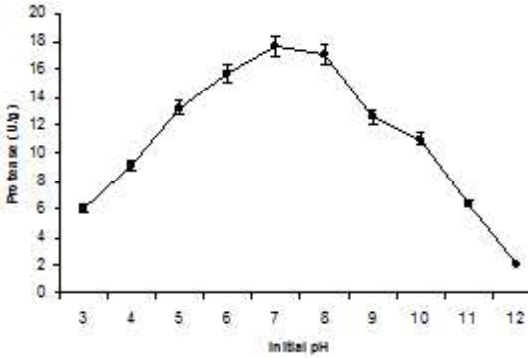

Fig. 2. Effect of initial pH on protease production

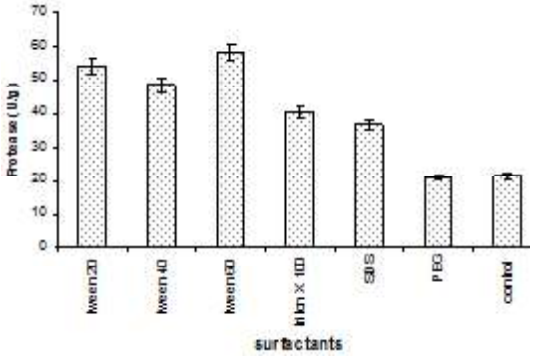

Fig. 3. Effect of surfactants on protease production

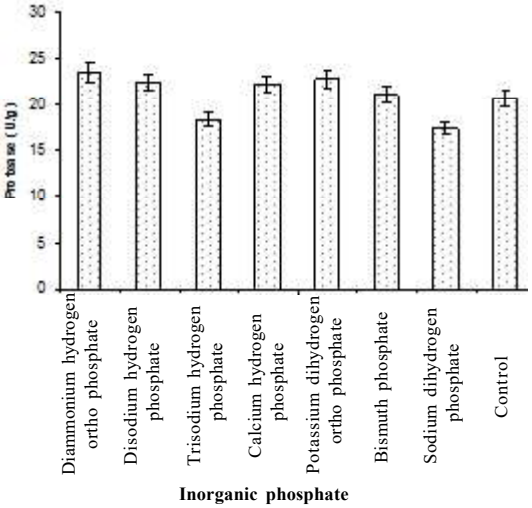

Fig. 4. Effect of inorganic phosphates on protease production

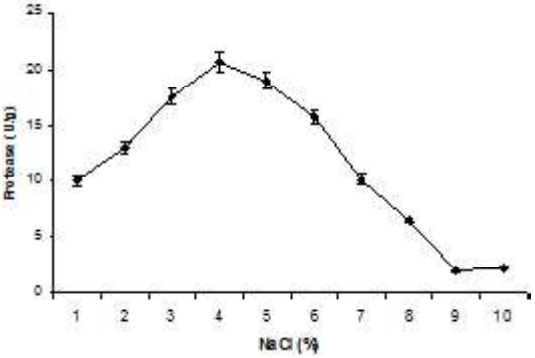

Fig. 5. Effect of different concentration NaCl on protease production

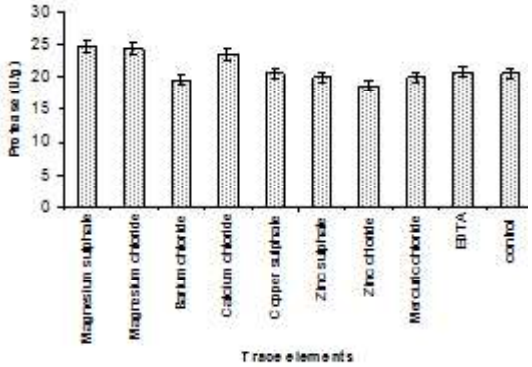

Fig. 6. Effect of different trace elements on protease production

subsequently inhibit the protease production. Similarly Kumar *et al*.[18] reported that the keratinase production by *Bacillus subtilis* (MTCC 9102) in solid-state fermentation using horn meal had its optimum temperature of 37°C. Supporting the present results, protease production using molasses as a substrate by *B. pantotheneticus* had the optimum temperature of 30°C[19].

Like temperature, pH also has direct effect on protease production. In this study, the effect of various pH on protease production showed the pH 7 (17.64 U/g) was the optimum for maximum protease production (Fig. 2). The acidic or alkaline pH has profound effect on this bacterium; they drastically reduce the protease production by this bacterium. Earlier studies demonstrated the highest protease production at pH 7 by selected proteolytic microorganisms. Elibol and Moreira[20] found that maximum protease production by marine bacterium *Teredinobacter turnirae* was at pH 7. Likewise, Agrawal *et al*.[21] reported that protease production under SSF by *Beauveria feline* was higher at pH 7.

The effect of carbon sources supplementation on protease production showed that it is essential to supply carbon sources with *O. niger* meal, because it improves the protease production to a higher level especially in maltose (61.36 U/g) and xylose (59.9 U/g) supplied medium. The other carbon sources gave only 50% protease production when compared to maltose and xylose (Table 1). Similar to this study, Prakasham *et al*.[22] reported that, the protease production under SSF by *B. clausii* sp improved in higher extent with the addition of maltose and xylose. Maltose is a better carbon source that improved protease production in *Bacillus* sp [23]. The protease production by *B. clausii* I-52 [24] was also an added evidence for the positive effect of maltose on protease synthesis. However, glucose, sucrose, fructose and some other carbon sources reduced the protease production and this may be due catabolic repression [25].

Nitrogen sources induced protease production inferred that, beef extract found to produce maximum amount (32.2 U/g) of protease than other tested nitrogen sources (Table 1). On the other hand among selected nutrients, inorganic nitrogen sources supplemented with trash fish meal drastically reduced the protease production. The drastic inhibitory effect of beef extract on protease production was also proved in *Bacillus* sp. [26]. But solid-state protease production by B. subtilis using Imperata cylindrical grass was improved with beef extract [27]. Similar improved production was also reported in statistical experiments in the case of protease production by *Bacillus* sp. and *Bacillus* sp. RKY3[28,29]

Surfactants are also a critical medium component for enhancing the fermentation condition. In the present study, SSF of protease showed that surfactants such as Tween 60 (58.2 U/g), Tween 20 (54.13 U/g) and Tween 40 (48.53 U/g) played main role in positively enhancing the protease production (Fig 3). Among the tested surfactants only polyethylene glycol (20.96 U/g) had negative impact on protease production when compared with control (21.5 U/g). Surfactants are mainly used in fermentation medium to enhance the surface tension. They are also used to stimulate the enzyme production [30]. Because they are active on cell wall and cytoplasmic membrane and make the cell permeable to these extracellular enzymes and other protein materials bound in cell membranes[31]. In the present study the protease production is more specific to non-ionic surfactants such as tweens. This results correlate with the maximum protease production by *Bacillus* sp. to Tween 20[32]. Zeng *et al*.,[33] in their study on extracellular enzyme production by Penicillium simplicissimum reported that, protease production increased much with different concentrations of Tween 80.

One of the important minor nutrients in fermentation media is phosphates. The effect of phosphate supplementation on protease production showed that, except trisodium ortho phosphate (18.36 U/g) and sodium dihydrogen phosphate (17.46 U/g) all other phosphates showed positive influence, but slightly enhanced the protease production. Among the phosphate tested diammonium hydrogen phosphate (23.49 U/g) produced maximum amount of protease over the control (20.7 U/g) (Fig 4). In agreement with the positive effect of majority of phosphates, Mehta *et al*.[34] reported that the protease production by *Streptomyces* sp. was greatly improved by addition of various concentrations of di potassium hydrogen orthophosphate.

Bioprocessing with halophilic microbes entirely requires NaCl for their successive growth

and biochemical production. In the present study, the bacterium used is estuarine in nature and it absolutely required 4% NaCl (20.7 U/g) for maximum protease production (Fig 5). Moreover the salinity in the ocean normally presents in this range and estuarine salinity is fluctuating near to ocean, but it depends upon tidal action. Shanmughapriya *et al*.,[35] reported that protease production by marine *Roseobacter* sp. (MMD040) have 2% NaCl as optimum for higher protease production. Concurrent to this study, some bacterium of marine origin requires more amount of NaCl. Patel *et al*.[36] reported that, *Bacillus* sp. isolated from seawater of western India requires 10% NaCl for optimum protease production.

Metal is also a minor nutrient, which also has the tendency to influence the protease production. The present study on the effect of metal ions on solid-state production resulted that, magnesium chloride (24.6 U/g), magnesium sulphate (24.23 U/g) and calcium chloride (23.38 U/g) have found to improve the protease production over the control (Fig 6). Although the metal ions are essential, but the type of metal ions requirement is depends upon the type of protease produced. Similar to this present study Vidyasagar *et al*.[37] reported that halophilic archaeon *Halogeometricum borinquense* requires more specifically $CaCl_2$ for maximizing the protease production. Similarly Rahman *et al*.,[38] reported that *Pseudomonas aeruginosa* strain K prefers K^+, Mg^{2+} and Ca^{2+} as the suitable metal ions for maximizing the protease production.

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