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ORIGINALARTICLE

Extracellular Protease Production from a Marine, Newly Isolated Halotolerant Bor S17b13 from Mangrove-Associated Soil In Western India

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ABSTRACT
In the present study, focus is on the production of extracellular protease and determines optimum parameters. Bor S17B13, a halotolerant, gram-positive, aerobic bacillus sp. isolated from a mangrove-associated soil sample in Maharashtra, India. On the basis of 16s rRNA gene sequencing, Bor S17B13 isolate was closely related to the Priestiaaryabhattai strain*. This sequence was submitted to NCBI and accession No. OM743775, Priestiamegaterium strain B21 (Bor S17B13 isolate). Interestingly, Bor S17B13 shows a diauxic growth pattern with a dual nitrogen source and a single carbon source. The highest level of protease production was observed during the second exponential phase, along with lust growth. The inoculum size most preferred was 9% (v/v) on bases of growth parameters. Plate studies show that the isolate can grow in a wide range of NaCl concentrations (0-20%) and at pH 7. Protease production was also observed; this wide range of NaCl% indicates the "Halotolerant" nature of Bor S17B13. Isolate was further screened for various carbon, nitrogen, and cation sources for optimium protease production. Contrary to expectations, the Bor S17B13 isolate produces more protease when subjected to fructose (179 U/ml), than it does to glucose (135 U/ml) in the medium. Among the nitrogen sources, optimum production was with casein enzymatic hydrolysate (124 U/ml), and gelatin was a very poor producer compared to casein. In this set of experiments, ammonium chloride supports the hypothesis of the repression of enzyme production by ammonium ions. Cations promote enzyme growth; in the set of experiments, K_2HPO_4 (163 g/ml) produced the maximum protease, followed by KH_2PO_4 (132 g/ml) and $CaCl_2$ (58 g/ml). Strong inhibition of enzymes was observed with $MgSO_4$ (6 U/ml) compared to the other ions. Specializing medium for a potential isolate plays a critical role in up-scaling the product to an industrial scale, minimising economic losses, and significantly lowering desired enzyme production costs.*
KEYWORDS: *Halotolerant, Protease, Carbon sources, Nitrogen sources, Cation, Priestiamegaterium strain B21*

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INTRODUCTION

Microorganisms have been investigated for their potential to release polymer-degrading enzymes such as amylase, protease, and cellulase, which would have significant use in the agricultural, detergent, and paper-pulp sectors. The global market, according to the global market share report of 2020–2027 by Grand View Research (Report ID: 978-1-68038-844-2), for enzymes is projected to grow by more than $9 billion. Protease is used in a wide variety of industries, including medicines, textiles, chemical processes, paper-pulp, foodstuffs, and agro-chemicals. The role that microbial enzymes play in safe, eco-friendly, and cost-effective biotech processes is driving an ever-increasing demand for them. The industry is expected to grow throughout the forecast period as a result of growing product demand from and use in the biofuel, cleaning supplies, cattle feed, and foodservice sectors.Proteolytic enzymes have been synthesized and characterized by researchers from several microbiological sources. Microbes account for two-thirds of commercial protease worldwide [1]. Bacterial proteolytic proteases seem to be the most extensively studied enzyme ever since the field of enzymology was established. Because of their high productivity, less time and space requirements, high potential for genetic manipulation, and low cost, proteases of

microbial origin have found widespread usage in biotechnological applications. Microbe proteolytic enzymes are comparatively small, dense, and spherical in structure [2]. Halotolerant microbes have drag attention as they have ability to bear extreme condition and still can grow with minimum media and physiological condition. The present work has focus on the production of extracellular protease and determining optimum parameters for growth and protease production from a noval halotolerant isolate Bor S17B13 that is gram positive bacilli closely related to *Priestiaaryabhattai* on the basis of 16s rRNA gene sequencing.

MATERIAL AND METHODS

Halophilic bacterium Bor S17B13 was isolated by enrichment from soil sample from coastal borivali (monari creek area), Maharashtra, western India. 1g soil samples were inoculated in enrichment media. 50ml broth flasks with samples were shaken at 120rpm. Enrichment media (Complete Medium Broth{CMB}) contains glucose, 10g; peptone, 5g; yeast extract, 5g; K_2HPO_4, 5g; NaCl, 100g and 200g, as per the experiment, and autoclaved Na_2CO_3 (20%, w/v) to adjust pH to 7. Inoculated flasks were incubated at 37 °C ±2 for 48 h. After growth, one loop-full of culture was streaked to Complete Medium Plate (CMP). Then obtained isolates were screened for protease, amylase and cellulase on respective medium plates and from them maximum producer of protease, was selected for secondary screening.A loopful of culture from the plate was transferred into 25 ml of sterile gelatin broth (GB) (gelatin, 10; casein hydrolysate, 10; glucose, 10; K_2HPO_4, 2.5; NaCl, 0% w/v; pH 7, g/l adjusted by adding a suitable volume of separately autoclaved 20% w/v Na_2CO_3) to prepare the inoculum. Ten percent of active culture was inoculated into 100 ml of gelatin broth in a 250-ml flask and incubated at 37°C ±2 on a 180-rpm rotary shaker. Extracted broth was measured for growth (600 nm) at various times intervals. The sample was then centrifuged for 10 minutes at 6000 rpm in a cooling centrifuge (4°C). After centrifugation, supernatant (cell-free extract) contained crude enzyme. This supernatant was preserved at cooling temperature for future experiments.

Identification of isolate, NCBI sequence submission and Phylogenetic tree construction

The identification of isolate 16s rRNA was performed, and the sequence was submitted to the NCBI data base. Then, using the neighbour joining method in NCBI BLAST, a phylogenetic tree was constructed.

Enzyme assay for protease

Anson-Hagihara's methodology was employed to screen the protease activity, using casein as a substrate for optimization and characterization research [3]. The enzyme (0.5 ml of crude enzyme) was added to 3 ml of casein substrate solution (0.6% w/v casein solution prepared in Borax-NaOH for pH 9). After 10 minutes of incubation at 37°C ±2, the enzyme reaction was terminated by adding 3.2 ml of the TCA mixture (0.11 M trichloroacetic acid, 0.22 M sodium acetate, and 0.33 M acetic acid). The mixture of the terminated reaction was incubated for 30 minutes in water bath at 37°C ±2. Using Whatman's No. 1 filter paper, the precipitates were removed. The filtrate's absorbance was measured at 280 nm. Under assay conditions, one unit of alkaline protease was defined as the quantity of enzyme that releases 1 µg of tyrosine per minute. As a benchmark for calculating enzyme units, tyrosine (0–100 µg) was used [4].

Growth kinetics and enzyme kinetics of BOR S17B13

The growth and enzyme kinetics of Bor S17B13 was checked at different time intervals. The culture was inoculated in GB medium (NaCl, 0% w/v; pH 7) and incubated at 37°C ±2 under shaking conditions (180rpm). Culture samples were withdrawn aseptically at different time intervals. Cell density and Enzyme activity was monitored as described above.

Optimization of inoculum size

For obtaining a higher amount of growth and the desired product, inoculum size is an important factor. As previously stated, inoculum broth was prepared; when the optical density of the broth reached approximately 0.6 to 0.7 O.D. at 600 nm. Inoculum culture (Bor S17B13) was added from range 1% to 10% in respective gelatin broth (NaCl, 0% w/v; pH 7), and then O.D. (600 nm) was taken at 48hr.

Effect of carbon sources on protease production

The effects of various carbon sources on enzyme production were investigated. The carbon sources, such as glucose, sucrose, xylose, galactose, lactose, starch, and fructose, were added into the gelatin broth (NaCl, 0% w/v; pH 7) as sole source of carbon of about 1% w/v. Here, 9% of activated culture of Bor S17B13 was inoculated in gelatin broth, and incubated at 37°C ±2 under shake flask conditions (180 rpm), and samples were withdrawn at definite time intervals (25 hr, 49 hr, and 73 hr) for estimating protease production.

Effect of nitrogen sources on growth and protease production

Following carbon sources, the next critical component is a nitrogen source. Here, the different nitrogen sources were investigated for their effect on protease enzyme production. The nitrogen sources included

casein (casein enzymatic hydrolysate), gelatin, yeast extract, peptone, and ammonium chloride in minimal media (0% NaCl w/v, pH 7), these nitrogen sources were used as the sole source of nitrogen, resulting in a final concentration of about 1% w/v. Nine percent of activated culture of Bor S17B13 was inoculated in broth and incubated at 37˚C ±2 under shake flask conditions (180 rpm), and samples were withdrawn at definite time intervals (25 hr, 49 hr, and 73 hr) for protease production estimation.

Effect of cations on protease production

Although cations are often known to stimulate the production of enzymes and provide them with stability, their impact on enzyme secretion was evaluated. For these experiments in gelatin broth, different salts were used: $CaCl_2$, $MgSo_4$, K_2HPO_4 and KH_2PO_4 at a concentration of 0.5%, w/v. Nine percent of activated culture of Bor S17B13 was inoculated and incubated at 37ºC ±2 under shake flask conditions (180 rpm), and samples were withdrawn at definite time intervals (25 hr, 49 hr, and 73 hr) for protease production estimation.

RESULTS AND DISCUSSION

Halophiles microbes have drag attention as they have ability to bear extreme condition and still can grow with minimum media and physiological condition. Their significance in various industrial and biological systems has been highlighted. The present work has focus on the production of extracellular protease and determining optimum parameters for growth and protease production from a halo-philic isolate Bor S17B13. In the present study, 62 isolates were obtained from two cites borivalimonari creek and jhow island mangrove ecosystem, Maharashtra. Their colony morphology was studied and their primary screening for industrially important enzymes (protease, amylase and cellulase) was performed on all of the isolates mentioned. Protease is one of them and has more demand in diverse fields, so further from primary screening, Bor S17B13, the maximum producer of protease, was selected for secondary screening of the protease enzyme [5]. Similar protease producer microbes about 33 Isolates associated to *Bruguieracylindrica*, a mangrove plant of North Sumatra, Indonesia [6].

Identification of isolate, NCBI sequence submission and Phylogenetic tree construction

Sequence was submitted to NCBI and accession No. OM743775, *Priestiamegaterium* strain B21 (Bor S17B13 isolate).

Phylogenetic tree: Bor S17B13 (*Priestiamegaterium* strain B21)

The present study focused on extracellular protease production and optimal growth and protease production and media optimization for higher yield of enzyme from a novel halo-philic isolate Bor S17B13, a gram-positive bacilli closely related to *Priestiaaryabhattai* based on 16s rRNA gene sequencing as mention below (Figure 1).

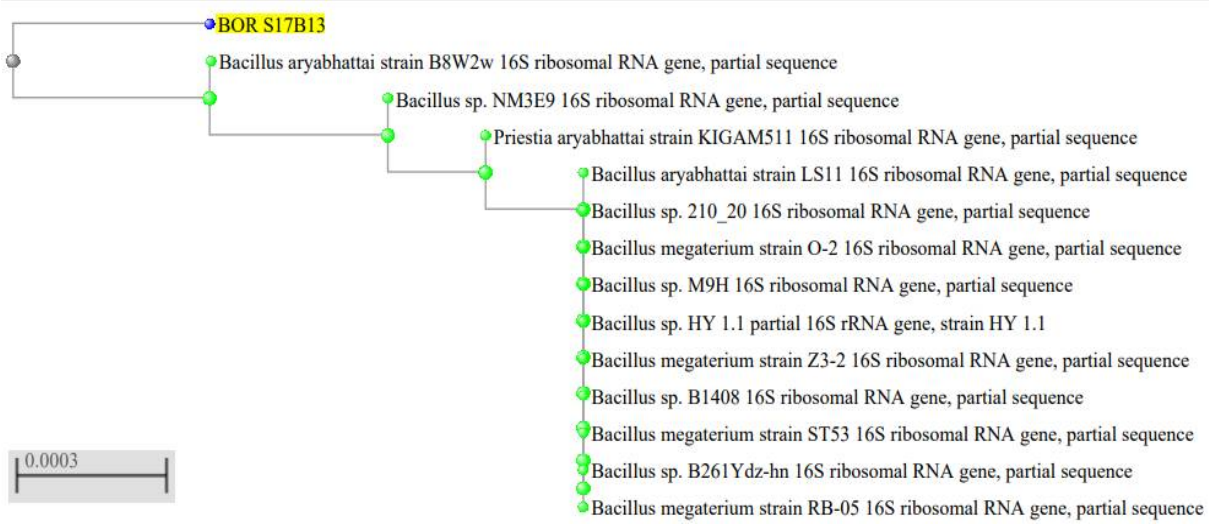

Figure 1. Phylogenetic tree with Neighbor joining method and scientific name of sequences listed represent closest homology with *Priestiaaryabhattai*

Growth and enzyme kinetics

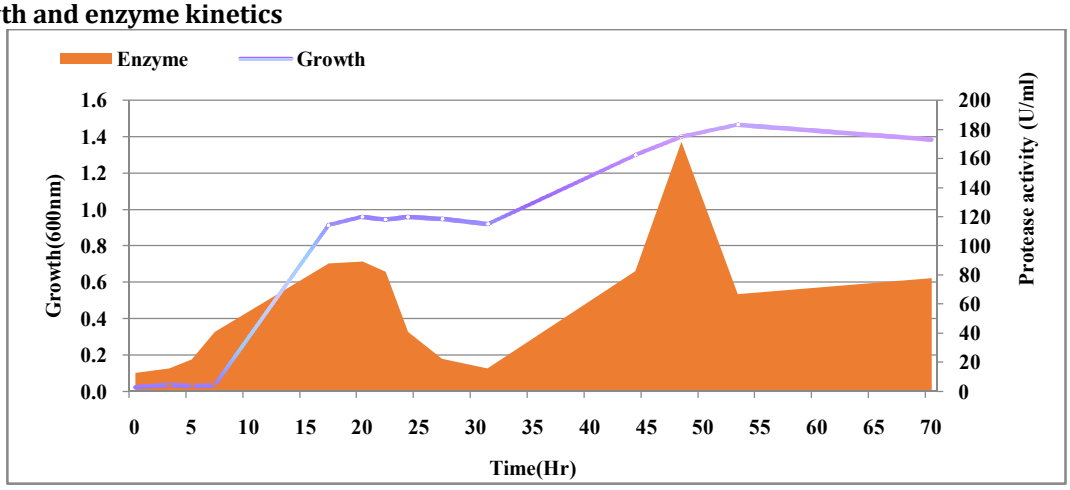

Figure 2. Growth kinetics (600 nm) and protease production (280 nm) of Bor S17B13 Samples were withdrawn at predetermined time intervals for 70 hr.

The growth kinetics and enzyme kinetics of Bor S17B13 was studied. It reveals that growth of the organism enter exponential phase after 7h and protease production increases with growth till exponential phase. The above graph (Figure 2) indicates a "Diauxic growth curve" pattern of growth. This type of growth is also known as the "biphasic growth curve". There was a clear second lag phase 27h to 44h were growth and enzyme production were drastically reduced. Here two nitrogen sources (Gelatin and Casein enzymatic hydrolysate) were added in the growth medium for obtaining flourishing growth, similar two nitrogen sources were used were gelatin was added along with casein hydrolysate in gelatin broth in ratio of 1% w/v each [7].

It was interesting to know that other then routine well known diauxic growth which is observed due to use of two carbon sources. In a study bacteria show a diverse range of consumption patterns, from diauxic growth to simultaneous consumption to switchable growth, when cultured in a batch medium containing a mixture of two growth-limiting substrates. They have previously demonstrated that all substrate consumption patterns may be explained by a simple model including mainly enzyme induction and dilution. Microbial development on mixed substrates is a dynamic counterpart to the population expansion of competing species [8]. Here in our study it may be due to two nitrogen sources and glucose as carbon source were used. A study supporting our hypothesis where they obtained diauxic growth pattern when two nitrogen salts like ammonium chloride and SCN along with glucose as one carbon source were supplied [9]. After second lag phase protease production is maximum during 48hrs (172 U/ml) at end of second exponential phase after that there was downfall in growth and protease production. Quite similar results obtained with *Pseudoalteromnas sp.*, as protease was detected at end of exponential phase [10]. The results of this study show that the diauxic growth pattern boosts protease production and growth during the second exponential phase more than the first (Figure 2).

Inoculum size

When it comes to industrially important products, optimizing inoculum size is crucial. Different percentages of inoculums were added to a set of gelatin broth, ranging from 1% to 10% (Figure 3), and the effect on bacteria growth was studied. The flask with 9% inoculums grew the most at an absorbance of 600 nm. A new isolate *Bacillus cereus* strain shows maxium growth in 5% inoculum [11]. Similarly in a study inoculum size was 5% (v/v) [12].

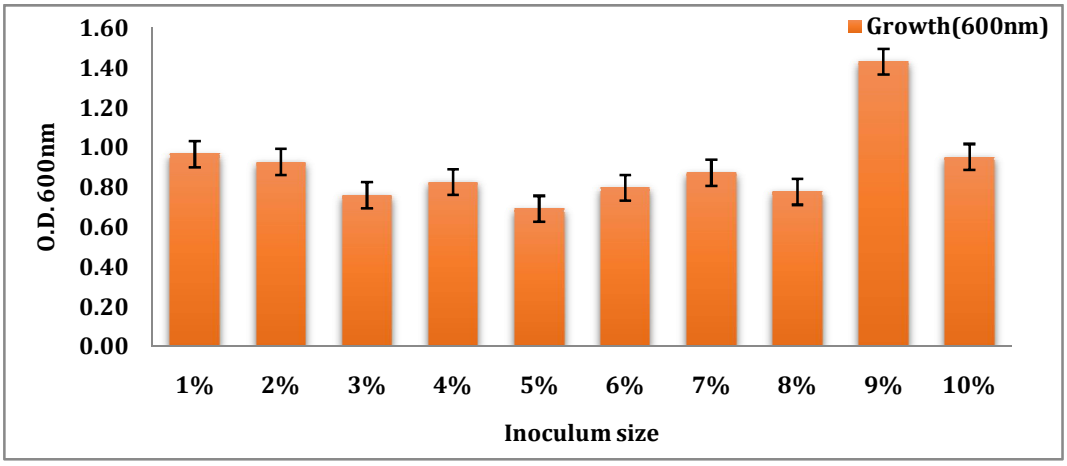

Figure 3. Optimization of inoculums size

Effect of carbon sources protease production

Different sugars were used to enhance protease production. Here (Figure 4) eight different forms of carbon sources were used at a concentration of 1% in gelatin broth as a single source of carbon.

In this study, the main focus is on protease production, and the approach is to increase production using different sugar sources. Mostly glucose as carbon source is widely used and also up taken quickly by many bacterial sp. In a study, *Pseudomonas fluorescens* and *Bacillus subtilus* utilize glucose as best source for protease production [13].

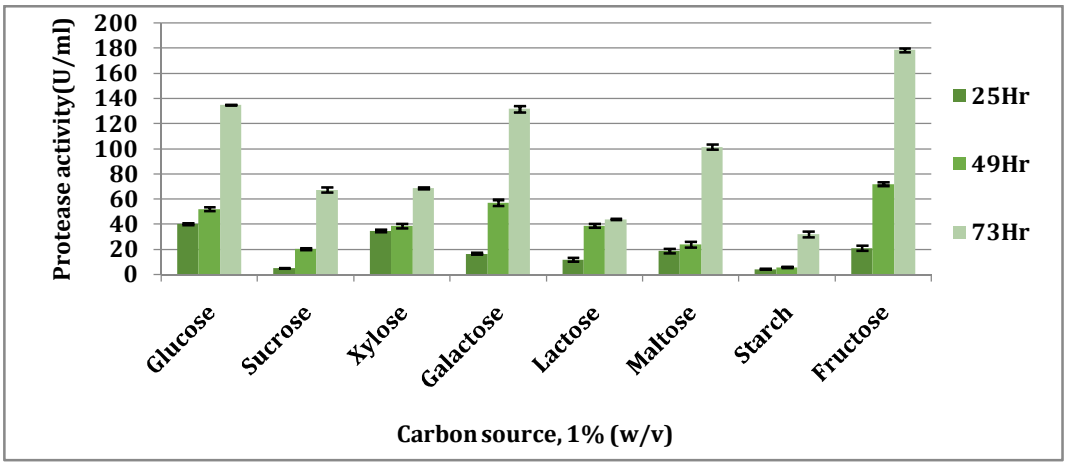

Figure 4. Comparative study of different types of carbon sources for Bor S17B13 (Data are expressed as mean ± SD)

Interestingly our case, Bor S17B13, has the highest production of protease with fructose as a source rather than glucose, which is well known. Similar results supporting our finding was obtained in alkaliphilic *Bacillus* sp. isolate name, NPST-AK15 from soda lake [14]. Few more similarity were *Bacillus licheniformis*, from salter sediment where after xylose, fructose supported maximum protease production [15]. In other study, in *Bacillus cereus* strain, 3rd most favorable carbon was fructose [11]. Maximum protease production was obtained at 73 h for all carbon sources in the experiment. The optimal protease enzyme was produced by fructose (179 U/ml), followed by; glucose (135 U/ml); galactose (132 U/ml); maltose (101 U/ml); xylose (69 U/ml); sucrose (67 U/ml); lactose (44 U/ml), and starch (32 U/ml).

In comparison to the others, the glucose broth produced the maximum protease at 25 h. Glucose and galactose had nearly similar amounts of protease produced at 73 h, followed by maltose. Furthermore, at 73 hours, sucrose and xylose produced nearly similar amounts of protease. In the present study starch favors the least enzyme production. Similar results were obtained in a study were different *Bacillus Sp.* isolated produce low protease enzyme in presence of starch compare other carbon sources [16].

Effect of nitrogen sources on protease production

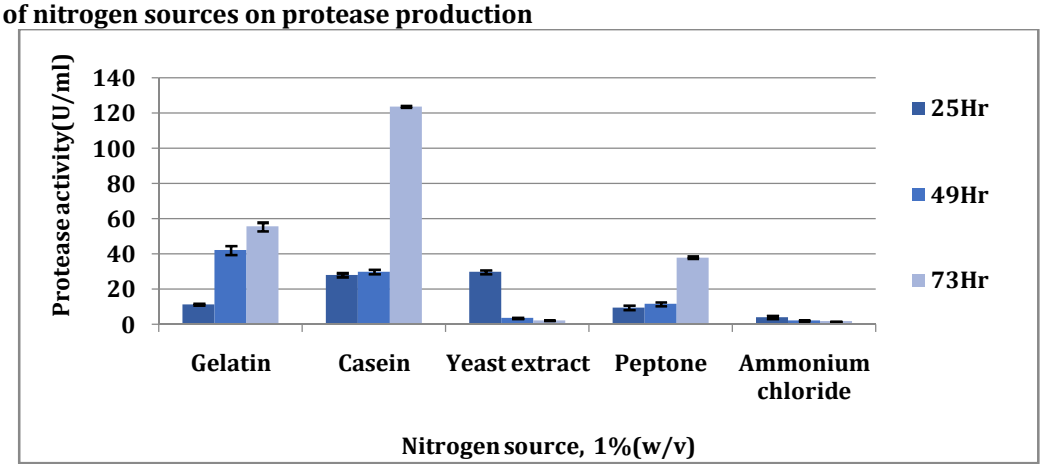

Figure 5. Comparative study of nitrogen sources for protease production (Data are expressed as mean ± SD)

Nitrogen sources have importance in bacterial growth and enzyme production. Here five different nitrogen sources (Figure 5) were used. Glucose was used as carbon source along with minimal salts as mention previously. All of the above nitrogen sources were used at 1% w/v concentration. Optimum protease was produced by casein enzymatic hydrolysate (124 U/ml) at 73h. A study supporting our result was an isolate AKS-4, bacterial isolate from soil has optimum results with casein as sole source of nitrogen [17]. Similar results were found in *Bacillus subtilis*, a psy-chrotrophic bacterium that also produces maximum protease with casein [18].

Then in our finding second most favorable for protease was gelatin (56 U/ml) followed by peptone (38 U/ml) both at 73h. In a study isolates from soil, *Serratiamarscens*, *Pseudomonas fluorescens*, *E. coli*, and *Bacillus subtilis*, all protease producers, grew best in peptone and urea [13]. A study display, *Bacillus licheniformis* a protease producer had optimium production using urea [15]. A halotolerant isolate (NPST-AK15) from soda lake, maxium protease yield was obtained from yeast extract [14]. Similar results were obtained in protease producer, *Bacillus licheniformis* where yeast extract gave optimum results [19]. Our maximum production for yeast extract was 30 U/ml and ammonium chloride was 4 U/ml, both at 25h. ammonium chloride in our study (Figure 8) support hypothesis phenomenon of repression of enzyme production by ammonium ions ([20][21][7][22]).

Effect of cations on protease production

Usually, cations are known for enhancing enzyme production; here, in this present study, various cations (Figure 6) effects were studied [4].

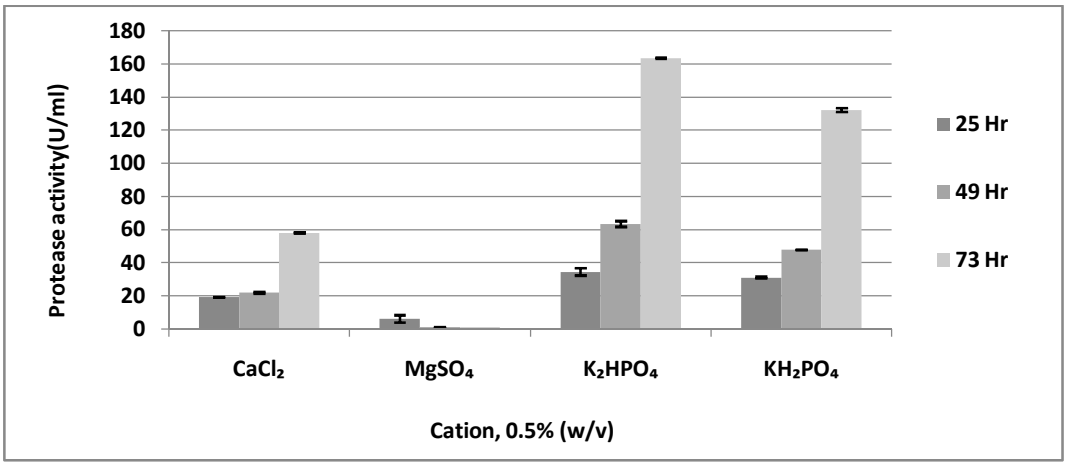

Figure 6. Comparative study of nitrogen sources for protease production (Data are expressed as mean ± SD)

Gelatin broth was used and 0.5% w/v of cations was added. From all cations maximum protease produced was produced at 73h by K_2HPO_4 (163 U/ml) followed by KH_2PO_4 (132 U/ml) and then was $CaCl_2$ (58 U/ml). At 25 hours, $MgSO_4$ (6 U/ml) produced the least amount of product compared to the

other cations. Enzyme production was max at 73h in all cation except for $MgSO_4$. Optimum production in this study was with K_2HPO_4. similar results were obtained were K_2HPO_4 favored protease enzyme production [19];[7]. As it shows in our case, K_2HPO_4 and KH_2PO_4 used for enhancing protease production similar results were obtained in a protease producer, *Bacillus mojavensis*[23]. Many studies favor Ca^{2+} ion for supporting protease production and increasing its yield [11];[13];[14].

CONCLUSION

The current work focuses on extracellular protease production from the halophilic isolate Bor S17B13. 62 isolates were collected from BorivaliMonari Creek and the Jhow Island Mangrove Ecosystem, Maharashtra. All of the isolates were screened for industrially important enzymes (protease, amylase, and cellulase). Protease is in high demand in many fields, so Bor S17B13, the highest protease producer, was chosen for secondary screening. From the experiment it was concluded 9% inoculums gave lust growth and was selected for further experiments. Interestingly in our case, Bor S17B13, has the highest production of protease with fructose as a source rather than glucose, which is well known. Similar results supporting our finding was obtained in alkaliphilic *Bacillus* sp. isolate [14]. Optimum protease was produced by casein enzymatic hydrolysate (124 U/ml) at 73h. A study supporting our result was an isolate AKS-4 utilising casein and producing maximum protease [17]. As cation are known for their enhancing ability in enzyme production. From all cations maximum protease produced was at 73h by K_2HPO_4 and $MgSO_4$ (6 U/ml) produced the least amount of product compared to the other cations. Compared to other ions in the experiment, MgSO4 ion, protease production was drastically reduced in our findings. In contrast to this result, MgSO4 showed positive effect on protease production in *Bacillus mojavensis* SA-strain [23]. Ca ion also supported growth in our study. Maximum production protease enzyme in our study was 179 U/ml by fructose as source. Diauxic growth pattern of was interesting characteristics of Bor S17B13.

FUTURE PROSPECTIVE

Over the course of the experiment, define that specialisation for each isolate obtained for product is a must, as microorganisms have very diverse source utilisation abilities. Furthermore, according to Grand View Research, demand for protease production is increasing on a daily basis, with the enzyme industry expecting an increase until 2027. Research on enzymes is projected to grow by more than $9 billion. Economic perspective: enzyme production is rich, and more studies will be facilitated in the subject.

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