

Optimization and Production of Amylase Enzyme from Crab Gut Microflora

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ABSTRACT

Crab receives bacteria in the gut from their aquatic environment through water and food that are populated with bacteria. Being rich in nutrients, the environment of the crab gut confers favourable conditions for the microorganisms. Enzymes are biological catalysts exceedingly esoteric with extraordinary catalytic power and embrace remarkable specificity. The objective of the present study was to improve the synthesis of amylase that was isolated from *Bacillus* sp., a type of crab intestine bacteria. In addition, many variables, including pH, temperature, and substrate concentration, were tuned. To generate higher and better enzyme activity, additional ions in the reaction media were categorised as activators or inhibitors. Based on this fact, crab waste can be effectively utilized for the production of amylases. The results illustrated in the table indicate that the optimum temperature for amylase activity was 33 ° C. The optimum temperature for amylase activity from MBK2 was similar to or quite higher than that of the optimum temperature of amylase from MBK1. The enzyme was highly active at pH 4.0 and it lost that optimum temperature for amylase activity was 33 ° C. The optimum temperature for amylase activity from MBK2 was similar to or quite higher than that of the optimum temperature of amylase from MBK1. The enzyme was highly active at pH 4.0 and it lost its activity considerably at pH 7. This pH optimum was higher than the pH optimum (11) of MBK1 and MBK2.

Keywords : Amylase, Starch, Rice-bran, *Bacillus* sp.

INTRODUCTION

All living things have the gut microflora as a necessary component. Numerous endogenous and external variables impact the physiological and biochemical characteristics of the microorganisms themselves and define the quantity and species composition of the microbial population. Various microorganisms from the environment infiltrate living things. The bacteria present in the aquatic environment have an impact on the composition of the gut biota and vice versa since the host and microorganisms share the ecosystem, but only those bacteria survive and find

appropriate living circumstances in that organism. This is because of natural selection. Crab receives bacteria in the gut from their aquatic environment through water and food that are populated with bacteria. Being rich in nutrients, the environment of the crab gut confers favourable conditions for the microorganisms. The importance of gut bacteria in the nutrition and well-being of their hosts has been established for homeothermic species, such as birds and mammals. Bacterial species of the gut can influence the health and development of the host. By creating an ecological niche for these species, the milieu of crustaceans' digestive tracts promotes favourable development for

bacteria. It has been demonstrated that the micro biota of crustaceans is heavily reliant on bacterial colonisation during early growth, environmental factors, nutritional composition, and dietary alterations. These bacteria have a higher chance of surviving because they feed on the food that the host animal absorbs, digestive fluids, and pieces of mucosal epithelium that have shed their scales. Microbes are identified in classical microbiology using both gross and microscopic morphologies. Colony size, form, and surface characteristics were all part of the gross morphology. With proper sample preparation and light microscopy, the shapes attributed to bacterial cocci (round), bacilli (rods), or spirochetes (corkscrew) may be easily seen. One of the most effective and often used methods for discriminating bacteria above the genus level is gram staining. Enzymes are biological catalysts exceedingly esoteric with extraordinary catalytic power and embrace remarkable specificity. A wide range of living things, including bacteria, plants, animals, and people, create amylases. Amylases are secreted by bacteria and fungi outside of the cells to perform extracellular digestion. In both academic and industrial settings, microbial amylases derived from bacteria, fungi, and yeast has been employed. The amount of amylase generated also varies according to the microbe's place of origin, with strains from environments rich in starch or amylase naturally producing more enzymes . Generally, enzymes are elected over chemical catalysts. Therefore, thermo-phillic microorganisms are conceived to be potentially good alternative sources of thermo-stable thermophiles . Thermo-stable enzymes have been delineated to have inflated stability to organic solvents, acidic and alkaline pH, and detergents . Enzymes are recognised as a crucial resource used by

the food, chemical, and related sectors to build a wide range of biotechnology goods. They have so far been acknowledged as a priceless catalyst for numerous organic transformations as well as the creation of quality chemicals and medications . Due to their low cost, high productivity, chemical stability, preservation of the environment, adaptability, and wide availability, microbial enzymes are frequently utilised in industrial processes. The pace of amylase production, particularly in fermentation processes, is greatly influenced by variables including pH, temperature, and supplies of carbon and nitrogen. Microorganisms are amenable to genetic engineering; therefore strains can be modified to produce larger amylase yields . Agro-industrial residues are generally considered the best substrates for the solid-state fermentation process because the metabolites are produced concentrated and purification processes are less costly . Solid state fermentation by agro-industrial waste makes an attractive method as support carbon sources for the production of industrial enzymes and various value-added products such as Organic acids, flavour compounds, biofuel, and secondary metabolites. When agro residues are used as primary substrates in solid-state fermentation, there is a limitation in the availability and accessibility of nutrients. Therefore, supplements of sugars and nitrogenous compounds in the form of diluents should be provided to enhance microbial growth and enzyme productivity. Moreover, for maximum production formation, microorganisms also need to facilitate optimum cultural conditions such as incubation time, growth temperature, and, initial pH of diluents, moisture content and inoculum level. The major factors that affect the microbial synthesis of enzymes in a solid-state fermentation system include the selection of a suitable substrate and

microorganism, the weight of the substrate, inoculum concentration and the moisture level of the substrate. Thus it involves the screening of several agro-industrial materials for microbial growth and product formation. In the industrial setting, they are employed in the manufacturing of digestive aids, cheese, baking, brewing, de-hairing, and tenderising of meat. It is also preferable to avoid adding calcium during starch hydrolysis for many industrial operations, such as brewing or syrup manufacture, as its presence lowers product quality and necessitates further calcium removal. Reduced danger of contamination, lower internal cooling costs, and higher diffusion rate are some benefits of employing thermo-stable - amylases in industrial processes. Nevertheless, practically all known - amylases have Ca^{2+} in their structure and need calcium to be added during hydrolysis to maintain their stability and activity. The creation of extracellular enzymes by *Bacillus* sp., one of the many non-pathogenic microbes capable of creating helpful enzymes, makes them particularly intriguing. With a vast number of them becoming commercially accessible, microbial amylases satisfy industrial demands and have almost entirely replaced the chemical hydrolysis of starch in the starch processing sector. The capacity to create amylases in large quantities and the simplicity with which they may be modified to produce desired products are the main benefits of employing microbes for amylase synthesis. Using a plasma enzyme test to identify diseases in various body sections. The pancreas produces the digesting enzyme - amylase, and in some pancreatic disorders, notably acute pancreatitis, this enzyme is present in plasma in elevated concentrations. The easiest enzyme to test is -amylase since it uses starch as a substrate and turns it into reducing sugars as a by-product. Being one of

the few proteins small enough to pass past the glomerulus of the kidney and be eliminated in the urine is a peculiar property of pancreatic -amylase in macro-amylasaemia, an uncommon disease. The plasma-amylase activity increases as a result of the plasma-amylase forming complexes with other proteins and becoming unable to be eliminated in this manner. However, due to the highly distinct clinical manifestations of macro-amylasaemia, acute pancreatitis could not be mistaken for it. The major factors that affect the microbial synthesis of enzymes in an SSF system include the selection of a suitable substrate and microorganism, the particle size of the substrate, inoculum concentration and moisture level of the substrate. Thus, it involves the screening of a number of agro-industrial materials for microbial growth and product formation. Temperature and pH are known to be important parameters in the production of enzymes from bacteria; hence, the thermal and the pH stability of the enzyme, which is a function of the exposure time, must also be taken into account. The goal of the current study was to improve the synthesis of amylase that was isolated from *Bacillus* sp., a type of crab intestine bacteria. In addition, a number of variables, including pH, temperature, and substrate concentration, were tuned. To generate higher and better enzyme activity, additional ions in the reaction media were categorised as activators or inhibitors. Additionally, we wanted to assess the enzymes' ability to remove stains by employing low-cost raw materials to extract enzymes from the amylase that the detergent industry uses extensively. Investigating the cleaning effectiveness of animal amylase mixed with laundry detergent and its unique compatibility as a very effective novel additive was the major contain of the enzyme application raised in this study.

MATERIALS AND METHODS

SAMPLE COLLECTION

Samples of marine crab species, blue swimmer crab (*Portunuspelagicus*) (Herbst, 1783) and three-spotted crab (*Portunussanguinolentus*) (Herbst, 1783) were collected from the Mannapad Estuary, Tuticorin district, which had been transported to the laboratory under iced condition. Crabs 12+1 cm in length were selected for this study. They were cleaned in chlorinated water and the length and breadth of their carapaces were measured. The surfaces of the crabs were sterilized using 70% ethanol. The intestines were aseptically dissected from the musculature and placed into a 2-mL sterile centrifuge tube.

ISOLATION OF GUT BACTERIA

The *Bacillus* species strains (MK1 and MK6)

used in the present study were isolated as potent amylolytic bacteria from the gut of *Portunussanguinolentus* and *Portunuspelagicus* (figure.1). Approximately 9 mL of distilled water was then sterilized. To this 1mL stock, the solution was added and the following dilutions were prepared i.e. 10⁻¹, 10⁻², 10⁻³, 10⁻⁴, 10⁻⁵, 10⁻⁶, 10⁻⁷ from each dilution 0.1 mL of sample was spread into the Petri plates with nutrient agar medium (peptone-5.0; NaCl-5.0; HM peptone B-1.5; yeast extract-1.5; agar-15.0; pH-7.4±0.2). The plates were incubated at 37°C in an inverted position for approximately 24 hours. After incubation, the number of colonies that formed was counted. A single colony was isolated and streaked on the prepared agar slants. Agar slants were maintained at 4°C in the refrigerator for further experimental studies.



Figure.1 plate showing the gut microflora of the crab species

BIOCHEMICAL ANALYSIS

KB002-HiAssorted™ Biochemical test (By kit)

The biochemical test was carried out for Citrate utilization test, Lysine utilization test, Ornithine utilization test, Urease detection test, Phenylalanine deamination (TDA), Nitrate reduction test, H₂S production test, Glucose utilization test, Adonitol, Lactose, Arabinose, Sorbitol according to company instruction (Hi Media Mumbai) 50 µl of the isolated organism was inoculated into each well in the test kit by surface inoculations

method. The kit was incubated at 37 ° C for 24 hrs based on the principle of pH change after substrate utilization. On incubation, organisms undergo metabolic changes which were indicated by colour changes in the media that can be either interrupted visually or after the addition of a reagent.

KB009- HiCarbo™ Utilization test (by Kit)

The carbohydrate test is based on the principle of pH change and substrate utilization. On incubation, the organism undergoes metabolic changes which were indicated by a spontaneous colour change in the media. The organisms were analysed for

the utilization of (i) Lactose, (ii) Xylose, (iii) Maltose (iv) Fructose (v) Dextrose (vi) Galactose (vii) Raffinose (viii) Trehalose (ix) Melibiose (x) Sucrose (xi) L-Arabinose, (12) Mannose, (13) Inulin, (14) Sodium gluconate, (15) Glycerol, (16) Salicin, (17) Dulcitol, (18) Inositol, (19) Sorbitol, (20) Mannitol, (21) Adonitol, (22) Arabitol, (23) Erythritol, (24) α -Methyl-D-glucoside, (25) Rhamnose, (26) Cellubiose, (27) Melezitose, (28) α -Methyl-D-mannoside, (29) Xylitol, (30) ONPG, (31) Esculin hydrolysis, (32) D-Arabinose, (33) Citrate utilization, (34) Malonate utilization, (35) Sorbose 50 μ l of broth containing the sample was inoculated into the well provided in the kit and incubation was carried out at 37 °C for 24 h

IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF BACTERIA

16S ribosomal RNA gene sequencing

Sequencing the purified PCR products with the help of the Rajiv Gandhi Centre for Biotechnology. Nucleotide sequences were compared with sequences in the National Centre for Biotechnology Information (NCBI). GenBank database using the BLASTn program database using the sequence matching program.

Phylogenetic analysis

Once the sequencing was done, the resultant nucleotide sequence was subjected to n-BLAST analysis in National Centre for Biotechnology Information (NCBI) and deposited in the GenBank database for universal accession.

QUALITATIVE TEST OF AMYLASE-PRODUCING STRAIN

By performing a starch hydrolysis test on a starch agar plate (peptic digest of animal

tissue-5.0; meat extract-3.0; starch, soluble-2.0; agar-15.0; pH-7.20.1), bacteria were isolated and their amylolytic potential was evaluated. Starch agar plates with the microbial isolates were streaked, and the plates were incubated at 37°C for 24 h. Following incubation, the starch agar plate was treated with freshly prepared 1% iodine solution. The blue colour zone around the growth denotes a poor outcome, whereas a clear zone of hydrolysis around the growth denotes a good outcome. These isolates were further examined because they exhibited distinct zones of hydrolysis and were thought to be amylase producers.

Substrate

Two different types of substrates were used one standard starch and another one agro-industrial waste to be used as substrate viz., rice bran was procured from the local market and powdered to obtain a particle size of 1.0 to 2.0 mm. SSF was performed with all two substrates and their enzyme production was checked by assay.

SUBMERGED FERMENTATION

Bacterial inoculums were inoculated into 50mL of the culture medium. The fermentation medium comprised starch (10 g/L), Peptone (10 g/L), yeast extract (20 g/L), KH_2PO_4 – (0.05 g/L); $\text{MnCl}_2 \cdot \text{H}_2\text{O}$ (0.015 g/L); $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.25 g/L); $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.05 g/L); $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.01 g/L). Submerged fermentation is used to make amylase. 10 mL of bacterial inoculum was added to 500 mL of the production medium for inoculation. The flask was put into a rotating shaker incubator and run for 24 hours at a particular speed, temperature, and pH. After incubation, the fermented broth was centrifuged in a cooling centrifuge for 15 minutes at 10,000 rpm. The amylase concentration in the supernatant was measured.

pH, temperature, and incubation time effects on growth and amylase production

The effect of pH value and temperature on amylase production was carried out using the following pH range (4-11) and incubation temperature (28, 33, 38, 43 and 48°C) for bacterial isolates *Bacillus* sp. For the test isolation, 100 mL of sterile medium (starch-10g/L; peptone-10g/L; yeast extract-20g/L; KH_2PO_4 -0.05g/L; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ -0.015g/L; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ -0.25g/L; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ -0.05g/L; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ -0.01g/L) was produced in several conical flasks and infected for 24-72 hours with 2% inoculum (starch/rice bran)

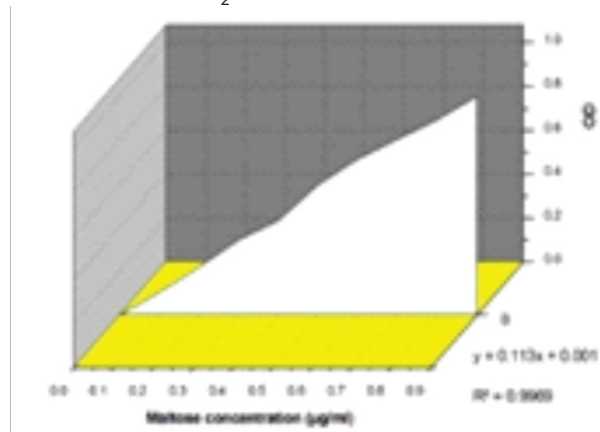
QUANTITATIVE TEST FOR AMYLASE

Based on the methods, amylase activity was estimated. According to Gomes *et al.*, amylase was mainly measured using soluble starch, 1% (w/v), as substrate in phosphate buffer (pH 6.5). The reaction mixture, which included 0.2mL of adequately diluted enzyme solution and 1.8mL of the substrate solution, was incubated at 50°C for 10 min. By adding 3mL of dinitrosalicylic acid (DNS), the process was halted. The Miller technique was used to determine the amount of reducing sugar released (1959). Absorbency of the solution was made at 540 nm using UV-Vis Spectrophotometer against a blank prepared by a control fermentation medium. The absorbency of the enzyme concentration of reducing sugar was determined by comparing the OD value with a standard graph prepared using maltose. In all circumstances, the quantity of enzyme releasing 1 g of reducing sugar as maltose per minute under test conditions is referred to as one unit (U) of enzyme activity.

STANDARD PREPARATION

Maltose working solution:

180mg of maltose is weighed and made up to 100mL with d. H_2O .



Maltose standard graph for amylase enzyme

Maltose standards are shown in the graph above. Given that the line is straight, the photometry was successful in demonstrating that the variation in optical density relies on the concentration of maltose as the concentration of maltose rises. Using Microsoft Excel, the R (correlation coefficient), which measures the strength of the association between the two variables and aids in determining the linear regression between maltose concentration and absorbance, was computed. Its square value was $R^2 = 0.9969$. The concentration of maltose emitted during this experiment was calculated using the maltose standard curve equation, $y = 0.113x + 0.001$, where y stands for maltose absorbance and x for concentration.

RESULTS AND DISCUSSION

The study was performed to isolate, characterize and optimise of growth and enzymatic activity of amylolytic bacteria

isolated from the gut of *Portunussanguinolentus* and *Portunuspelagicus*. Enrichment of samples, selective plating, microscopy, biochemical tests and the different enzymatic assay was applied for this purpose.

Morphological characterization

The selected antagonistic isolate MBK1 was gram-positive rod-shaped bacilli with square ends. They are single rod-shaped or appear in short chains. MBK2 was an aerobic gram-positive, round shape, white, smooth edges and raised elevation, spore-forming motile bacteria. Gram-positive, rod-shaped bacteria that differentiate into heat-resistant endospores under aerobic conditions were placed in the genus *Bacillus*. Bacteria belonging to the genus *Bacillus* have a long and distinguished history in the realms of biotechnology. Manufacture of amylases and proteases for industrial applications began early this century, but significant production and usage were delayed until after the 1950s when the advantages of including the alkaline protease of *Bacillus licheniformis* in washing detergents were realized. This was followed by developments in the starch processing industry based on the α -amylase from *Bacillus licheniformis*, particularly the conversion of starch to high-fructose corn syrups as sucrose replacements in foods and beverages. described the genomic information that can be employed in the development of other researchers, including comparative analyses employing genes and genomes, origin and dispersion of strains. It was reported as a *Bacillus cereus* isolated from the digestive tract in a saline environment. Reported that all the microbes were grouped into six families and seven genera, which showed a good relationship between the bacterial populations associated with crabs; *Bacillus* was the dominant genus, followed by *Staphylococcus*, *Pseudomonas*,

Enterobacter, *Aeromonas*, *Alcaligenes* and *Acinetobacter*. reported from the gut of fifteen mangrove crab species 523 bacterial strains were isolated and classified into ten taxonomic groups including the highest number of strains *Bacillus* (113).

Biochemical characterization

The isolate MBK1 exhibited positive lysine utilization, ornithine utilization, urease detection, H₂S production test, adonitol, lactose, arabinose, sorbitol and starch. Further, this isolate showed negative for the following reactions, such as citrate utilization test, phenylalanine deamination (TDA), nitrate reduction and glucose. The isolate MBK2 exhibited positive lysine utilization, ornithine utilization, urease detection, adonitol and starch. Further, this isolate showed negative for the following reactions, such as citrate utilization test, phenylalanine deamination (TDA), nitrate reduction, H₂S production test, glucose, lactose, arabinose and sorbitol. These results suggested that all the isolates belong to the genus *Bacillus* sp (figure.3).

Analysis for carbon utility test

Among the 35 carbon sources, this MBK1 strain used and ferment the Lactose, Dextrose, Galactose, Mannitol, Adonitol, Arabitol, Erythritol, α -Methyl-D-glucoside, ONPG, Esculin hydrolysis, D-Arabinose, Citrate utilization and Malonate utilization. However, they could not hydrolyze Xylose, Maltose, Fructose, Raffinose, Trehalose, Melibiose, Sucrose, L-Arabinose, Mannose, Inulin, Sodium gluconate, Glycerol, Salicin, Dulcitol, Inositol, Sorbitol, Rhamnose, Cellobiose, Melezitose, α -Methyl-D-mannoside, Xylitol and Sorbose. The MBK2 strain was used and ferments the Lactose, Xylose, Maltose, Fructose, Dextrose, Galactose, Raffinose, Trehalose, Melibiose, Sucrose, Mannose, Inulin, Sodium gluconate, Glycerol, Salicin, Dulcitol, Inositol, ONPG,

Esculin hydrolysis, D-Arabinose, Citrate utilization and Malonate utilization. However, they could not hydrolyze L-Arabinose, Sorbitol, Mannitol, Adonitol, Arabitol, Erythritol, α -Methyl-D-glucoside, Rhamnose, Cellobiose, Melezitose, α -Methyl-D-mannoside, Xylitol and Sorbose. (figure.4)

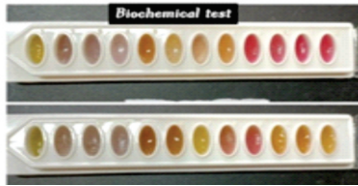


Figure.3 Biochemical characterization of MBK1 and MBK2

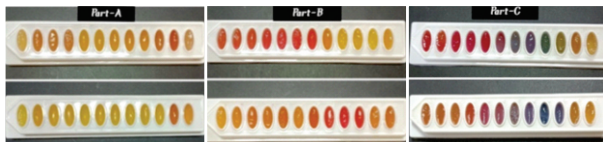


Figure.4 Characterization of carbohydrate utilization for MBK1 and MBK2

Molecular identification

The 16S rRNA gene of the MBK1 & MBK2 strains were amplified by PCR techniques using the 16S rDNA universal primers and both the strands were sequenced. The resultant nucleotide sequences were subjected to n-BLAST analysis in the National Center of Biotechnology Information (NCBI) and deposited in the GenBank database for universal accession. The 16S rRNA

sequence of isolated strains MBK1 & MBK2 was subjected to alignment using BLAST to find out the sequence similarities. With the help of the BLAST Nucleotide tool, the sequence alignment drawn by the slanted cladogram phylogenetic tree of MBK1 and MBK2 showed in Figures 5 & 6. The strain MBK1 closest sequence to the *Bacillus thuringiensis* strain with 99.29% similarity, accession number MT435558 and the MBK2 strain is closely similar with 99.86% KX610819 *Bacillus cereus*.

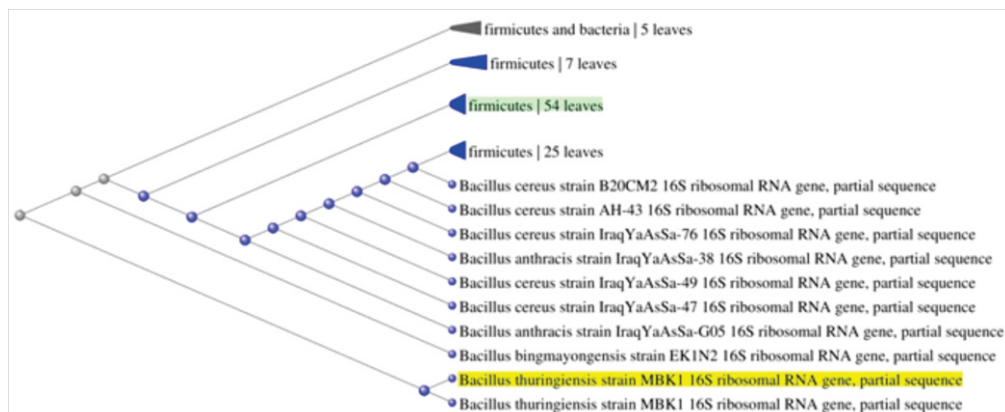


Figure:5 Phylogenetic tree of MBK1

(July 2022)

OPTIMIZATION AND PRODUCTION OF AMYLASE

06/03/2023, 12:59 Bacillus cereus strain MBK2 16S ribosomal RNA gene, partial sequence - Nucleotide - NCBI

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Nucleotide

GenBank

Bacillus cereus strain MBK2 16S ribosomal RNA gene, partial sequence

GenBank: OP526970.1

FASTA Graphics

Go to

LOCUS

OP526970

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DNA

linear

BCT 17-FEB-2023

DEFINITION

Bacillus cereus strain MBK2 16S ribosomal RNA gene, partial sequence.

ACCESSION

OP526970

VERSION

OP526970.1

KEYWORDS

SOURCE

Bacillus cereus

ORGANISM

[Bacillus cereus](#)
Bacteria; Bacillota; Bacilli; Bacillales; Bacillaceae; Bacillus;
Bacillus cereus group.

REFERENCE

1 (bases 1 to 720)

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COMMENT

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Sequencing Technology :: Sanger dideoxy sequencing
##Assembly-Data-END##

FEATURES

Location/Qualifiers

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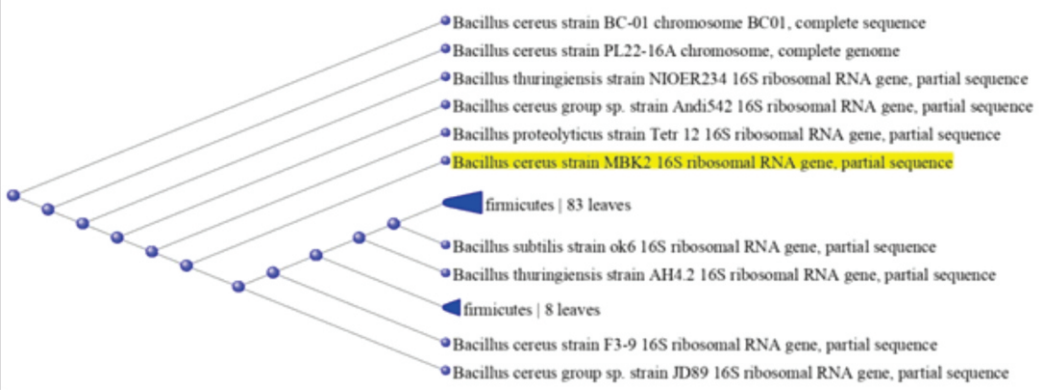


Figure: 6. Phylogenetic tree of MBK2

Selection of probiotic microorganisms that produce amylase

After the screening, it was discovered that only 2 of the 6 bacterial strains produced the most amylase. The strain from these two that produced the zone most effectively was chosen for further study. Fogarty and Kelly (1979), as well as Iverson and Millis (1974), described the use of starch agar and idoine for the detection of amylase (hydrolytic enzyme) generating bacteria. Starch hydrolysis may be observed on plates as a clear zone encircling a colony. The amylase generated during the organisms' development has hydrolyzed starch around the colony, hence testing negative when inundated with iodine is the mechanism behind the clean zone that was seen (Figure.7). The unhydrolyzed portion of the plate tested positive for starch (amylose), which is why it was blue-black. According to Akpan *et al.* (1999), a quick screening approach like Remazol Brilliant Blue (R.B.B.) will be more effective because the starch hydrolysis method is time-consuming and difficult for direct separation of intact cells. After all, the cells die after being flooded with iodine. R.B.B. starch agar offers a quick and easy method for searching for amylolytic bacteria. It doesn't call for floods or previous slant-based colony reproduction. The zones stand out vividly against the blue-black background.

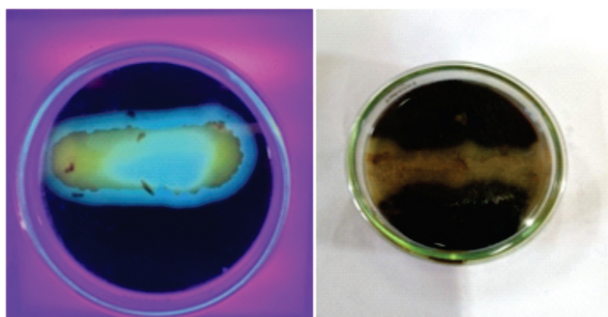


Figure. 7 Striking of Bacterial strains on starch agar plate showed clear surrounding zone after flooding the plate with iodine solution

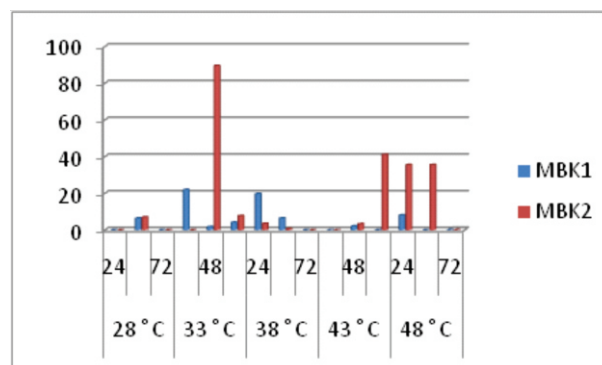


Figure 8; Effect on enzyme production with pH – 4 at different temperatures of MBK1 & MBK2 strains with different time intervals

Figure 8, shows the effect of temperature of pH 4, and starch as a substrate on the activity of α -amylase whereby, maximum enzyme activity was observed on MBK2 strain at 33° C at 48 h. MBK1 showed the highest amylase activity at 21.91 ± 0.07 at 33° C, 24 h. MBK2 showed the highest amylase activity 89.55 ± 0.08 at 33° C at 48 h. The result of the temperature effect on the growth of selected bacterial strains MBK1 and MBK2 showed maximum growth at 28° C 6.42 ± 0.04 of 48 h incubation and 7.00 ± 0.24 of 48 h incubation; 33° C 21.91 ± 0.07 of 24 h incubation and 89.55 ± 0.08 of 48 h incubation; 38° C 19.79 ± 0.09 of 24 h incubation and 3.5 ± 0.12 of 24 h incubation; 43° C 2.16 ± 0.51 of 48 h incubation and 41.07 ± 0.20 of 72 h incubation; 48° C 8.07 ± 0.23 of 24 h incubation and 35.52 ± 0.55 of 24 h and 48h incubation.

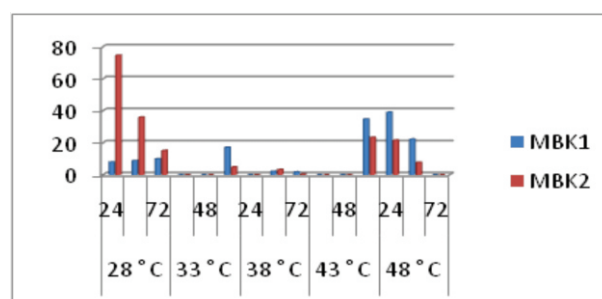


Figure 9; Effect on enzyme production with pH – 4 at different temperatures of MBK1 & MBK2 strains with different time intervals

Figure 9, shows the effect of temperature of pH 7 on the activity of α -amylase whereby, maximum enzyme activity was observed on MBK2 strain at 28°C at 24 h. MBK1 showed the highest amylase activity at 39.01 ± 0.47 at 48°C, 24 h. MBK2 showed the highest amylase activity 74.64 ± 0.31 at 28°C at 24 h. The result of the temperature effect on the growth of selected bacterial strains MBK1 and MBK2 showed maximum growth at 28°C 9.91 ± 0.21 of 72 h incubation and 74.64 ± 0.31 of 24 h incubation; 33°C 17.04 ± 0.13 of 72 h incubation and 4.82 ± 0.04 of 72 h incubation; 38°C 2.08 ± 0.08 of 48 h incubation and 3.21 ± 0.04 of 48 h incubation; 43°C 34.77 ± 0.16 of 72 h incubation and 23.38 ± 0.08 of 72 h incubation; 48°C 39.01 ± 0.47 of 24 h incubation and 21.6 ± 0.32 of 24 h incubation.

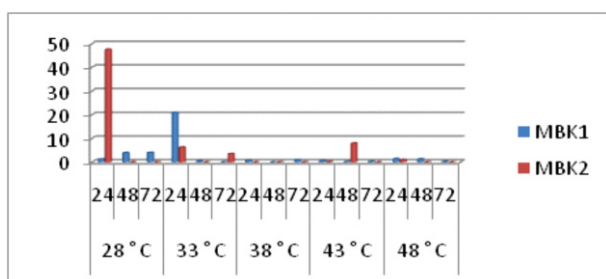


Figure 10; Effect on enzyme production with pH – 4 at different temperatures of MBK1 & MBK2 strains with different time intervals

Figure 10, shows the effect of temperature of pH 11 on the activity of α -amylase whereby, maximum enzyme activity was observed on MBK2 strain at 28°C at 24 h. MBK1 showed the highest amylase activity 20.92 ± 0.39 at 33°C, 24 h. MBK2 showed the highest amylase activity 47.58 ± 1.01 at 28°C at 24 h. The result of the temperature effect on the growth of selected bacterial strains MBK1 and MBK2 showed maximum growth at 28°C 4.01 ± 0.04 of 72 h incubation and 47.58 ± 1.01 of 24 h incubation; 33°C 20.92 ± 0.39 of 24 h incubation and 6.25 ± 0.31 of 24 h incubation; 38°C 0.98 ± 0.05 of 72 h incubation and nil activity at the temperature; 43°C 0.66 ± 0.07 of

24 h incubation and 7.94 ± 0.39 of 48 h incubation; 48°C 1.44 ± 0.07 of 24 h incubation and 1 ± 0.08 of 24 h incubation.

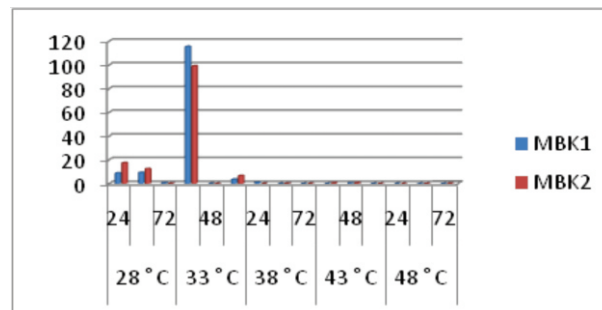


Figure 11; Effect on enzyme production with pH – 4 at different temperatures of MBK1 & MBK2 strains with different time intervals

Figure 11, shows the effect of temperature of pH 4 on the activity of α -amylase whereby, maximum enzyme activity was observed on MBK1 strain at 33°C at 24 h. MBK1 showed the highest amylase activity at 115.69 ± 0.15 at 33°C, 24 h. MBK2 showed the highest amylase activity 99.15 ± 0.07 at 33°C at 24 h. The result of the temperature effect on the growth of selected bacterial strains MBK1 and MBK2 showed maximum growth at 28°C 9.29 ± 0.82 of 24 h incubation and 17.484 ± 0.23 of 24 h incubation; 33°C 115.69 ± 0.15 of 24 h incubation and 99.15 ± 0.07 of 24 h incubation; 38°C 1.03 ± 0.03 of 24 h incubation and 0.11 ± 0.02 of 72 h incubation; 43°C 0.44 ± 0.03 of 48 h incubation and 0.54 ± 0.05 of 48 h incubation; 48°C no activity at the incubation temperature and 0.20 ± 0.05 of 72 h incubation.

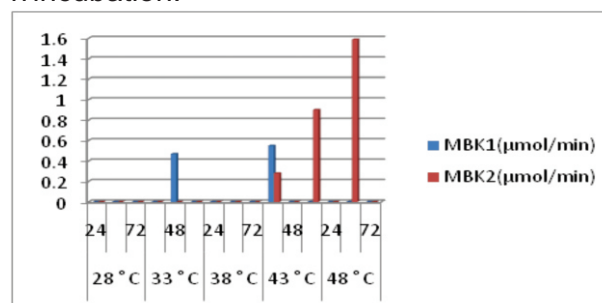


Figure 12; Effect on enzyme production with pH – 7 at different temperatures of MBK1 & MBK2 strains with different time intervals

Figure 12, shows the effect of temperature of pH 7 on the activity of α -amylase whereby, maximum enzyme activity was observed on MBK2 strain at 48°C at 48 h. MBK1 showed the highest amylase activity 0.55 ± 0.07 at 43°C, 24 h. MBK2 showed the highest amylase activity 1.59 ± 0.8 at 48°C at 48 h. The result of the temperature effect on the growth of selected bacterial strain MBK1 showed maximum growth at 33°C 0.47 ± 0.15 of 48 h incubation; 43°C 0.55 ± 0.07 of 24 h incubation, and no production at 28°C, 38°C and 48°C. The result of the temperature effect on the growth of selected bacterial strain MBK2 showed maximum growth at 43°C 0.90 ± 0.05 of 72 h incubation; 48°C 1.59 ± 0.8 of 48 h incubation and no activity at 28°C, 33°C and 38°C.

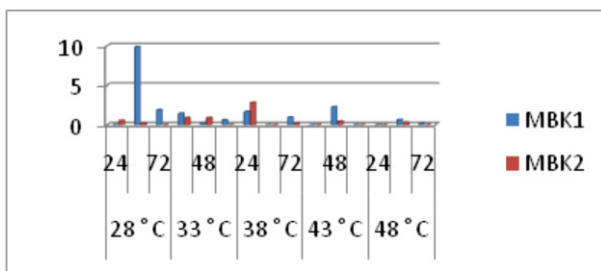


Figure 13; Effect on enzyme production with pH – 11 at different temperatures of MBK1 & MBK2 strains with different time intervals

Figure 13, shows the effect of temperature of pH 11 on the activity of α -amylase whereby, maximum enzyme activity was observed on MBK1 strain at 28°C at 48 h. MBK1 showed the highest amylase activity 9.88 ± 0.84 at 28°C, 48 h. MBK2 showed the highest amylase activity 2.82 ± 0.31 at 38°C at 24 h. The result of the temperature effect on the growth of selected bacterial strains MBK1 and MBK2 showed maximum growth at 28°C 9.88 ± 0.84 of 48 h incubation and 0.55 ± 0.07 of 24 h incubation; 33°C 1.44 ± 0.7 of 24 h incubation and 0.89 ± 0.23 of 24 h incubation; 38°C 1.66 ± 0.2 of 24 h incubation and 2.82 ± 0.31 of 24 h incubation; 43°C 2.29 ± 0.07 of 48

h incubation and 0.44 ± 0.04 of 48 h incubation; 48°C 0.64 ± 0.07 of 48 h incubation and 0.33 ± 0.12 of 48 h incubation.

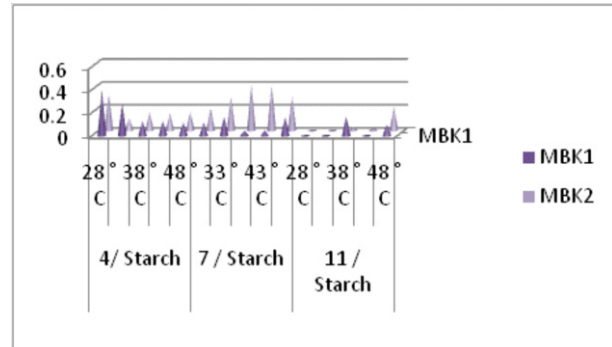


Figure 14; Effect on protein estimation with pH – 4,7&8 at different temperature of MBK1 & MBK2 strains

Figure 14, showed the effect of various temperatures on incubation at pH 4 and substrate as a starch. The result showed the highest protein estimated on pH 4 at 28°C MBK1 - 0.403 and MBK2-0.329; pH 7 at 33°C MBK1 - 0.170 and 43°C MBK2 - 0.404; pH 11 at 38°C MBK1 - 0.171 and 48°C MBK2 - 0.205.

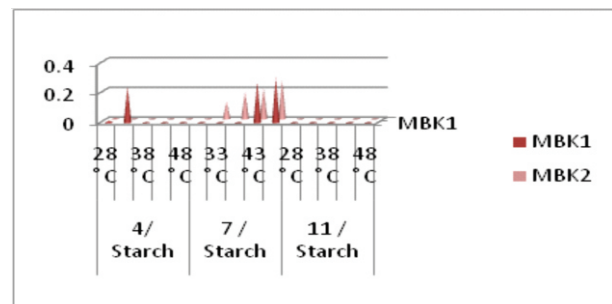


Figure 15; Effect on protein estimation with pH – 4,7&8 at different temperature of MBK1 and MBK2 strains

Figure 15, showed the effect of various temperatures on incubation at pH 4 and substrate as rice bran. The result showed the highest protein estimated on pH 4 at 33°C MBK1 - 0.244 and MBK2 - 0.008; pH 7 at 48°C MBK1 - 0.308 MBK2 - 0.263; and no protein on pH 11.

(Filho *et al.*, 2018) described the genomic information that can be employed in the

development of other researchers, including comparative analyses employing genes and genomes, origin and dispersion of strains. It was reported as a *Bacillus cereus* isolated from the digestive tract in a saline environment. –(Zote *et al.*, 2018) reported that all the microbes were grouped into six families and seven genera, which showed a good relationship between the bacterial populations associated with crabs; *Bacillus* was the dominant genus, followed by *Staphylococcus*, *Pseudomonas*, *Enterobacter*, *Aeromonas*, *Alcaligenes* and *Acinetobacter*. In present study, the crab digestive flora contains *Bacillus thuringiensis* (OP518457) and *Bacillus cereus* (OP526970) strains. (K *et al.*, 2017) reported from the gut of fifteen mangrove crab species 523 bacterial strains were isolated and classified into ten taxonomic groups including the highest number of strains *Bacillus* (113). Several reports have described amylase production from this particular genus isolated from various sources (Božić *et al.*, 2014) (R, 2017)–(Tamamura *et al.*, 2014). Bacteria belonging to the genus *Bacillus* have a long and distinguished history in the realms of biotechnology. Manufacture of amylases and proteases for industrial applications began early this century, but significant production and usage were delayed until after the 1950s when the advantages of including the alkaline protease of *Bacillus licheniformis* in washing detergents were realized. This was followed by developments in the starch processing industry based on the α -amylase from *Bacillus licheniformis*, particularly the conversion of starch to high-fructose corn syrups as sucrose replacements in foods and beverages.

The production of extracellular amylase was found to be significantly influenced by the composition of the medium and the culture conditions. Carbon is not only an essential

component of the medium but also one of the elements required for bacterial growth and metabolism, which in turn stimulates the production of enzymes. *Bacillus* sp. produced amylase at the highest level when starch was the only carbon source used in the current situation. Aygan *et al.*, 2008 reported that an enzyme obtained from *Bacillus* sp. AB68 was active in a broad temperature range between 20 and 90°C, with an optimum of 50°C. temperature is an important limiting factor for the storage of enzymes. (Deb *et al.*, 2013) reported the effect of temperature on enzyme activity ranging from 35°C - 70°C at optimum pH. Yordan Stefanov described 31% of the studied *Bacillus* strains showed amylolytic activity after cultivation on a starch agar (61% of *Bacillus cereus*, 36% of *Bacillus thuringiensis* and 3% of *Bacillus sphaericus*). (Vijayaraghavan *et al.*, 2015) also revealed maximum amylase production by *Bacillus cereus* IND4 was attained by response surface statistical optimization methods employing different process parameters under SSF. The selection of an ideal agro-biotech waste for enzyme production depends upon several factors, mainly related to the cost and availability of the substrate material (Singh Nee Nigam *et al.*, 2009) In the present study, maximum enzyme production (115.69 $\mu\text{mol}\cdot\text{min}^{-1}$) was observed with rice-bran than starch 89.55 $\mu\text{mol}\cdot\text{min}^{-1}$. The results described here are highly significant because no reports so far evidenced the use of crab waste and rice bran as the substrate for the production of amylases. Based on this fact, crab waste can be effectively utilized for the production of amylases. The results illustrated in the table indicate that the optimum temperature for amylase activity was 33°C. The optimum temperature for amylase activity from MBK2 was similar to or quite higher than that of the optimum temperature of amylase from MBK1. The

enzyme was highly active at pH 4.0 and it lost its activity considerably at pH 7. This pH optimum was higher than the pH optimum (11) of MBK1 and MBK2.

CONCLUSION

The results obtained in the present study indicated that *Bacillus* sp. could be a potential strain for amylase production in submerged fermentation using starch and rice bran-like easily available substrates. The optimization processes parameters such as pH (4), temperature (33°C) and inoculum size 1% for attaining a higher yield of amylase.

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