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Research Article

Marine-derived Producer Bor S17B13 and its Response to Variations in Salt (NaCl) Concentration and pH in the Growth Medium

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Abstract

In the current investigation, our primary focus is on the synthesis of extracellular protease, and we aim to identify the factors that produce the most favorable results. Bor S17B13 is a halo-tolerant, gram +ve, bacillus sp. isolated from soil associated mangrove vegetation in the Indian state of Maharashtra. Based on the sequencing of the 16s rRNA gene, it was discovered that strain Bor S17B13 had a connection to the Priestia aryabhattai strain. It is interesting to note that Bor S17B13 reveals a biphasic growth pattern despite having two different sources of nitrogen and just one source of carbon. During the second log phase, which was also the time in which lavish growth was noted and the maximum level of protease production was recorded at the same time, The growth rate achieved with an inoculum concentration of 9% v/v was optimal. Optimization for the isolate revealed that it can grow on 0-20% NaCl concentrations and even produce the protease enzyme, confirming the Bor S17B13 isolate's halotolerant nature. Maximum production of protease was at 0% NaCl (w/v) (171 U/ml), and optimum growth was also seen at the same concentration. Growth and protease activity were greatest at pH 7 (172 U/ml), but they can grow in the pH range of 7 to 9. When up-scaling a product to an industrial level, the use of a specialised medium for a possible isolate plays a very important and critical function. This helps to avoid economic losses and significantly lowers the manufacturing costs of the enzyme product.

Keywords: Mangrove Ecosystem; Halotolerant; Protease; NaCl; Growth pH; Optimization

Introduction

Microorganisms produce enzymes such as protease, cellulase, and amylase. Proteases from them have wide applications in the agricultural, medicine, chemical processing, cleansing, and paper-pulp industries. The global market for enzymes is expected to rise by $9 billion by 2027, according to GVR (Grand View Research report ID: 9781680388442). Microbial enzymes' importance in safe, eco-friendly, cost-effective biotechnological processes are fueling demand. Bio-fuel, cleaning supplies, livestock feed, and foodservice will drive industry growth over the course of the period.

Proteolytic proteases produced by bacteria appear to be the most well investigated enzyme since enzymology was first developed. These enzymes have attracted attention not only for the crucial roles they play in metabolic processes, but also for the extensive ways in which they are put to use in numerous commercial ventures [1]. Due to the fact that these microbial proteases possess all of the desirable features for industrial applications, they are favored over proteases derived from plants and animals [2]. Proteases obtained from these microorganisms are widely diluted for use in a variety of commercial settings and are majorly extra cellular

[3]. Microorganisms having halotolerant nature have attracted a lot of attention because of their capacity to survive in harsh environments while still being able to thrive with minimal growth medium and physiological requirements. The current research is focused on the production of extracellular protease as well as the determination of the optimal growth conditions and protease production conditions for a novel halotolerant isolate known as Bor S17B13. Based on the sequencing of its 16S rRNA gene, this isolate is a Gram +ve bacilli and is related to *Priestia aryabhattai*. The NCBI sequence submission accession number was OM743775, named *Priestia megaterium* strain B21 (Bor S17B13 isolate).

Material and Methods

Halophilic bacteria Bor S17B13 was isolated from monari creek in borivali, Maharashtra, western India. Enrichment media were inoculated with 1g sample soil. 50 ml broth flasks were agitated at 120 rpm. Enrichment media contain 1% glucose, 0.5% peptone, 0.5% yeast extract, 0.5% K_2HPO_4, 10% and 20% NaCl, and 20% autoclaved Na_2CO_3 to adjust pH to 7. The flasks were kept at 37°C for 48 h, and then one loop culture was streaked on a CMP (Complete Medium Plate) for obtaining isolated colonies [4]. Then, isolates were scrutinised for protease, amylase, and cellulase on corresponding medium plates, and the highest protease producer was chosen for secondary screening. A loopful of culture from the plate was put into 25 ml of gelatin broth (GB) (gelatin, 1%; glucose, 1%; casein hydrolysate, 1%; K_2HPO_4, 0.25%; NaCl, 0% w/v; pH 7, g/l) adjusted by adding autoclaved 20% w/v Na_2CO_3) to prepare the inoculums [5]. In a 250-ml flask, 10% of the active culture had been inoculated into 100 ml of GB and incubated at 37°C ± 2 on a 180-rpm rotational shaker. Extracted broth growth (600 nm) was monitored at intervals. The sample was centrifuged at 4°C for 10 minutes at 6000 rpm. After centrifugation, the supernatant contains crude-enzyme. As in our studies other researches were carried out where focus was on marine isolates have screening for industrially important enzymes as demand is increasing and market is valuation is clearly visible ([6-8]).

Enzyme assay for protease

Anson-Hagihara's methodology was used for protease assay were casein was used as a substrate [9]. Crude enzyme of about 0.5 ml was added in 3 ml of casein solution (0.6% w/v with borax-NaOH buffer system for pH 9). After that mixture was incubated at 37˚C ± 2 for 10 min and termination of reaction was done acting TCA mixture (Trichloroacetic acid (0.11 M), sodium acetate (0.22 M), and acetic acid (0.33 M)) and incubated for 30 min. After that, with the help of Whatman's filter-paper no. 1, filtrate was separated from precipitate, and the filtrate's absorbance was measured at 280 nm. The enzyme assay was calculated as one-unit of alkaline protease, which means the quantity of enzyme that releases 1 µg of tyrosine/min, and enzyme units tyrosine (0-100 µg) was used (Thumar and Singh, 2007).

Growth kinetics and enzyme kinetics of Bor S17B13

At a variety of time intervals, both the rate of growth and the enzyme assay of Bor S17B13 were analyzed. GB media (NaCl, 0% w/v, and pH 7) was used for the inoculation, and the culture was then incubated at 37°C while being agitated (180 rpm). At various times, sterile culture samples were removed using appropriate precautions. Following the procedures outlined above, growths as well as enzyme activity were measured.

Optimization of media for growth and protease production

Optimization of inoculum size

The inoculum size has a crucial role in determining the final yield and growth of the organism. Inoculum was used when its O.D. reached 0.6-0.7 at 600 nm. The optical density (at 600 nm) was measured 48 hours after adding inoculum culture (Bor S17B13) at concentrations ranging from 1% to 10% inoculums in gelatin broth (NaCl, 0% w/v; pH 7).

Effect of salt (NaCl) on protease production

Various salt concentrations (NaCl, 0%, 5%, 10%, 15%, and 20% w/v; pH 7) were used in gelatin broth to study the impact of salt on growth and enzyme secretion. By adding the appropriate volume of separately autoclaved 20%, w/v Na_2CO_3, the pH concentrations of the medium were adjusted. Gelatin broths were inoculated with 9% of activated culture (Bor S17B13). The incubation process was performed at 37°C ±2 with 180 rpm shake flask conditions, and samples were withdrawn at predetermined intervals to estimate protease activity and growth up to 70h.

Effect of pH on protease production

To identify the effect of pH on growth and enzyme secretion, the isolate, Bor S17B13 was grown in various growth pH

concentrations (pH 7, pH 7.5, pH 8, pH 9) in gelatin broth at 0% NaCl concentration. Nine percent of this activated culture of Bor S17B13 was inoculated into 100 ml of gelatin broth and incubated at 37°C ±2 under shake flask conditions (180 rpm), with samples withdrawn at appropriate time intervals up to 70h. In this experiment, the growth pH was made complementary to the substrate pH (casein solution). For illustration, crude enzyme produced in a medium with pH 9 will be processed with casein at pH 9 for the enzymatic process.

Results and Discussion

Halophile bacteria attract interest because they can grow in harsh conditions with minimal-medium and physiological changes. They are important in industrial and biomedical applications. This study investigates extracellular protease synthesis and growth characteristics from a halo-tolerant isolate Bor S17B13. 62 isolates were collected from Borivali-Monari and Jhow Island Mangrove Environment, Maharashtra. All isolates were screened for industrial enzymes (protease, amylase, and cellulase) and colony morphology [4]. Protease is one among them and is used in many sectors, hence Bor S17B13, the highest protease producer, was chosen for secondary screening. Other studies have shown that microbes that synthesise protease and are isolated from *Bruguiera cylindrica*, which is a mangrove plant native to North Sumatra in Indonesia, number about 33 [11]. This work examined extracellular protease generation, optimal growth, and media optimization for increased enzyme yield from a new halo-toletant isolate Bor S17B13, +ve gram, bacilli.

Inoculum size

Figure 1: Optimization of inoculums size.

Industrially significant products require careful consideration of inoculum size and optimization. Inoculum was added to a series of gelatin broths at varying concentrations, from 1% to 10% (Figure 1). Maximum growth in our studies was observed in using 9% inoculums size. From this all further experiments were carried using 9% inoculums size. Other studies indicate 1% inoculum as optimum for *Bacillus licheniformis* isolated from saltern sediments [12] in our studies 1% was second most favorable size. Few other studies have used 10% inoculums size for protease production ([5,10]).

Effect of salt (NaCl) on protease production

Figure 2: Growth and protease production in Bor S17B13 at 0% (A), 5% (B), 10% (C), 15% (D), and 20% (E) NaCl (w/v).

At pH 7, the effect of salt on protease production was studied in gelatin broth supplemented with 0%, 5%, 10%, 15%, and 20% w/v NaCl. Growth was lustrous in the range of 0% and 5% w/v salt concentration broth, but growth was slow when compared with 10% w/v, 15% w/v, and 20% w/v salt. Although growth was observed in all cases, it occurred at different times. The optical density of broth reached "1" O.D. in 17 hours for 0% w/v NaCl, 27 hours for 5%(w/v) NaCl, and 54 hours for 10% (w/v) NaCl concentration broth, respectively. Growth was very less with 15% (w/v) NaCl and 20% (w/v) NaCl concentration broth. Protease production was optimum (171 U/ml) with 0% (w/v) NaCl (Figure 2A) at 48h then for 5% (w/v) NaCl (49 U/ml) (Figure 2B) at 44h, followed by 10% (w/v) NaCl (26 U/ml) (Figure 2C) at 70h. At 15% (w/v) NaCl (3 U/ml) and 20% (w/v) NaCl (7 U/ml) the enzyme production reduced drastically (Figure 2D) (Figure 2E) respectively.

Growth and protease production were correlated in these studies. Figure 2A shows that growth and protease production go linearly up to 48 h, after which enzyme production decreases with an increase in growth. Figure 2B shows a similar pattern. Figure 2C shows that growth and protease production increase with time. In figure 2D, growth and protease production are the least, but they follow the pattern of protease production increasing with an increase in growth. While in figure 2E, initially the production of protease is linear, then after 44 h as growth increases, the production of enzyme increases, but later at 53 h to 70 h, growth shows a decline pattern, but enzyme production is still increasing.

Figure 3: Growth in Bor S17B13 at 0%, 5%, 10%, 15%, 20% NaCl (w/v).

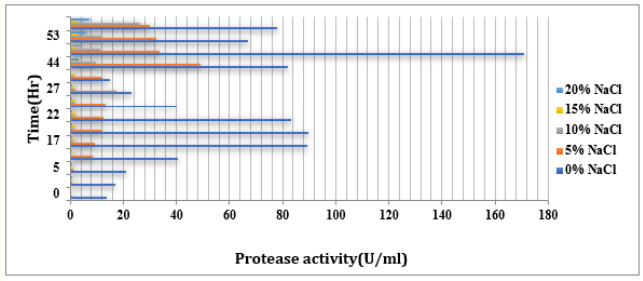

Figure 4: Comparative study on effect of NaCl conc. on protease production.

The isolate Bor S17B13, was able grow in 0-20% (w/v) NaCl, above graph (Figure 3) clearly shows biphasic growth of the isolate at the 0% (w/v) NaCl parameter; growth was slightly reduced at the 5% (w/v) NaCl parameter then, a second lag phase was prominently visible at in-between 25-35h, after which growth was increased with a peak at 44 h. There was a noticeable reduction in growth when 10% (w/v) NaCl broth when compared to 0% (w/v) NaCl broth. Very less growth was observed in 15% and 20% (w/v) NaCl. Protease production (Figure 4) was optimum (171 U/ml) with 0% (w/v) NaCl at 48h then for 5% (w/v) NaCl (49 U/

ml) (Figure 4) at 44h, followed by 10% (w/v) NaCl (26 U/ml) at 70h. At 15% (w/v) NaCl (3 U/ml) and 20% (w/v) NaCl (7 U/ml) the enzyme production reduced drastically along with low growth.

This demonstrates that optimum growth was achieved at 0% (w/v) NaCl. Grow in 0-20% (w/v) NaCl indicating that the isolate is "Halotolerant" in nature. Similar trend was observed in *Bacillus sp.* NPST-AK15, a halotolerant alkalophilic strain isolated from hypersaline soda lakes which could grow in 0-20% (w/v) NaCl conc. [13]. A halotolerant *Bacillus licheniformis* strain BA17, could also support grow at 0-20% (w/v) NaCl conc. [14]. Similar, Po2 halotolerant isolate was obtained by were optimal concentration was 10% (w/v) NaCl conc. but could survive 20% (w/v) [5].

Effect of pH

Bor S17B13 was studied for the effect of different pH on the growth and enzyme production in gelatin broth with 0% NaCl (w/v), as per the previously analyzed data (Figure 3 and 4).

Figure 5: Growth and protease production in Bor S17B13 at growth pH variations: pH 7 (A), pH 7.5 (B), pH 8 (C), pH 9 (D).

Bor S17B13 was studied for the effect of different pH on the growth and enzyme production in gelatin broth with 0% NaCl (w/v). Various pH (7, 7.5, 8, and 9) were investigated in the figure above (Figure 5). From the results pH 7 showed a diauxic growth curve, optimum protease production was at pH 7 (172 U/ml) at 48 h, then pH 8 (133 U/ml) at 27 h, followed by pH 9 (113 U/ml) at 44 h, and pH 7.5 (106 U/ml) at 70 h.

In pH 7 (Figure 5A), the production of proteases and growth were associated. Growth and protease synthesis are parallel up to 48 hours, beyond which time enzyme production declines as growth increases. In pH 7.5 (Figure 5B), growth and protease production were linear, for example; as growth increased, enzyme production increased, and vice versa. While in pH 8 (Figure 5C), growth and protease production were linear till 31 h; after that, growth increased but protease production decreased remarkably during 44 h to 48 h, followed by an increase in both in growth and protease after 48h up to 70h. For pH 9 (Figure 5D), growth was very low until 48 h, and then there was a sharp increase in growth, while protease production was low till 31h, then sudden busting of protease production was seen between 31 h and 48 h, and protease decreased drastically between 48 h and 70 h.

Figure 6: Effect of pH variation on growth of Bor S17B13.

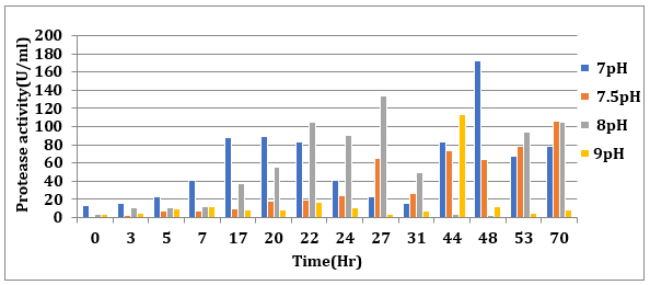

Figure 7: Comparative study on effect of pH variation on protease production of Bor S17B13.

The effect of pH (7, 7.5, 8, and 9) variation on the growth (Figure 6) and enzyme (Figure 7) of Bor S17B13 was studied. In broth with pH 7 and pH 7.5, there was a diauxic growth pattern. While in pH 8 graph, diauxic growth was not clearly separated, it has a linear, stationary phase like normal four phase growth graph. Growth was very low with in broth with pH 9. Growth increased after 53h of incubation. In the present study, pH 7 showed a diauxic growth curve, optimum protease production was at pH 7 (172 U/ml) at 48 h, pH 7.5 (106 U/ml) at 70 h, then pH 8 (133 U/ml) at 27 h and pH 9 (113 U/ml) at 44 h.

Other studies supporting our growth pH are; *Bacillus thuringiensis*, a protease-producing bacteria, was optimized from paddy field soil evidences optimum protease production at pH 7 [15]. In a study, *Bacillus megaterium*, a thermostable protease was having optimum enzyme activity at 7.5 pH [16]. The maximum protease activity of an isolated *Bacillus cereus* strain -AT in solid-state fermentation was pH 8 [17]. Our Isolate indicates protease production from pH 7 to pH 9 of the growth medium and optimum for growth and protease production was pH 7.

Conclusion

Bor S17B13, which could grow in 0-20% (w/v) NaCl, demonstrated biphasic growth at 0% NaCl. Growth was slightly reduced at 5% NaCl, then a second lag phase was prominently visible at 25-35 h, after which growth increased with a peak at 44 h. 10% NaCl broth reduced growth compared to 0%. 15% and 20% (w/v) NaCl grew less. Protease production was highest at 48 h with 0% (w/v) NaCl (171 U/ml), 44 h with 5% (49 U/ml), and 70 h with 10% (26 U/ml). Enzyme production and growth were greatly reduced at 15% (w/v) NaCl (3 U/ml) and 20% (7 U/ml). This shows optimal growth at 0% (w/v) NaCl. The isolate is "halotolerant" because it grows in 0-20% (w/v) NaCl. The bacterial growth and protease enzyme kinetics of Bor S17B13 were examined at pH 7, 7.5, 8, and 9. Diauxic growth was observed in pH 7 and pH 7.5 flasks. The pH 8 parameter did not separate the diauxic-growth pattern. while the pH-9 broth had limited growth and production. In this study, pH 7 revealed a diauxic growth curve, with optimum protease production. After 48 hours with 0% (w/v) NaCl concentration, protease production peaked at 172 U/ml and decreased as salt concentration increased. From the standpoint of optimization, pH 7 was best for growth as well as protease production. These above parameters play very important role when enzyme is produced at industrial scale.

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