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Study of Amylolytic Action by Metabolites Isolated from Endosymbiotic Bacteria *Bacillus cereus*

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Abstract: *Bacillus cereus* is an endosymbiotic bacteria that has been isolated from pink perch (*Nemipterus japonicus*). Pink perch is a common food fish of high demand. This study was conducted to check whether the bacteria have amylolytic action or not. For this, the bacteria were isolated from the gut of the fish after alcohol sterilization. Optimisation of growth conditions, pH and temperature were performed. Growth curve also obtained by incubating bacterial colonies for various time periods. Bacterial extract had been used for the remaining studies. By using the DNS method, its amylolytic action was carried out and Amylase degradation capacity of the isolated bacteria further confirmed by adding iodine solution to the starch - agar petri plate containing bacterial colony. Optimum pH and temperature for maximum amylase activity were also identified. Gut endosymbionts generally have a role in digestion of fishes. Here the amylolytic action of *Bacillus cereus* has been proven hence, it may take part in carbohydrate degradation of pink perch. Thus, the bacteria can help the growth of the host.

Keywords: Amylolytic action, *Bacillus cereus,* Pink perch, Optimization, Endosymbionts

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Introduction

Even before large multicellular organisms developed, microorganisms were there. Development of organisms provides a new livable, stable environment for microorganisms. Hence, large organisms establish complex interactions with microorganisms through their evolution. In symbiosis, symbionts maintain a close relationship with the host, thereby they can function better and it is also beneficial to the host. The term symbiosis was first defined by Anton de Bary in 1875 as "the living together of differently named organisms" and redefined as "a system in which there is a reciprocal benefit for the symbionts". These mutually beneficial living arrangements are common, and have frequently evolved to be essential to the livelihood of both partners. If

microbial symbionts are absent, most of the plants and animals cannot survive. Mechanisms for achieving and maintaining the associations in symbioses changes as change in the role of the microbe in the host biology, in the evolutionary history of the relationship, and in the genomic features of the microorganism.

Fishes also exhibit symbiosis with many bacteria. In fish, there are two types of bacteria present in its gut. They are autochthonous (able to colonize on the mucosal surface) and allochthonous (free living). Autochthonous is more important for fish. They are capable of providing nutrition to the fish and also help in prevention of certain diseases. Other than this, various other microbes also inhabit the fish gut. Host habitat has a major role in determining the gut microbes (Kim *et al.,* 2007).

Pink perch (*Nemipterus japonicus*) is a least studied group of fishes. It is a marine fish which is commonly found in the Pacific and Indian Ocean. Pink perch commonly known as Rani fish and it comes under the family Nemipteridae. It is found usually in schools, at coastal waters and mud or sand bottom. Pink Perch gets its name from the pink hue and yellow stripes present with scales on its skin. It is a fish with high economic value and it is one of the most delicious fish. It has high health value and many studies related to products obtained from pink perch were done. But, there are almost no studies done related to ways to improve health and production of this particular fish. Symbiosis can improve the fish's growth and health. Hence, by studying symbiosis, we can improve fish production. Also, studying functional microbiomics of fish gut can improve the yield in aquaculture. This study was conducted to check whether the bacteria *Bacillus cereus* that has been isolated from pink perch (*Nemipterus japonicus*) have amylolytic action or not.

Materials and Methods

Sampling:

The pink perch (*Nemipterus japonicus*) was procured from a fish market at Thrissur, Kerala, India. After surface sterilization fish was dissected and the intestine was extirpated. The isolation of endosymbiotic bacteria was done from the surface sterilized chopped intestine and inoculated into the sterilized media. Isolation of pure bacterial strain was done by pour plate technique.

Subculture of selected isolates was performed in agar plates following standard bacterial isolation by repeated streaking on agar and stored in nutrient broth for the growth of pure culture bacteria, Gram staining and microscopic examination were done to observe the general morphology of the cell. The identification of bacterial strain was done by the employment of 16 S rRNA sequencing. DNA isolation from microbial samples was done using the HiGenoMB Microbial DNA isolation kit developed by HiMedia.

PCR Protocol:

Polymerase chain reaction (PCR) is done to amplify specific cloned or genomic DNA sequences.

Composition of the Taq Master Mix:

- Taq DNA polymerase is supplied in a 2X Taq buffer.
- 0.4Mm dNTPs.
- 3.2Mm Mgcl2.
- 0.02% bromophenol blue.

Primer Details:

Primer details are shown in Table 1.

Purification of PCR production:

Removed unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit (Millipore). The PCR product was sequenced using the primers. Sequencing reactions were performed using an ABI PRISMR BigDye TM Terminator Cycle Sequencing Kits with AmpliTaq@ DNA polymerase (FS enzyme) (Applied Biosystems).

Sequencing protocol:

Single-pass sequencing was performed on each

Table 1: Primer details

Primer Name	Sequence details	Number of Base
27F	5`AGAGTTTGATCMTGGCTCAG 3`	20
1492R	5`TACGGYTACCTTGTTACGACTT3`	20

A **B**

Fig. 1: A- Pink perch; and B- Pink perch gut containing nutrient broth.

template using below 16s rRNA universal primers.

Bioinformatics protocol:

The 16S rRNA sequence was run in blast using NCBI. The phylogeny analysis of query sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment. The program MUSCLE 3.7 was used for multiple alignments of sequences (Edgar, 2004). The resulting aligned sequences were cured using the program G blocks 0.91b. This G block eliminates poorly aligned positions and divergent regions (Talavera and Castresana, 2007). Finally, the program PhyMI. 3.0 aLRT was used for phylogeny analysis and HKY85 as a Substitution model. PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. The program TreeDyn 198.3 was used for tree rendering (Dereeper *et al.,* 2008).

Optimization of cultural conditions for maximizing bacterial growth were done, and the growth curve of bacteria was estimated by analyzing bacterial growth in colorimeter. Absorbance of bacterial culture was taken in 600 nm and found log, lag and stationary phase. The bacterial concentration (cells/ml) was found by the equation-- bacterial cell cultures OD 600 of 1.0 = 8×10^8 cells/ml. Isolated enzyme from bacterial broth and screening was done using the DNS method. For identifying the amylase degradation capacity of isolated organisms**,** 0.1% Starch agar was prepared in a clean 250 ml conical flask. The media was autoclaved and sterilized media was poured into the petri plates. The isolated bacteria were streaked in the solidified medium. The plates were incubated at 37 ℃ for 24 h. After incubation, the surface of the plates was flooded with iodine solution and then examined for starch hydrolysis.

Results and Discussion

The fish that has been used for the experiment is shown in Figure 1A. Gut containing nutrient broth is shown in Figure 1B. Gut endosymbiotic bacteria that were isolated from the pink perch is shown in Figure 2. In Figure 3, a nutrient agar plate showing isolated bacteria is given.

Genomic DNA and PCR amplification result is shown in Figure 4. Figure 5 illustrates sequencing results and from this, it is clear that the isolated organism is *Bacillus cereus*.

A growth curve has been obtained by incubating bacteria for various periods. It is shown in Figure 6. From this, it is clear that maximum growth obtained after 27 h of incubation. After 29 h, there occurred a decline in

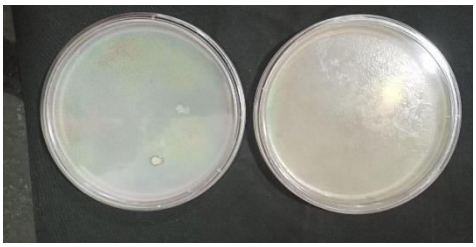

Fig. 2: Isolation of gut endosymbiotic bacteria from pink perch.

Fig. 3: Nutrient agar plate showing isolated bacteria.

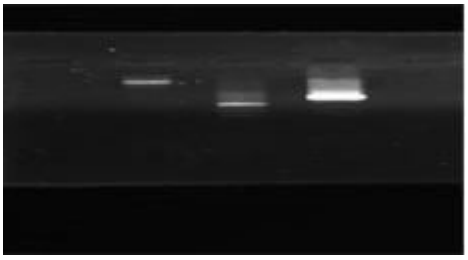

Fig. 4: Genomic DNA and PCR amplification of MCAR/VC/ZOO/AMB/2022.

TAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAATATTTTGAACTGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTC
ACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTG
GGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAA
GGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTAC
GTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCA
CGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGA
GGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGC
CGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAA
ACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGAAAA
CCCTAGAGATAGGGCTTCTCCTTCGGGAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAG
CGCAACCCTTGATCTTAGTTGCCATCATTAAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGC
CCCTTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGAGCTAATCTCATAAAACCGTTCTCAGTTCGGAT
TGTAGGCTGCAACTCGCCTACATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCA
CACCACGAGAGTTTGTAAC

Result: ***Bacillus cereus***

Fig. 5: Sequencing result of *Bacillus cereus.*

bacterial growth.

Bacterial cultural conditions such as Temperature and pH were analyzed. Bacteria were incubated at various temperatures ranging from 20 to 60 °C to find out the most favorable temperature for bacterial growth from the observations. The results are shown in Table 2. It was evident that maximum bacterial growth may be obtained by allowing the bacteria to grow at 37℃. However, bacterial strains can grow in a wide range of temperatures. Similarly, the bacterial growth media was maintained in different pH by administering buffers to it. pH was maintained by three different buffers; acetate (3.6-

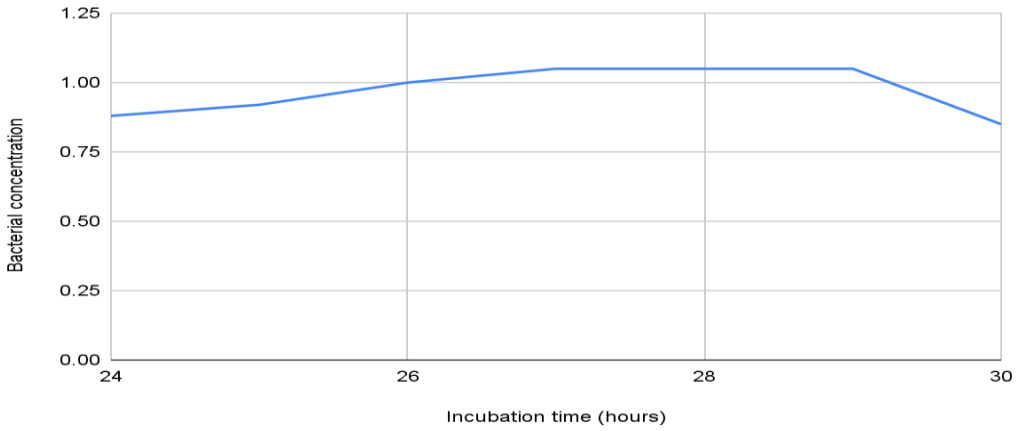

Fig. 6: Bacterial Growth Curve.

Table 2: Effect of Temperature in bacterial growth

Temperature °C	Bacterial concentration cells/ml $\times 10^8$	OD at 600nm
3	0.64	0.08
37	5.04	0.63
60	0.8	0.10

5.6), phosphate (5.8-7.4), and bicarbonate (9.2-10.6). Three different pH (4,7,10) was taken as a parameter to find out the effect of pH on bacterial growth, however, bacteria showed maximum growth in pH 10 and growth was reduced immensely in neutral and low pH that suggests that isolate prefer basic pH (Roselin *et.al*., 2012). The results are given in Table 3.

As shown in Table 4, Enzyme degradation percentage was maximum when 8 ml of enzyme was added. Generally, as the concentration of enzymes increases, the percentage of degradation also increases. The effect of temperature on enzyme degradation was also analyzed. Results are shown in Table 5. At room temperature (37°C) the degradation of enzymes was maximum. It is minimum when it is placed at cold temperature (3°C). Effect of pH on enzyme degradation given in Table 6. At pH 4 (acidic pH) maximum degradation was obtained. As pH increased, the percentage of degradation decreased. In all animals amylase appears to be similar, although there are slight differences in the pH optima and activities. Dhage (1968) and Phillips (1969) suggested that amylase activity in the intestine of herbivorous carp is much more intense than in carnivorous fishes. In the present investigation, a considerable population of amylolytic bacteria was detected in the fish species studied, and certain bacterial strains are known extracellular amylase producers. There is a possibility of introduction of these microflora in fish digestive tracts along with the food ingested, but, whether they form a persistent population in the gut is doubtful However, the amylolytic bacteria have been detected in fish gut after 24 h of starvation in our study, and it seems that some of the flora forms a persistent population. After a few minutes of addition of iodine solution, a typical starch hydrolysis reaction was observed which shows a

Table 3: Effect of pH in bacterial growth

pH	Bacterial concentration cells/ml $\times 10^8$	OD at 600 nm
4	0.56	0.07
7	4.64	0.58
10	4.96	0.62

Table 4: Table showing activity of variation

Enzyme concentration (ml)	Percentage of degradation (%)
4	69.23
6	73.08
8	80

Table 5: Effect of temperature in enzyme degradation

Temperature °C	Percentage of degradation (%)
3	44.61
37	76.15
60	62.31

Table 6: Effect of pH in enzyme degradation

pH	Percentage of degradation (%)
4	62.30
7	59.23
10	50

Fig. 7: Plate shows the amylase degrading capacity of isolated organism.

clear zone surrounding the microbial colony indicating the degradation of starch to reduce sugars by microorganism.

Figure 7 shows that the *Bacillus cereus* has the amylase degrading capacity. The regions where the bacteria present appeared as clear regions due to the degradation.

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