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Research Article

ENZYME PRODUCTION ABILITY BY *BACILLUS SUBTILIS* AND *BACILLUS LICHENIFORMIS* - A COMPARATIVE STUDY

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ABSTRACT

Objective: B.subtilis and B.licheniformis have been reported as one of the potential protease producer. Hence, an attempt has been made to optimize and compare the enzyme production by entrapment and fermentor conditions.
Methods: Growth profile of both the strains has been studied. Optimization of culture conditions with respect to pH, temperature, agitation, carbon, nitrogen sources and metals has also been analyzed. Immobilization studies have been conducted for the strains. Enzyme production ability in fermentor has been premeditated.
Results: Growth profile for both the strains was found to be similar. Strains were optimized for maximal enzyme production. Fermentation has yielded higher production of enzyme (8080 U/ml) compared to immobilization (850 U/ml). Conclusion: B.subtilis was more stable in enzyme production with immobilization and fermentation when compared to B.licheniformis.

Keywords: B.subtilis, B.licheniformis, Immobilization, Fermentation.

INTRODUCTION

Most of the enzyme market is dominated by hydrolytic enzymes, such as lipases, proteases, amylases, amidases, and esterases [1]. Proteases have a variety of functions and exhibit potential applications in industrial sectors. Probably, proteases are an important hydrolytic enzyme which was studied extensively [2]. The largest share of enzyme market was dominated by protease. These enzymes have been used to degrade protein products in various industrial processes [3]. Although variety of microbes involve in protease production, *Bacillus sps* is the major protease producing organism. Immobilization of cells and used for enzyme production was an excellent alternative for the usage of free cells. Advantage of immobilization includes limiting cell wash out, high productivity, ease of separation, prevention of cells from shear forces and repeated usage [4].

This paper presents the studies on comparative production of protease by *Bacillus subtilis* and *Bacillus licheniformis* with various physical and chemical parameters. Production in immobilized cells with batch and continuous and enzyme secretion in fermentor by *Bacillus subtilis* and *Bacillus licheniformis* were studied and analyzed.

MATERIALS AND METHODS

Bacteria and culture conditions

Bacillus subtilis and *Bacillus licheniformis* was obtained from National chemical laboratory, India and maintained in slopes at 4˚C. The basal medium contains peptone (5 g/l), yeast extract (1 g/l), sodium chloride (0.5 g/l) and casein (0.1%, w/v). The medium pH was adjusted to 8.0 with 0.1N NaOH. Cultivation of the organism was done with 25 ml of medium in 250 ml Erlenmeyer flask. After overnight incubation sample was drawn and measured for growth and protease production. All the experiments were carried out in triplicates and repeated twice.

Measurement of protease activity

Proteolytic activity was measured by using casein as a substrate. Two ml of Casein (2%) was added with 0.5 ml of culture supernatant and allowed to react for 10 min at 37˚C. After incubation, the reaction was stopped by the addition of 2.5 ml of 110 mM of trichloro acetic acid. The reaction mixture was incubated in room temperature for 30 min. The mixture was centrifuged at 5000 rpm at 4˚C for 10 min. The clear supernatant was mixed with 3 ml of 500 mM sodium carbonate and 0.5 ml of 1N folin ciocalteau's reagent and the absorbance was measured at 660 nm. One unit of enzyme activity is defined as the amount of enzyme required to liberate 1µM of tyrosine per min under standard assay conditions [5].

Growth curve

Growth kinetics and secretion of enzyme was studied in basal medium of 100 ml with 10% inoculum at pH 8.0 in 250 ml Erlenmeyer flask. The flask was incubated at room temperature in static condition up to 72 hrs. Sample was withdrawn at every 4 hrs interval, centrifuged at 10,000 rpm for 15 min at 4˚C and analyzed for the change in medium pH, biomass, total protein content and enzyme activity [6].

Protein assay

Protein concentration was determined by Lowry's Method using Folin Ciocalteu reagent [7] with Bovine serum albumin as a standard, by measuring the absorbance at 660 nm.

Optimization of process parameters for maximal protease production

Effect of pH and temperature

The effect of medium pH and incubation temperature on growth and enzyme production was determined by growing the strains *B.subtilis* and *B.licheniformis* in different range of pH (4.0 to 12.0) at 37˚C. Similarly, the influence of temperature was determined by growing the strains in varying temperatures (4, 20, 30, 37, 40, 50 and 60˚C) at a constant pH 8.0. Growth and protease production was measured after overnight incubation [8].

Effect of agitation speed

Influence of agitation speed on growth and protease production of *B.subtilis* and *B.licheniformis* was determined by incubated at 37˚C with different agitation speed (50, 100 and 150 rpm) at a constant pH 8.0. Growth and protease production was measured after overnight incubation [9].

Effect of carbon and nitrogen sources

Various carbon sources (glucose, lactose, starch, sucrose and maltose at 1%), nitrogen sources (beef extract, soya bean meal, peptone, casein and malt extract at 0.5%) and inorganic nitrogen sources (ammonium sulphate, ammonium oxalate, ammonium nitrate, ammonium chloride and ammonium acetate at 0.5%) were added to the basal medium as a sole source, both the strains *B.subtilis* and *B.licheniformis* was incubated at 37˚C for overnight and analyzed for growth and enzyme production [10, 11].

Effect of metals

Influence of metals on growth and enzyme production of *B.subtilis* and *B.licheniformis* was determined by adding various metals (0.1%) including calcium chloride, magnesium chloride, zinc chloride, ferric chloride and sodium chloride in the basal medium and incubated at 37˚C at a constant pH 8.0 [12].

Immobilization studies

Cell suspension of both the cultures were obtained by centrifuging the overnight grown culture, mixed with 3% sodium alginate matrix and the beads were formed by adding drop by drop of this suspension to 0.2M $CaCl_2$ and kept for curing at 4˚C. Batch and repeated batch fermentation was carried out. Batch fermentation was done by analyzing cell leakage and enzyme production for 24 hrs and repeated batch culture for 9 days by changing the spent medium with fresh medium at every 24 hrs [13].

Fermentation

Seed culture was prepared for each strain in 1 liter erlenmeyer flask containing 250 ml of optimized media (glucose, 10 g/l; peptone, 5 g/l; $FeCl_3$, 1 g/l; NaCl, 0.5 g/l and glucose, 10 g/l; soyabean meal, 5 g/l; $MgCl_2$, 1 g/l; NaCl, 0.5 g/l for *B.subtilis* and *B.licheniformis* respectively), at a constant pH 10.0. Batch cultivation was carried out for enzyme production in 5 liter bioreactor with a working volume of 2.5 liter. After the seed culture inoculated, the culture was agitated at 100-200 rpm for *B.subtilis* and 300-400 rpm for *B.licheniformis* [14].

RESULTS AND DISCUSSION

Growth curve

Growth curve of both the strains *B.subtilis* and *B.licheniformis* was done with respect to protease production in the basal medium with pH 8.0 at 37˚C. The lag phase for *B.subtilis* was very short (4 hr), after that it showed exponential growth up to 32 hrs followed by stationary phase till 48 hrs and then it started to decline. *B.licheniformis* showed lag phase up to 8 hrs followed by exponential phase till 32 hrs and stationary phase ended up in 48 hrs. A previous report [4] showed the comparison of protease production by *Bacillus subtilis* and *Serratia marcescens*.

Optimization of culture conditions

Effect of pH and temperature

Both the strains were able to grow and produce enzyme with a wide range of pH (5.0 to 12.0). Maximum growth and production was found at pH 10.0 was taken as 100%. Similar report with *Bacillus sps* showed pH 10.0 was optimum for protease production [15]. Compare to *B.licheniformis*, *B.subtilis* showed higher activity in all ranges of pH. Even at pH 5.0, *B.subtilis* could produce 60% of activity. It is interesting to note that at pH 12.0 it produced 86% of activity. At pH 5.0, *B.licheniformis* showed more than 50% reduction in its activity and showed 80% of activity with pH 12.0. Protease production is highly depending on the medium pH as it strongly influences the transport of components across the cell membrane and enzymatic processes which in turn affects the cell growth and product formation [6]. This study suggested that both the strains are alkaline in nature (Fig. 1).

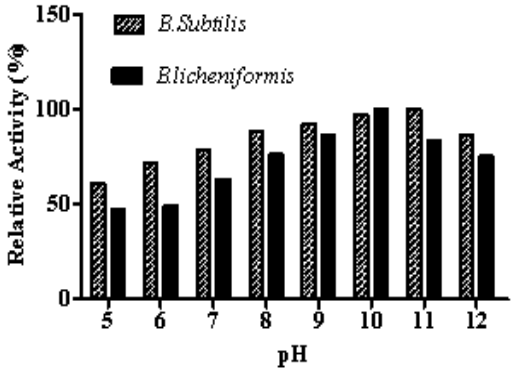

Figure 1: Effect of pH on enzyme production by *B.subtilis* and *B.licheniformis.*

Temperature is one of the critical parameter which has to control and maintain in an optimum condition for maximal enzyme production [16]. Optimum growth temperature for *B.subtilis* and *B.licheniformis* were found to be 50 and 60˚C respectively. Growth is in accordance with protease production that maximum activity was found at 50˚C for *B.subtilis*, 40˚C for *B.licheniformis* was taken as 100%. Similar reports [17, 18] showed 50 and 35±2˚C is optimum for *B.subtilis* and *B.licheniformis* respectively. Incubating at 4˚C drastically reduces the activity more than 50% for both the strains. At 60˚C, the activity was found to be 55 and 59% for *B.subtilis* and *B.licheniformis* respectively (Fig. 2).

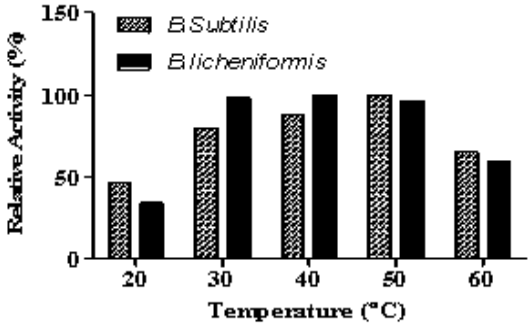

Figure 2: Effect of Temperature on enzyme production by *B.subtilis* and *B.licheniformis*

Effect of agitation speed

Optimum agitation condition for the growth and protease production was determined with three different agitation speed (50, 100 and 150 rpm). Both the strain had different requirements for agitation. *B.subtilis* grown and produce maximum enzyme with lower agitation speed (50 rpm), this is in accordance with [19] a previous report with *Bacillus sps* showed higher agitation speed reduces the enzyme production which may be due to the less requirement of oxygen, while *B.licheniformis* requires higher agitation speed (150 rpm) for maximum growth and enzyme production. Similar report with *B.licheniformis* [18] showed maximum enzyme production with maximum agitation speed (Fig. 3).

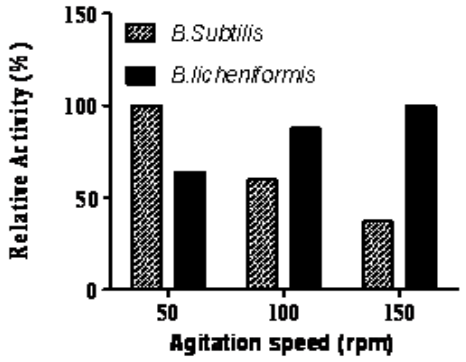

Figure 3: Effect of different agitation speed on enzyme production by *B.subtilis* and *B.licheniformis*

Effect of carbon, nitrogen sources and metallic salts

Five different carbon sources (1%) were tested for the enhancement of growth and enzyme production. Among the Sugars tested, glucose found to have a little enhancing effect in *B.subtilis* and *B.licheniformis,* lactose increases the enzyme activity, and this is in accordance with a previous report [20] showed little enhancing effect with glucose for *B.subilis* and maximum production with lactose for *B.licheniformis*. In contrast a study [6] showed reduction in the secretion of enzyme with the presence of glucose by *Bacillus sps*.

Among the organic nitrogen sources (0.5%) tested, peptone and beef extract increases the enzyme production in *B.subtilis* whereas peptone and soyabean meal enhances production in *B.licheniformis*, this is in accordance with Prakasam et al [6] showed enhancement in production with peptone by a *Bacillus sps*. Inorganic nitrogen sources found to have an inhibitory effect on enzyme production. Ammonium acetate had no enhancing and repressing effect in *B.subtilis.* This suggested that ammonium ions found have an inhibitory effect on enzyme production. Readily metabolizable nitrogen ions in medium will repress the enzyme synthesis [16]. While in *B.licheniformis*, ammonium acetate and ammonium nitrate had a little enhancing effect. Previous study with a *Bacillus sps* [11] showed enhancement of enzyme production with acetate. Similarly, Singh et al [8] and Sen and Satyanarayana [21] showed that the maximum enzyme production with ammonium ions by *B.licheniformis*. This study reveals that organic nitrogen was better for maximum enzyme production compared to inorganic nitrogen sources.

Five different metallic salts (0.1%) were tested for the enhancement for enzyme production. Among the tested salts, ferric chloride found to enhance the growth and enzyme production (2.5 g/l and 114% respectively) followed by calcuim chloride shows 78% production in *Bacillus subtilis*, these result is in accordance with Abidi et al [22] in contrast these salts found to reduce the production in *Bacillus licheniformis*, Similarly, Sangeeta Negi et al [23] shows reduction in enzyme production with ferric chloride. Magnesium chloride enhanced the growth and enzyme production in *B.licheniformis*. Other tested salts did not have significant effect on growth and enzyme production in both the *Bacillus sps*.

Immobilization

Batch fermentation was done up to 92 hrs to determine the cell leakage and enzyme production. Cell leakage constantly increased, enzyme production reached maximum in 34th hr and after that gradual decrease in production was seen in both the cultures. It was obvious that production was much higher in immobilized cells (5578 U/ml for *B.subtilis* and 1978 U/ml for *B.licheniformis*) compared to free cells (850 U/ml for *B.subtilis* and 742 U/ml for *B.licheniformis*).

In repeated batch fermentation, cell leakage and enzyme production was monitored for 9 days (until the beads disintegrated). At each cycle, the cell leakage was increased and the enzyme production was decreased. These results coincided with Adinaryana et al [13] studies.

Fermentor studies

Batch fermentation of both the strain was carried out for 48 hrs in a production medium. Dry cell mass was constantly increased over time. Glucose in the medium found to decrease in concentration with increase in fermentation time [14]. It might be due to the assimilation of glucose during fermentation process. Highest level released proteins and amino nitrogen content in the fermentation broth was observed clearly. The situation was found similar in shake flask experiment and fermentor. The level of release of soluble proteins and amino nitrogen group was quite comparable with shake flask and fermentor conditions for both *Bacillus* sps. Enzyme was found exactly similar with protein content in the medium. As protein content increases it also increases over time period. Yield was much higher (8080 U/ml for *B.subtilis* and 8000 U/ml for *B.licheniformis*) compared to shake flask experiment. Thus, the used production medium gave high levels of end product release.

CONCLUSION

Bacillus is considered to be an important species for protease production, on which *B.subtilis* and *B.licheniformis* have identified as potential protease producer in previous reports. Thus, we tried to optimize and comparative production of enzyme has been studied both in immobilization and fermentor conditions. We found that *B.subtilis* showed more production of enzyme in entrapment and fermentation and identified as highly stable compared to *B.licheniformis*.

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