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Investigation on influence of cultural conditions in submerged fermentation for protease enzyme by *Bacillus subtilis*

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ABSTRACT

Extracellular alkaline protease produced by an isolate from soil was preliminarily identified to be *Bacillus subtilis*. Studies on submerged fermentation revealed that maximum level i.e. 321 µg/ml/min of protease production was observed during early stationary phase. Optimization of fermentation medium for characterization of protease was carried out. Optimization parameter includes incubation period, temperature, pH, substrate concentration, carbon sources, nitrogen sources and tween 80 concentrations. The optimized conditions found for protease production were 40^0C at pH 10 (alkaline condition), with 10% inoculums, 2 ml tween 80, 1% sucrose, 1% ammonium chloride and 20% casein, after 72 h of incubation stimulates protease production. The protease profile of the selected isolate shows its potential for its industrial applications.

INTRODUCTION

Protease enzymes are essential components of all living beings including prokaryotes, fungi,plants and animals. Protease is most applicable enzyme on commercial level like detergents, leather industries, food and pharmaceutical industries[1,3,26]. It is also used in brewing, baking, meat tenderdization, peptide synthesis, medical diagnosis, cheese making, in medical treatment for inflammation and in unhairing of sheepskins. It has wide applications in bioremediation[4,5]. Protease occurs in animals, plants and micro-organisms. Most important groups of proteases includes, serine protease (E.C.3.4.21), cysteine (thiol) protease (E.C. 3.4.22), aspartic protease (E.C. 3.4.23) and metalloprotease (E.C. 3.4.24)[16]. Being in microbial origin, protease accounts about 60% of the total enzyme sale in the world with 2/3rd of this amount[6]. A variety of microorganisms such as bacteria, fungi, yeast and actinomycetes are known to produce these enzymes[7]. Selection of efficient micro-organism plays an important role in higher yield of enzymes producing enzyme on industrial level, isolation and characterization of new promising strains with cheap carbon and nitrogen source is a continuous process. Protease secreted from thermophilic bacteria have become increasingly useful for a range of commercial application due to faster reaction rate, increase in solubility of non gaseous reactants and products and minimum risk for contamination by mesophilic microorganisms[7]. *Bacillus sp.* and *Aspergillus sp.* are found to possess protease activity on a large scale basis. Molds of the genera *Aspergillus, Penicillium* and *Rhizopus* are especially useful for producing proteases, as several species of these genera are generally regarded as

safe[8]. During large scale production, heavy metals plays an important role for protease fermentation. For the production of enzymes for industrial use, isolation and characterization of new promising strain is a continuous process[23]. They are generally produced by using submerged fermentation due to its apparent advantages in down streaming besides the cost intensiveness for medium components[10,18,27]. In this context, overall yield of enzyme production is influenced by physicochemical conditions. Thus, in our laboratory, preliminary we carried out experimentation in order to determine various environmental factors in submerged fermentation for proteolytic activity under stander condition. This paper particularly focus on effect of different cultural conditions on protease fermentation by *Bacillus subtilis* isolated from dairy waste contaminated soil.

MATERIALS AND METHODS

Collection of soil nearby dairy effluents

Ten different soil samples were collected from the vicinity of dairy effluents of Jalgaon Jilha Sahakari Dudh Utpadak Sangh Maryadit, Jalgaon. All these samples were refrigerated and subsequently used for screening of protease producing strains.

Screening of proteolytic activity

Screening and enrichment of strains capable of protease production was made by inoculating one gram of soil sample in nutrient broth containing 1% casein followed by incubation at 37 ^{0}C for 48 hrs. Streaking, a loopful of inoculums from enriched sample on sterile skim milk agar plate containing 1% of casein. Casein degradation ability of the microorganism was confirmed by appearing a clear zone around the colonies after incubation of 48 h at 37^{0}C.

Selection of potent strains with proteolytic activity

Protease fermentation and Proteolytic assay

After screening, isolation procedure capability of each selected strain was tested in sterile liquid minimal medium as per suggested by Naidu and Devi[19]. The strain and the minimal medium showing maximum results for protease production were selected for further experimentation.

Protease activity was assayed by the modified method; minimal media with inoculums is incubated at 37^{0}C for 48h. After incubation, sample was allowed for centrifugation, supernatant were collected and used for protease assay.

Photoplate 1 : Proteolytlc activity shown by the screened isolate

Identification of isolates

Mixed microbial cultures were repeatedly streaked on sterile nutrient agar plates containing 1% casein. These cultures were preliminarily identified by doing Grams staining, motility; with other cultural characteristics like colour of colony, opacity, elevation, consistency for bacterial colonies. Identification was carried out under laboratory conditions[11-13].

Mass cultivation of proteolytic microorganism

Erlenmeyer flask (500 ml) containing sterile skim milk broth was inoculated with 10ml enriched inoculums and incubated for 48 h at 37^{0}C in static condition. The pellets were harvested after cultivation and then used in degradation studies.

Submerged fermentation for protease

Fermentation medium for protease studies was carried out in yeast extract casein medium as per given by Naidu and Devi et al[19].

Factors affecting proteolytic activity

Optimization parameters plays important role in large scale industrial fermentation processes. For developmental activities of fermentation products, most influencing factors on it includes, incubation period, incubation temperature on stability of protease, pH, substrate concentration, carbon source and nitrogen source and effect of Tween 80. These factors were studied in terms of experimentation in order to develop fermenta-

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tion on large scale. All the experimentation was carried out in triplicates and mean values were taken as final values.

RESULTS AND DISCUSSION

All forms of life on earth including prokaryotes, fungi, plants and animals have an integral constituent occupying and that is protease. Proteases occupy nearly 60% of enzyme sales, procured from microbial, plant and animal sources[15]. There are ever high exploitation of alkaline proteases in industrial sector like food processing, leather, detergent, pharmaceutical, diagnostic, waste management, silver recovery, medical purposes, feeds and chemical industries[9].

Microbial alkaline proteases dominated worldwide the overall enzyme market mainly two third of share of the detergent industry[2]. As compared to protease production from plants and animal sources, it is ease to produce protease by microbial fermentations.

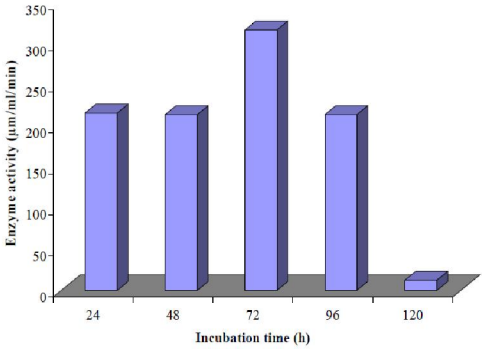

Figure 1 : Influence of incubation period on protease activity

Collection of ten different soil samples from Jalgaon Jilha Sahakari Dudh Utpadak Sangh Maryadit, Jalgaon, were done. All these samples were refrigerated and then allowed for subsequent screening, isolation and identification of protease enzyme. Screening, isolation procedure was followed for protease production. A strain which hydrolyses the skim milk agar and the zone of hydrolysis on skim milk agar ensures the confirmation of protease secreting ability of microorganism (Figure 1). The highest activity of protease was recorded after 72 h of incubation. All these strains were examined for stability in selected medium and maximum proteolytic activity. One of the bacterial strain showing satisfied results, easy cultivation was selected for fermentation process. Selection of potent strains with proteolytic activity was done by studying protease fermentation and carrying out proteolytic assay.

Protease producing capacity

Twenty three out of thirty bacterial isolates exhibited various degrees of proteolytic activities when cultivated on skim milk agar. Selection criteria when applied for maximum proteolytic activity, only one strain was found to exhibit maximum activity of enzyme.

Proteolytic assay

Proteolytic activity was assayed after carrying fermentation of protease enzyme at 37^{0}C for 48 h. Following 48 h of incubation, 5 ml fermented broth was allowed for centrifugation and supernantant collected was used for proteolytic assay.

Identification of bacterial isolate

Identification key was followed for identification of *B.subtilis*[11-13].

Optimizing parameters for proteolytic activity

Incubation period

Protease activity was found to be maximum after 72 hrs of incubation. The enzyme activity was found to be in decreasing order during 96 hrs of incubation (Figure 1). On the contrary findings of Kumar et al, 2002[23], reported that *Pseudomonas sp.*S22 showed a peak for protease production at 24 hrs of incubation and again peaks at 108 hrs.

Incubation temperature

There is a specific temperature for each microorganism to grow and produce enzymes. It is generally observed that, as there is increase in temperature which leads to increase in reaction rates. The reason behind this might be due to alteration or denaturation of active site. There are misleading results on idea of an optimum rate of enzyme reaction. This is because rate of enzyme activity observed at any temperature is due to products of two rates, the reaction rate and the denaturation rate. If any enzyme is observed for its activity for one second, it would give high activity at high temperatures but if same reaction is observed for one hour, then it would give low activity at these temperatures.

In experimentation with *Bacillus subtilis*, 40^{0}C temperature was showing its maximum protease activity

(310 µg/ml/min). After, 45^{0}C there is decline in enzyme activity i.e. 277 µg/ml/min (Figure 2). Same results were interpreted by Sen et al, 1993 using *B. licheniformis* and for *B. subtilis*.

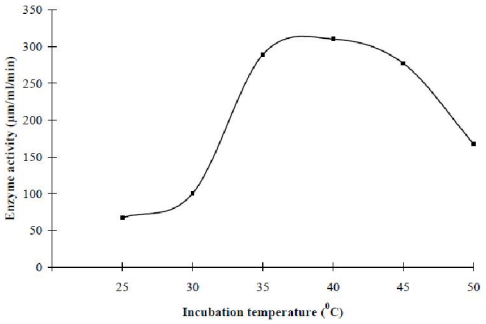

Figure 2 : Influence of temperature as an optimizing parameter for protease activity

Effect of pH

Culture medium pH strongly affects many enzymatic processes and transport of compounds across cell membrane. Maximum protease (317.88µg/ml/min) production was observed at pH 10 by *Bacillus subtilis* (Figure 3). As pH increases, decrease in protease level occurs. Same results were recorded by Kumar *et al*, 2002[23] who has reported that protease production was maximum at pH 7 and 9 for *Bacillus subtilis* strain S4 a *Pseudomonas* spp S22 respectively. Borris[25] and Sen, 1993[24] also reported alkaline protease active at pH range 9 to 13.

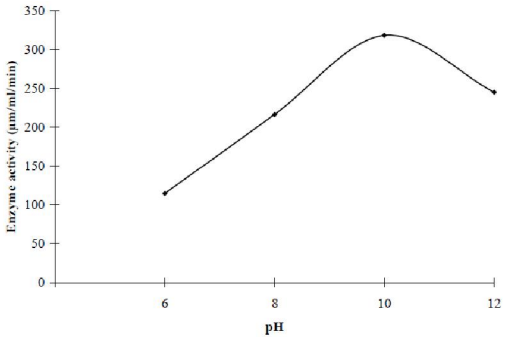

Figure 3 : Influence of pH on protease activity

Substrate concentration

Effect of different casein (Substrate) concentrations including in range of 5 to 25% on protease production was carried out under laboratory level. In parallel set of experimentation effect of casein concentration on total number of protein concentration was also carried out. At 20% optimized concentration of casein, enzyme activity was found to be 320.77 µg/ml/min (Figure 4)

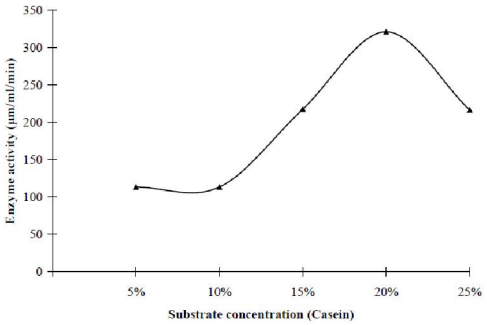

Figure 4 : Influence of different substrate concentrations on protease activity

Carbon source

At one percent concentration of glucose, lactose, sucrose, maltose and starch were tested for their effect on protease fermentation. It was found that sucrose accelerates production of protease while as starch showed its unfavorable condition for stimulation of protease (321 µg/ml/min, Figure 5). Addition of carbon source in the form of monosaccharide's and polysaccharides could influence production of enzyme[20]. Out of wheatbran and lentil husk, wheat bran showed highest protease production in *Bacillus spp*.

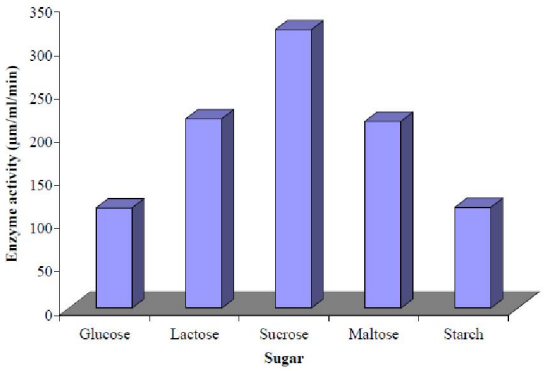

Figure 5 : Effect of various sugar concentrations on protease activity

Nitrogen source

Various nitrogen sources like sodium nitrate, ammonium chloride, urea, ammonium phosphate and casein were examined for production of protease by replacing 0.5 % yeast extract in the production medium. Caesin and peptone were recorded to be best nitrogen sources for protease fermentation by *Prevotella ruminicolo 23* by Wang and Hsu[21]. In parallel experimentation,[22] reported soyabean meal as best nitrogen source. In our experimentation by

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B.subtilis, ammonium chloride was found to be best nitrogen source for protease fermentation.

Effect of tween 80 on protease production

Surfactants plays an important role in cleaning, wetting, dispersing,emulsifying and anti foaming in many practical applications and products including detergents, emulsion etc. The stability of enzymes remains a critical aspect in pharmaceutical and industrial biotechnological applications. Tween 80 ranging from 0.5 % to 5% on protease fermentation. It was found that at 2ml concentration maximum protease activity 210.39 µg/ml/min was observed (Figure 7.0). Gouda M., 2006[28] studied two protease that were found to be very stable aginist non –ionic surfactants such as tween 80 and triton 100.

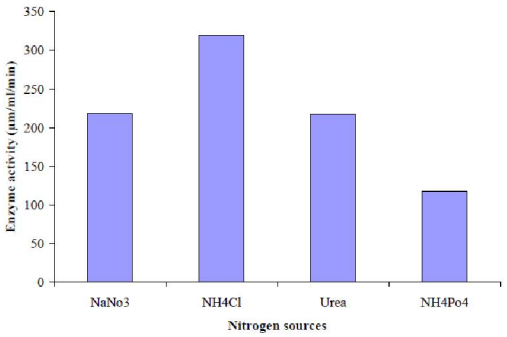

Figure 6 : Effect of various nitrogen sources on protease activity

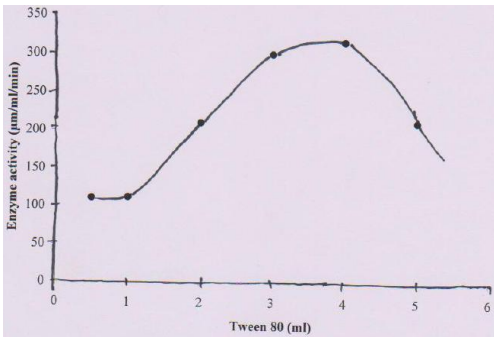

Figure 7 : Effect of tween 80 on protease activity

CONCLUSION

The results obtained in the present study indicated that *Bacillus subtilis* could be a potential strain for protase production by submerged fermentation with casein as a substrate. The enzyme production was influenced by various physiological and chemical natures of the substrate as well as medium ingredients during submerged fermentation. Among the various parameters screened for submerged fermentation like pH, temperature, incubation period, carbon and nitrogen sources played an important role for higher amount of protease production. After optimization of individual parameters separately when further experiment was carried out with all optimized parameters, protease production was increased as compared with previous one.

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