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Research Article

Impact of Substrate pH and Enzyme-Substrate Incubation Time on Protease from a New Halo-tolerant Bor S17B13 Found in the Mangroves of Western India

Priyanka Sawant and Jignasha Thumar*

Department of Microbiology and Biotechnology, Gujarat University, Ahmedabad, Gujarat, India

***Corresponding Author:** Jignasha Thumar, Department of Microbiology and Biotechnology, Gujarat University, Ahmedabad, Gujarat, India.

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Abstract

Since enzymes are bioactive chemicals, they are of interest to scientists all over the world. Demand for microbial enzymes is rising rapidly across a wide range of sectors as their importance in ensuring the security, sustainability and efficiency of biotech processes becomes more widely recognised. Media optimization is a powerful tool to enhance production capacity from biological sources (microorganisms). In this present study, samples were collected from mangrove-rich sources such as Borivali Monari Creek and Jhow Island, Maharashtra, western India. Samples collected were mangrove-associated soil, mangrove root, and sea water. The type of sampling was random. Samples were collected with prior permission from the mangrove cell maharashtra government. A total of 30 samples were processed, from which 62 isolates were obtained. These isolates were all screened for industrially important enzymes (protease, amylase, and cellulase), and the best protease producer was selected for further studies. The method of optimization was "ONE VARIABLE AT A TIME" (OVAT) and method is a well-known optimization technique. Optimization was carried out for parameters like inoculum size, pH of the growth medium, various carbon sources, varied nitrogen sources, and metal ions and cations. Casein was used as substrate, and casein was prepared in various pH buffers for the enzyme assay. At 5 minutes after incubation of enzyme substrate mixture, enzyme activity was at its peak, and substrate degradation in the medium was minimal as compare 65 minutes set. Enzyme activity decreased dramatically as substrate pH increased (between 11 and 12 pH). This optimization supports maximizing the yield of protease production and is of great benefit to the biotech industry. The present study shows that with time stability of enzyme reduces and residues of degraded substrate increases within the reaction mixture. Here in our experiment variation in substrate pH were studied, which showed that protease from Bor S17B13 isolate has optimum enzyme activity at substrate pH 10 at highly alkaline pH then 10 pH, activity of enzyme was reduced. A phenomenon called dissociation of substrate (casein) was seen at casein solution 13 pH.

Keywords: Mangrove Ecosystem; Halotolerant; Protease; Bor S17B13; Substrate pH; Enzyme Substrate; ES

Introduction

Researchers from all over the globe are interested in enzymes because of their role as bioactive molecules. The role that microbial enzymes play in safe, eco-friendly, and cost-effective biotech processes is driving an ever-increasing and ever-expanding demand for them in a variety of industries and other uses. From 2020 to 2027, the worldwide market for industrial enzymes is projected to expand from its 2019 valuation of USD 5.6 billion at a CAGR (compound annual growth rate) of 6.4% according to GVR (Grand View Research, report ID: 9781680388442). The industry is expected to grow throughout the forecast period as a result of growing product demand from and use in sectors such as biofuel,

cleaning supplies, cattle feed, and foodservice. Proteases obtained from these microorganisms are widely diluted for use in a variety of commercial settings and are majorly extra cellular [1]. There are several categories for acidic and basic proteases that originate from microorganisms. The availability of functional groups and peptide bond location are also used to categories them [2]. Proteases from bacteria and other microorganisms are the most often used enzyme in industry [3]. Microbes produce a plethora of intracellular proteases that play crucial roles in division, protein cycles, hormone control, and the cellular protein reservoir, whereas the extracellular proteases are important in hydrolysis process [4]. Bacillus and Streptomyces species found in the water system that produce alkaline proteases are promising hosts for peptides and chemical synthesis [5]. The present research focuses on optimization in terms of substrate pH and ES incubation time of the novel isolate Bor S17B13 from mangrove-rich soil. Mangroves ecosystem are rich in microbial community as ecosystem is rich in nutritive perspective and human interference is restricted so there are endless opportunity for novel isolates. Western stretch of India is known was mangrove rich ecosystem. Above it special permission is must for entering mangrove forest, which in our case permission was taken from mangrove cell maharashtra western India. Samples were collected from borivali monari creek and Jhow Island, Maharashtra, western India.

Material and Method

In this investigation, samples were collected from areas abundant in mangroves, including Borivali Monari Creek and Jhow Island in the state of Maharashtra in western India. Soil, root, and sea-water samples were all taken from the mangrove ecosystem. The sampling method used was “Random sampling”. Samples were collected with prior permission from the mangrove cell Maharashtra goverment . Thirty samples were analysed, and 62 isolates were found. We tested all of these isolates for three commercially relevant enzymes (protease, amylase, and cellulase), and ultimately the best protease producer isolate, Bor S17B13, was selected for further studies. The optimization strategy used was the well-known “ONE VARIABLE AT A TIME” (OVAT) method.

Isolation

Many Biotechnology companies are interested in halotolerant organisms and their enzymes for the same reasons. Samples were collected from mangrove rich forest from borivali monari creek and jhow island, Maharashtra. Type of sampling was Random. In this experiment, 30 samples were processed using enriched media. Isolation was carried out by processing 1g soil inoculated in enrichment broth. Eight of the samples were processed from Jhow Island, and the other 22 were processed from the coast of Borivali. All of the samples were tested with a 10% salt conc. and a 20% salt conc. (NaCl). Samples from Borivalimonari and Jhow Island were also checked for 7 pH and 9 pH growth parameters, respectively. From the samples mentioned above, a total of 62 isolates were obtained, and each of these was tested for bio-industrially significant enzymes such as cellulase, amylase, and protease.

Primary screening

Isolates were analyzed to determine whether or not they contained protease enzymes. The pH of the medium was adjusted by Na_2CO_3 (20%, w/v), which was pH 7 and pH 9 for Borivalimonari creek and Jhow Island isolates respectively. The media contain gelatin at a concentration of 10 g/l, peptone at a concentration of 5 g/l, agar at a concentration of 20 g/l, and sodium chloride at concentrations of 10% and 20%. Subsequently the plates were incubated for another 48 hours at room temperature after that plates were examined for hydrolyzed zone by overlay them with Frazier reagent (Composed of $HgCl_2$, 150g/L and concentrated HCl, 200 ml/L) for protease [6] and isolates were also screened for amylase and cellulase ([7-9]).

Secondary screening and Enzyme kinetics

To prepare the inoculum, a loop of culture from the complete medium plate was transferred to gelatin broth (25 ml). It contains 1% gelatin, 1% casein enzymatic hydrolysate, 1% glucose hydrolysate, and 0.25% K_2HPO_4; the pH is raised to 7 by adding autoclaved 20% (w/v) Na_2CO_3 separately. The broth was shaken at 37 °C (180 rpm). When the O.D. reached 0.6 to 0.7, 10% of the culture was used to inoculate 100 mL of GB broth at 37 °C on a shaking incubator at 180 rpm. The O.D. (600 nm) was obtained at various intervals of time to determine growth kinetics, and the culture broth was centrifuged. The supernatant centrifuged from the cell-free extract yielded crude enzyme. In our research anson-hagihara methodology was used for enzyme assay [10]. This crude enzyme’s growth was monitored over time. In this enzymatic experiment, crude enzymes with casein as substrate and a TCA mixture were used, as previously stated. The end-reaction filtrate was filtered with Whatman’s No. 1 and measured at 280 nm. Under

test conditions, one unit of protease releases 1 microgram of tyrosine per minute. Tyrosine (0–100 µg) was applied to calculate enzyme units [11,12].

Optimization medium based- OVAT

Media optimization is a powerful tool to enhance production capacity from biological origin (microorganisms) product. OVAT method is well known method vial performing optimization. Here "OVAT", means 'ONE VARIABLE AT A TIME '. This method is also known as OFAT(ONE FACTOR AT A TIME) were single variable is changed and all other variable are kept same. In this present study optimization was carried with OVAT method. Optimization was carried out for parameters like inoculums size (1-10%), pH (7-9) of growth medium, NaCl concentration (0-20% NaCl conc.), various carbon sources, varied nitrogen sources and metal ions/cations [13].

Optimization enzyme-substrate (ES) based-OVAT

Increasing the activity of protease enzyme-substrate-based optimization is required after media optimization. The time given to the enzyme substrate mixture makes a significant difference in determining an isolated enzyme's true potential. The pH of the substrate is also important in this case; this will give us an idea of the pH of the substrate at which our enzyme is most active, regardless of the pH of the growth medium.

Incubation time

While using crude enzyme for detection of protease activity, substrate casein was used for the assay and the reaction mixture was kept for 10min. and its activity was measured. Relative activity is when same enzyme is referred with parameter for increasing enzyme activity. In this experiment after inoculating active culture in 100mL GB broth and incubating at 37 °C on a rotary shaker at 180 rpm, as mentioned above the O.D. (600 nm) was taken at different time intervals for growth kinetics further the broth was centrifuged. Crude enzyme, also known as cell-free extract, was obtained after centrifugation in the form of supernatant. That crude enzyme was used for enzymatic assay , were crude enzyme (0.5 ml) was added to 3 ml of casein substrate solution and reaction mixture was incubated for different time with interval of 5 min up to 65 min at 37 °C water bath [14].

Substrate pH

In this experiment, casein is used as a substrate. The effects of different pH of substrate (casein) solutions were investigated in this study. In this experiment, after inoculating active culture in 100 mL of GB broth and incubating at 37 °C on a rotary shaker at 180 rpm. As mentioned previously, the crude enzyme was extracted after centrifugation. An enzymatic assay was performed using crude enzyme and casein as enzyme substrate reaction mixture. Here in study casein solution was prepared using 0.6% casein w/v and different buffer systems like, Phosphate buffer system K_2HPO_4-KH_2PO_4 for pH 6 and pH 7, KH_2PO_4-NaOH for pH 8, Borax-NaOH for pH 9 and pH 10, glycine-NaOH along with Na_2HPO_4-NaOH with different concentrations were used for pH 11 to 13.5. Experiments were performed for casein solution pH 6 to 13.5 and optimum pH was observed for higher enzyme activity [15].

Result and Discussion

Isolation

Two sites from mangrove rich area were selected for screening of potential isolates Jhow site and Borivali site from Maharashtra, India. From both the sites total 30 samples were processed from which eight were from Jhow site and twenty-two were from Borivali site. By processing this sample via enrichment method and then plate streaking, total 62 isolates were obtained.

Primary screening

Today enzyme industries have a big share in market of biological products. So above mentioned 62 isolates were screened for industrially demanding enzymes like cellulase, amylase and protease. From them protease enzyme producer were selected for further studies as protease enzyme stay sable for long time medium and wide application. The final selected isolate was Bor S17B13, which was maximum producer of protease from the isolates on the basis of hydrolysis zone on plate [6].

Secondary screening and Enzyme kinetics

Growth kinetics of isolate Bor S17B13 isolate from Borivali site.

The isolate was growing in a 10% NaCl containing broth, but growth was slow. Plate screening was done on a range of different

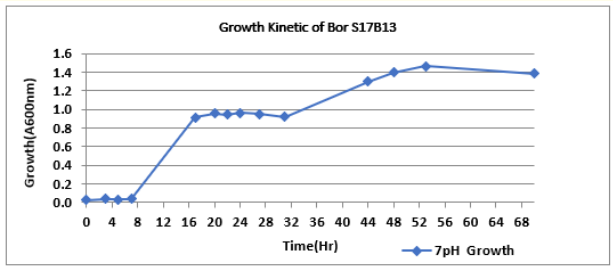

Figure 1: Growth kinetics of isolate Bor S17B13 isolate from Borivali site with 0% NaCl concentration.

salt (NaCl) concentrations. Isolate had lustrous growth at 0% NaCl concentration in the growing medium, which indicates isolate is halotolerant in nature. So growth kinetics and enzyme kinetics was studied in broth with 0% NaCl concentration and 7pH. Isolate exhibits lag, followed by a log phase lasting 27 hours. As seen in above graph (Figure 1) in between 27hr - 44hr there is a short lag phase then followed by second log phase then there is increase in cell density. The above graph (Figure 1) indicates a "Biphasic growth curve" pattern of growth.

Enzyme Kinetics of isolate Bor S17B13 for protease production

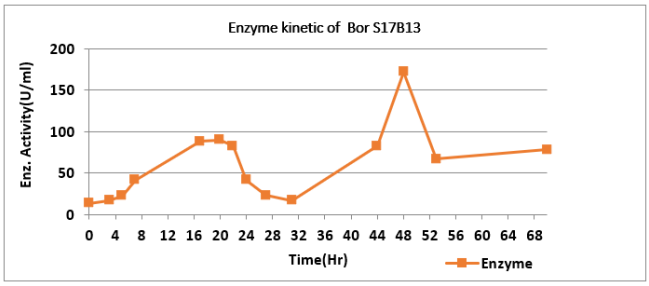

Figure 2: Enzyme kinetics of isolate Bor S17B13 isolate from Borivali site.

After primary screening, secondary screening (Figure 2) was performed on the final selected isolate, Bor S17B13. Isolate had lustrous growth at 0% NaCl concentration in the growing medium, which indicates isolate is halo-tolerant in nature. So growth and enzyme kinetics were investigated in broth containing 0% NaCl and pH 7. The assay was carried out in accordance with Anson-Hagihara's methodology. Readings at the 280 nm wavelength were taken at various time intervals up to 70 hours.

Results indicate there was a low amount of protease production during the lag phase. The enzyme production then increased until 22 hours into the exponential phase. Because the isolate has a diauxic growth pattern, the short lag phase in which protease production was drastically reduced was between 27 and 44 hours. After 44 hr, there is a hike in protease production during 48hrs (172 U/ml) which is maximum. Then there is down fall in enzyme production after 48hrs.

Optimization of culture media for increasing yield of protease enzyme

An "one variable at a time" (OVAT) approach (Figure 3) was used in this study to optimise the medium and increase enzyme production. Isolate Bor S17B13 was further selected for optimization to increase productivity. There were two types of approaches in the present study. One is optimization based on growth medium, which includes different salt (NaCl) concentrations, growth pH, carbon sources, nitrogen sources, cations, and crude sources. The other approach is enzyme process-based, where variation in substrate (casein) pH and time of incubation of enzyme substrate mixtures are important. Here, relative activity was studied with respect to time of incubation up to 65 minutes with an interval of 5 minutes, and enzyme activity was checked.

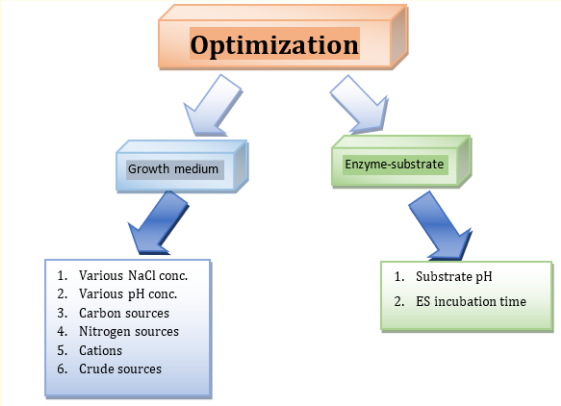

Figure 3: Layout and work flow for OVAT approach.

Optimization medium based - OVAT

In the present study medium based optimization was done. Optimization method was "One Variable At Time" also known as OVAT method. Where in optimumNaCl conc. was 0% NaCl and

growth was observed 0-20% NaCl indicating Halo-tolerant nature of isolate Bor S17B13. Optimum pH was 7. With respect to carbon source, fructose was best source and casein (Casein enzymatic hydrolysate) was optimum as nitrogen source. K_2HPO_4 was best supporting in medium for protease production. Diauxic/biphasic growth pattern was observed with two lag phase. Maximum protease production was observed in end of second exponential phase. Further studies were carried to increase enzyme –substrate affinity.

Optimization substrate base and their industrial importance - OVAT

Incubation time

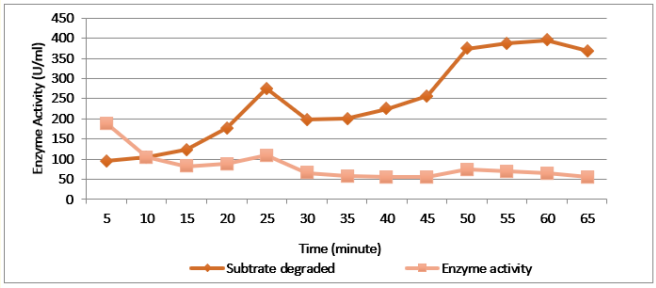

Figure 4: Effect of time on enzyme-substrate [ES] reaction and activity of enzyme.

When comparing the effect of enzyme activity to its own performance, the term "relative activity" is used. A single broth's crude enzyme was used for the parameter. The culture was grown in gelatin broth, and after 73 h of incubation, crude enzymes were withdrawn. After that enzyme activity assay was performed with the Anson-Hagihara method. Here the factor is the incubation time of the reaction mixture from 0 to 65 minutes (Figure 4). At an interval of 5 minutes, enzyme activity was optimum and degraded substrate in medium was minimum. All the sets were kept at 37°C with different time intervals. With the course of time emzyme activity reduces and degraded substrate increase. At 25 minutes incubation enzyme activity show spike and substrate degradation increase giving a spike indicating best time for incubation can be selected from 5 min. (189 U/ml), 10 min. (104 U/ml), 25 min. (110 U/ml) for enzyme –substrate [ES] incubation. This experiment shows the effect of the incubation time of an ES mixture on enzyme activity. In comparison to 5 minutes of incubation, enzyme activity at 65 min (57 U/ml) decreased by approximately 3.3 fold. A study that supported our experimental results observes the effect of pH on the stability of enzymes from *Streptomyces sp*. at different ratios of time incubation. Protease was mixed with a buffer at pH 9.0 for 60 minutes. The enzyme's relative activity was then measured, reaching its peak at a 10-minute interval [16].

Substrate pH

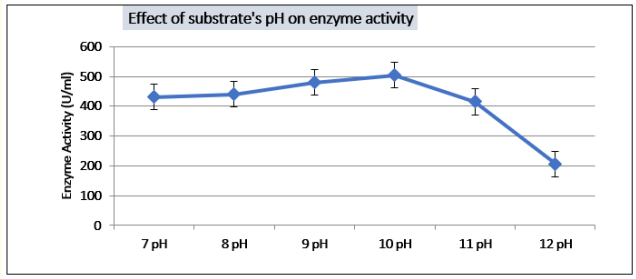

Figure 5: Effect of substrate's pH on enzyme activity.

Various types of substrates were used for protease enzyme assay. In the present study casein was used as substrate and casein was prepared in various pH buffers for the enzyme assay. Here same crude enzyme with different casein solution was examined. From the above graph (Figure 5) it clearly explains that at pH 10, enzyme activity was optimum as compare to other and with increase in substrate pH (11pH and 12 pH), enzyme activity reduced drastically. Study supporting our finding was an alkaline protease from the mutant strain of Bacillus licheniformis UV-9 for use in the development of cleaning agents and pH 11, activity was high [17].

Challenges faced during substrate based optimization

During the experiment every step from preparation of substrate till the final reading play crucial role on interpretation of the result.

As shown in Figure 6, the highest enzyme activity was found in casein solution prepared at pH 13. Challenges faced during the experiment were when casein was prepared at a higher alkaline pH, dissociation of the casein molecule occurred, and the release of free tyrosine increased, which indicates a very high amount of

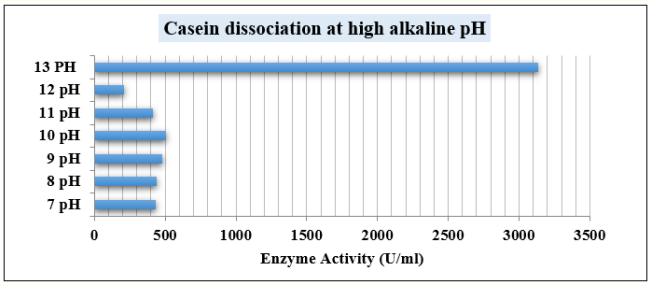

Figure 6: Effect of substrate's pH and casein dissociation at high pH.

enzyme activity during the experiment. As it is seen in figure 6, enzyme activity was low at pH 7 and increased at higher pH levels up to 10 pH. After that, enzyme activity was reduced from11pH and 12pH but a sudden spike was observed at pH 13 of the substrate. This can give the pseudo interpretation that the enzyme is very active at highly alkaline pH. A study supporting our experimental results explains dissociation and re-association of casein at high alkaline environment. Casein's micelle behavior in very alkaline environments (pH = 12–14 or more than 14) was examined. Turbidity, TEM imaging, and surface charge studies showed complete casein micelle breakup into submicelles at pH = 12 and 13. At pH ~ 14, sub-micelles unexpectedly re-associated, forming massive, aggregated aggregates. This re-association mechanism requires calcium phosphate. It neutralises negative charges and forms phospho-serine-calcium phosphate links to moderate protein interactions. Ca-depleted casein showed the significance of calcium phosphate [18].

Conclusion

Enzymes are recognised as being of curiosity to researchers all around the world due to the fact that they are bioactive substances. The relevance of microbial enzymes in assuring the safety, viability, and effectiveness of biotech processes is being increasingly recognised, which is driving an uptick in the rate at which demand for these enzymes is growing across a diverse variety of industries. The optimization of the media is a potent strategy that may be used to increase manufacturing capacity from microbial sources. A well-known strategy for optimising results is called the OVAT method. The inoculum size, the pH of the broth culture, the many different carbon sources, the many different nitrogen sources, as well as the metal ions &cations were all factors that were optimised. As a substrate, casein was utilised, and prior to the enzyme assay, casein was produced in a number of different pH buffers. The enzyme activity reached its maximum level five minutes after the incubation period ended, while the rate of substrate- degradation was low. The rise in substrate pH resulted in a substantial drop in enzyme activity (11 - 12 pH). At a pH of 13, dissociation of the casein molecule was seen in the casein solution. This optimization helps maximize the output of protease manufacturing, which is a huge boon to the biotechnology industry and is supported by this optimization.

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