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Studies on screening of *Bacillus* sp. for Protease Production

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ABSTRACT: Screening of bacteria and other organisms for protease production has gained momentum in recent times. Proteases are hydrolytic enzymes capable of degrading proteins into small peptides and amino acids for industrial applications. Proteases are widely used in food, pharmaceutical, leather, detergent industry and other industries. This review summarizes various aspects of proteases and their conditions of production. The enzymatic and physicochemical properties of alkaline proteases obtained from different sources under different environmental conditions are discussed.

Keywords: Screening, Protease, industry, environmental conditions

I. INTRODUCTION

The metabolic processes of the cells to form different parts under the influence of different enzymes that act as biocatalysts to initiate various biochemical reactions has bought new revolution in different industrial sectors. Till date 3000 different enzymes have been recognised and many of them are used in biotechnological and industrial applications. Enzymes are commercially utilized in the different process like detergent, food, pharmaceutical, diagnostics, and fine chemical industries [1]. Very less efforts and little progress have been noteworthy for commercial success in biotechnological enzyme production processes [2]. Based on the response to different NaCl concentrations Halophiles inhabit extreme environments and they can be classified into three groups on the basis of their response to NaCl. Slight halophiles which breed optimally at 2–5% NaCl, moderate halophiles show rapid growth at 5–20% NaCl and severe halophiles which optimally grow at 20–30% NaCl. Halophilic enzymes have exclusive enzymatic functions compare to non-halophilic enzymes. It requires high salt concentrations in the range of 1–4 M for higher activity and longer stability [3]. Approximately 60% of the total enzyme sales in the world account for microbial proteases [4]. Proteases are one of the most significant groups of industrial enzymes with wider applications that range from meat tenderization to silver recovery [5]. However, the production of intracellular and extracellular enzymes on an industrial scale is dependent on microorganisms that play a vital role in this technology [6,7]. Hence, to get maximum benefit, selected organisms are grown in fermenters under optimum conditions and can be further used to make different products [8–9].

Bacillus species are the essential producers of extracellular proteases, and industrial sectors mostly use *Bacillus subtilis* for the production of various enzymes. However, not all the enzymes are used for industrial process, proteases are the main enzymes produced from microbial sources, of which only few are recommended as commercial producers. *B. subtilis* also known as hay *Bacillus* and grass *Bacillus* is found mainly in soil and a rod-shaped organism, which can form a tough, protective endospore and can have the tendency to withstand severe environmental conditions. They are obligate aerobes or facultative anaerobe and include both free-living and pathogenic species [10,11]. Protease enzymes occupy a significant position in the enzyme technology as they are widely used in different industries [12]. *B. subtilis* is widely used for the production of specific chemicals and industrial enzymes [13,14,15]. Proteases have found their use in contact lens cleaners and enzymatic debriders [16] and help in the natural healing process in local management of skin ulceration by efficient removal of necrotic material [17]. These enzymes have found their use in efficient management of waste as well.

Bioconversion of wastes into useful biomass by microorganisms and their enzymes is a new tool, and new protease-producing microorganisms and perfected fermentation technology are required to meet the demand for this enzyme [18]. Hyperactive strains are being sought for use in different industries [19]. *Bacillus* species were found highly stable to high thermal and pH stability. Now a days, isolation and characterization of new promising strains using cheap carbon and nitrogen source is being tested all over the globe [20].

Suganthi *et al* 2013 argued that protease producing halotolerant bacterium was isolated from saltern pond sediment (Tuticorin) and identified as *Bacillus licheniformis* (TD4) by 16S rRNA gene sequencing. Protease production was increased by optimizing the culture conditions. The nutritional factors such as carbon and nitrogen sources, NaCl and also physical parameters like incubation time, pH, agitation, inoculum size were optimized for the maximum yield of protease. Studies on the effect of different carbon and nitrogen sources revealed that xylose and urea enhances the enzyme production. Thus, with selected C–N sources along with 1 M NaCl the maximum protease production (141.46 U/mg) was obtained in the period o 24 h incubation at pH 8 under 250 rpm compared to the initial enzyme production (89.87 U/mg).

Gaurav *et al* 2015 reported the production of thermostable protease and its characterization in *Bacillus* species, which is a thermotolerant bacterium; *Bacillus subtilis* is widely used for isolating protease enzyme. Gelatin was used as the substrate in nutrient agar medium for screening and showed the maximum zone of activity (22 mm) after overnight incubation and addition of the indicator. Under submerged fermentation conditions, a high level of protease production was found at 45 °C after 36 h at pH 10, with continuous agitation (180 rpm). The presence of galactose and peptone in the medium enhanced enzyme production by 0.5% when compared with other carbon and nitrogen sources. Thus, such additions can augment protease production and their application in various industries. The avenues in the field of protease screen have opened new ways to commercially utilize this natural service. Hence, the objective of the present study was to review the applications of protease, optimal conditions for its productions, and screening of protease.

A. Habitat of Protease producers

Protease producers are almost found in every type of environment. There are reports from dumping site at Langol, Manipur, India [23]; Egyptian soda lake [24]; Alkaline lonar lake Maharastra, [25]; hot springs, Jordan [26]. The samples collected from leather factories [27,28], food processing industrial effluent [29], wood factory [30], detergent industry [31], milk processing plant [32] and industrial waste such as tannery waste [33,34, 35], also contain protease producers. Sugarcane molasses [36], Thai fish sauce [37], Thua nao [38] vegetable waste [39], animal dung [40,41,42], degraded and fresh meat [43] and buffalo hide [44], mangrove sediment sample [45], compost containing dead animal's remnants [46], vermicompost pit soil sample [47], sewage sludge sample [48], meat waste contaminated soil [49] and soil from poultry waste site [50], fruiting body of the edible mushroom *Pleurotus citrinopileatus* [51]. Thirty-nine, protease producing, *Streptomycetes* were isolated from sewage of tanneries and soil around it, agricultural soil, agricultural fields, water sources from a fish farm, sediments with neutral and alkaline pH and Qaroun lake [52].

1. Indicators for protease secretion

a. pH. The transportation of various components across the cell membrane is dependent on the pH of the medium. The molecular basis of pH affecting bacterial metabolism in culture broth is difficult to understand. However, the proton motive force in chemiosmosis is influenced by the pH of medium, it may be feasible that under optimum pH range, the relative metabolic efficiency is maximum [53]. Therefore, pH needs to be neutralised as it is reported that medium with neutral initial pH for alkaline protease production by *P. chrysogenum* IHH5 [54], *S. roseiscleroticus* [55], *B. Cereus* [56] *Bacillus polymyxa* [57], *Bacillus aquimaris* VITP4 [58] and *P. Aeruginosa* MCM B-327 [59] revealed better results. However, at pH 6.3–6.5 protease production by *Bacillus* sp yielded optimum results. The results may vary from one species to the other, MIG [60] and *B. cereus* SIU1 [61], slightly alkaline medium (pH 8.0–8.5) has been reported to be optimum for protease production by *B. licheniformis* IKBC-17 [62], *B. subtilis* IKBS 10 [63], *Bacillus macerans* IKBM-11 [64], *Bacillus amovivorus* [65] and *Aspergillus niger* [66]. The varied results obtained by different researchers did not lead to any solid conclusion about the reason behind this kind of observation. It was observed that the optimum pH for growth is 9.0 for the majority of the isolates, while the optimum pH with regard to enzyme secretion varied between pH 8.0–10.0 for protease production, [67]. Few bacterial species like *Bacillus* sp. [68], *Bacillus* sp. strain APP1 [69], *Bacillus proteolyticus* CFR3001 [70], *V. pantothenticus* [71] and *Pseudomonas fluorescens* [72] showed better results at 9 pH.

Higher initial pH, 10.0 for *A. oryzae* 637 [73] and *B. licheniformis* TISTR 1010 [74], 10.5 for *B. circulans* [75] and 10.7 for *Bacillus* sp. 2–5 [76] have also been reported for maximum protease production by the workers.

b. Temperature. Temperature is a significant parameter that has to be controlled and varied from organism to organism for maximum cell growth and enzyme production. The optimum temperature requirement for different species for protease production by different microorganisms varies greatly. Maximum production of protease by *A. niger* was obtained at 45 °C [77]. High optimum temperature of 50 °C has been reported for *Bacillus* sp. strain APP1 [78] and *B. subtilis* BS1 [79]. *B. licheniformis* IKBC-17, *B. subtilis* IKBS-10. The optimum temperature of alkaline protease production by *B. cereus and B. polymyxa* has also been reported as 60 °C [80]. The temperature of 40 °C has been also been reported to be best for production of protease by *Bacillus* sp. [81], *B. licheniformis* GUS1 [82], *V. pantothenticus* [83] and *S. Roseiscleroticus* [84].

The 30°C optimum temperature for protease production by *P. aeruginosa* PseA [85], *B. Licheniformis* [86], *B. Coagulans* [86], *B. Cereus* [87], *P. aeruginosa* MCM B-327 [88], *P. chrysogenum* IHH5 [89] and *A. oryzae* 637 [90] has been reported. Optimum temperature of 25 °C has been reported for *B. circulans*[91], *Microbacterium* sp. [92] and 28°C for *B. Cinerea* [93]. *P. fluorescens* was competent of producing protease in the range of 27–57 °C with yield maximum at 37 °C [94]. A temperature of 37 °C was found as optimal temperature for protease production by a number of *Bacillus* species such as *B. amovivorus* [95], *B. proteolyticus* CFR3001 [96], *B. aquimaris* VITP4 [97] and *B. subtilis* strain Rand [98].

c. Incubation period. The incubation period depends upon the type of microorganism and other culture conditions such as inoculum size, metabolic state of cell pH and temperature. This affects the enzyme production considerably and it differes from 24 h to a week. Protease production by *Bacillus pumilus* started 16 h after incubation, increased gradually and reached a maximum at about 28 h [99]. For *B. subtilis* [100] and *B. licheniformi* [101] maximum growth and enzyme production was observed after 2 days. *Bacillus* sp. *Pseudomonas fluorescens* produced maximum protease after 24 h of incubation, decreased from 48 to 168 h [102]. The maximum protease production from *V. Pantothenticus* [103], *B. subtilis* [104] and *B. licheniformis* [105] was recorded after 72 h.

The enzyme production by *Penicillium chrysogenum* slowly augmented with time and the highest enzyme activity was reported after 72 h of incubation [106]. Also, maximum protease production by *A. flavus* and *Aspergillus terreus* was reported after 72 h [107].

B. Solid State fermentation and Protease production

The process in which solid substrate not only supplies the nutrient to the culture but also serves as an anchorage for the microbial cells is known as Solid State fermentation. This is very much researched in areas with abundance of biomass and agroindustrial residues, as these are cheap raw materials, superior volumetric output, simpler machinery, use of economical substrate, low energy requirements and low waste water output, simple technique, low capital asset [108].

However, SSF processes present some limitations, such as the SSF technique is mainly restricted to process involving fungi, the restricted range of microorganisms that are able to grow under reduced moisture levels, control of conditions is again very complicated and there is no defined concentration of media components [109].

C. Substrate

The substrates can be used with little pre-treatments that include milling and washing [110]. Cost and accessibility are significant considerations, and hence, the selection of an suitable solid substrate plays an vital role in the development of efficient Solid State Fermentation processes. Maximum protease production was seen in watermelon rind among melon rind, rice, lentil and corn husks used for protease production by *Bacillus* sp. [111]. Wheat bran has also been used for protease production by *Beauveria feline* [112]. Rice bran was reported to be the best substrate for protease production by a *Bacillus* sp. [113]. Broken rice of different varieties was used for protease production by *A. niger* [114]. Defatted soybean cake was used for protease production by a *Penicillium* sp. [115]. A *B. subtilis* isolate was shown to be able to produce extracellular protease in solid-state fermentation using soy cake as culture medium [116]. Soybean was used for production of alkaline protease by *Teredinobacter turnirae* [117]. Among the different agro-industrial waste products and kitchen waste materials, viz. mustered oil cake, wheat bran, rice bran, *Imperata cylindrica* grass, banana leaves, potato peels and used tea leaves screened as substrates/solid supports for the production of alkaline protease by *B. subtilis* DM-04, potato peel followed by *I. cylindrica* grass supported maximum protease production.

Potato peel and *I. cylindrica* grass mixed in a ratio of 1:1 (w/w) significantly enhanced the protease production as compared to individual substrate [118]. *A. oryzae* NRRL 2217 was capable of producing maximum protease on mixed substrate coconut oil cake: wheat bran in mass ratio of 1:3 [119]. Wheat bran enriched with fish scales and egg shell in a ratio of 1:2:0.005 (w/w) was used for protease production by *Penicillium* sp [120].

Horse gram husk was used Govarthanan *et al*. [121] as substrate while producing protease from *Bacillus* sp. and reported maximum (240 U/ml) protease production with maltose as a source of carbon. Pigeon pea waste, pineapple waste, orange peel waste, sugarcane bagasse, green gram, chick pea, red gram, black gram husks and wheat bran, wheat bran, rice bran, raw potato starch and raw sweet potato starch were tested for protease production by *Bacillus* sp [122]. Green gram husk has also been reported to support maximum protease production by *B. circulans* [123]. The mixture of two or even three different substrates gives better enzyme yields than each of the substrates used in isolation [124]. The bacteria [125] and fungus [126] has been used widely in industrial scale to produce protease [127]. Coffee pulp, coffee cherry husk, coffee parchment husk, silver skin and coffee spent wastes were tested for protease production by *A. oryzae* and coffee cherry husk was found to be most appropriate [128].

Substrate Particle size. The availability of surface area play a prominent role for the attachment of microbes, transfer of different nutrients and substrates and growth of microbial strain and product production [129]. Very fine substrate particles have larger surface area for microbial attack, however, too small particles may cause substrate agglomeration which may impede with ventilation and may thus result in poor growth. Larger particles provide better aeration effectiveness but provide limited surface for microbial attack. Therefore, it may be necessary to provide compromised particle size [130]. The soybean coarse size (2 mm) was found to be optimal size of the substrate for higher protease production by *T. Turnirae* [131].

Moisture content. An optimal level of moisture is required for maximum enzyme productivity. High enzymatic titre (240 U/g) was attained when the initial moisture level was 22.4% [132]. An augment in moisture level may decrease the porosity of the substrate such as wheat bran, thus limit oxygen transfer, while lower moisture content causes decrease in solubility of nutrients of substrate, lower degree of swelling. An increase in moisture content causes a decrease in the porosity of the substrate, thereby decreasing the gas exchange. Low moisture content leads to sub-optimal growth and a lower degree of substrate swelling which also decreases enzyme production [133].

II. CONCLUSION

The screening of Bacteria for Protease Production has gained considerable attention in this biotechnological era. There is a need to explore new areas in which the already identified microorganisms can be utilized for industrial scale to meet the demands of ever growing population. The new habitats that are extreme can be also searched for the identification of these organisms. . The performance of protease is influenced by several factors, such as pH of production medium, ionic strength, temperature and mechanical handling. Further, the genetic and protein engineering studies need to be conducted to meet the requirements of harsh conditions during industrial processes.

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