Characterization, Purification, and Production of Alkaline Amylase from Bacteria via Solid-State

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Abstract

Amylase is used in a variety of industries, including detergents, food, fermentation, textile paper, and pharmaceuticals. Bacteria generating amylase enzyme was investigated in this study from the gut of fish (*Channa punctatus)*. A shake flask fermentation was used to make the extracted amylase enzyme, and ammonium sulfate precipitation was used to purify it. The activity of the enzyme was determined using a synthetic carbohydrates starch substrate. The partially purified enzyme exhibits maximum activity at the optimum pH 7-11, temperature 28-37°C, and substrate concentration of one percent Starch, and the result also shows that maximum alkaline amylase synthesis occurs in solid-state conditions at pH 11, incubated for 5 days at room temperature. Under the solid-state fermentation process, bacteria produce the most amylase. The influence of activators and inhibitors on amylase enzyme production was also investigated, with activators $MgCl_2$ and $CaCl_2$ increasing enzyme activity and inhibitors SDS and EDTA decreasing enzyme activity.

Keywords: Alkaline amylase, *Bacteria*, solid-state fermentation, enzyme activity.

Introduction

Fish are aquatic creatures, cold-blooded (ectothermic) vertebrates, breathe with gills, and swim with fins belonging to phylum Chordata generally found in fresh and saltwater. The study of fish is called ichthyology. Fishes are highly nutritious. They are low in calories, cholesterol, and fat, but are a good source of high-quality protein good for diabetic patients. The protein in fish is highly digestible and with well-balanced in amino acids. They are also a good source of vitamins A, B, D, calcium, iodine, and fluorine. They are loaded with Omega-3 polyunsaturated fatty acids. Eicosa-pentanoic acid and docosahexaenoic acid are two polyunsaturated fatty acids found in fish. The aquatic environment is populated with bacteria which are received by the Fish through water and food (1). Water from where fish were collected is heavily polluted, so the food and water on which fish feed are also highly polluted. This is the reason why we take fish for bacteria isolation because due to polluted habitat the gastrointestinal tract of fish contains more heterotrophic bacteria.

Gastrointestinal tract bacteria play several important roles in fish, such as digestion, immunity, protection from pathogenic bacteria, and brain development. The alimentary tract of fish can be divided into anterior and posterior regions. The anterior part consists mouth, buccal cavity, and pharynx. The posterior part consists foregut, midgut, or intestine, and hindgut or rectum. It is reported that microorganisms inhabiting the gut of fish helps in the digestive process by producing enzymes for the breakdown of different substrate for example chitin, cellulose, starch, protein, and phytate. The bacterial ecology of the gastrointestinal system, in general, has a lot of enzymatic capability, and it appears that the fish digestive tract has a lot of it. The enzymatic mass in the digestive tract interferes significantly with a significant portion of the host animals' metabolism (2).

Different gut microbes having considerable cellulose, amylase, and protease activity had been isolated from freshwater fish**.** There are several enzymes isolated from the fish gut are Cellulase, Phytase, Amylase, Zymase, Hydrolytic enzyme, Endogenous enzyme, Exogenous enzyme, and Extracellular enzyme (3).

Materials and Methods

For the experiment, fish were collected by gill net with the help of local fishermen from Ghaila Bridge of Gomti River at Lucknow during the months of January to March. To isolate the gut microbiota fishes were starved for 24 hours to remove the bacteria that were transit in nature. Fish were sacrificed and dissected in laminar airflow to avoid contamination.

Microbial culture

An intestinal swab has been taken and serially diluted (10 times) in normal saline (.85%). Samples were poured on nutrient agar media from each dilution. The culture was placed in an incubator for 24h at 37°C. To obtain pure culture bacterial colonies were streaked on nutrient agar media from mix culture.

Screening for Amylotic activity

The isolated bacteria were kept on the starch agar plates. The clear zone of hydrolysis shows the amylotic activity of bacteria. Then zone of hydrolysis becomes visible when 1% of iodine solution was layered on the agar plates (4).

Identification and Strain improvement of bacteria

The isolated bacteria were further identified through the biochemical test and 16s rRNA sequencing. The isolated and identified bacteria was further treated with UV (ultra violet) rays and EtBr (Ethidium Bromide) treatment. This is given as 2min, 4min, 6 min, 8min, 10min and 1µg/ml, 2µg/ml, 3µg/ml, 5µg/ml for UV and EtBr respectively (5).

Media optimization

Various medium components were used in the production of best amylase from bacteria source as follows-

Composition	Quantity in gm
NaCl	0.8gm
KCl	0.8gm
CaCl2	0.1gm
Na_2HPO4	2gm
MgSO4	0.2gm
FeSO4	0.1gm
Glucose	8.0gm
NH4Cl2	2gm
peptone	2gm
Beef extract	2gm
Yeast extract	2gm
Urea	2gm
Distilled water	1000
pH	11

Production of enzyme

Amylase production was carried out in solid-state fermentation. One ml of 24hrs old bacteria culture was transferred into 100 ml of sterile potato dextrose broth at pH 11. And left it at room temperature for 5 days (6-8).

Enzyme recovery

In a chilled centrifuge, the bacterial culture was spun at 10,000 rpm for 10 minutes at 4°C. The resultant supernatant was utilized to estimate alkaline amylase using a crude enzyme (9-14).

Amylase enzyme assay

The amylase enzyme assay was done by DNS assay (dinitrosalisilic assay)

Alkaline Amylase Enzyme Characterization

The enzyme was incubated at different pHs of the starch substrate from 5 to 11 for the determination of optimum pH (15-18). The enzyme and substrate reaction were carried out at different temperatures ranging from 22 to 50°C. The reaction mixtures were incubated at respected temperatures and enzyme activity was determined to find out the optimum temperature of amylase (19-23). The activators used were MgCl2 and CaCl2 and inhibitors used as SDS and EDTA. All the chemical used in the incubation mixture at 1mM concentration and the enzyme assay was carried out (24-27).

Optimization of Amylase

Different organic and inorganic (peptone, beef extract, yeast extract, urea, and ammonium sulfate) nitrogen sources at a concentration of 0.2% were used the optimum nitrogen source was found by analyzing the result of amylase production (28). Different carbon sources (maltose, lactose, sucrose, and mannitol) used at a concentration of 0.8% were used. The optimum carbon source was found by analyzing the result of amylase production (29)**.** Different metal ion sources (MgSO4, CaSO4, FeSO4, and CUSO4) used at a concentration of 0.04% were used. The optimum metal ion source was found by analyzing the result of amylase production (30).

Results and Discussion

Isolation and purification of bacteria strain

Seven different bacterial isolates were selected from mixed culture plates obtaining after serial dilution of bacteria, the colony were differentiated on the basis of colony morphology and named as DJCP 01, 02, 03, 04, 05, 06 and 07. All the seven bacteria strain were inoculated on respected NAM media plates.

Screening of bacteria strain

All the seven bacteria isolates were screened for the production of alkaline amylase by starch-iodine test on the minimal agar medium with 1% starch. The isolated DJCP 07 showing maximum zone of hydrolysis. The observations can be seen below in Figure 1.

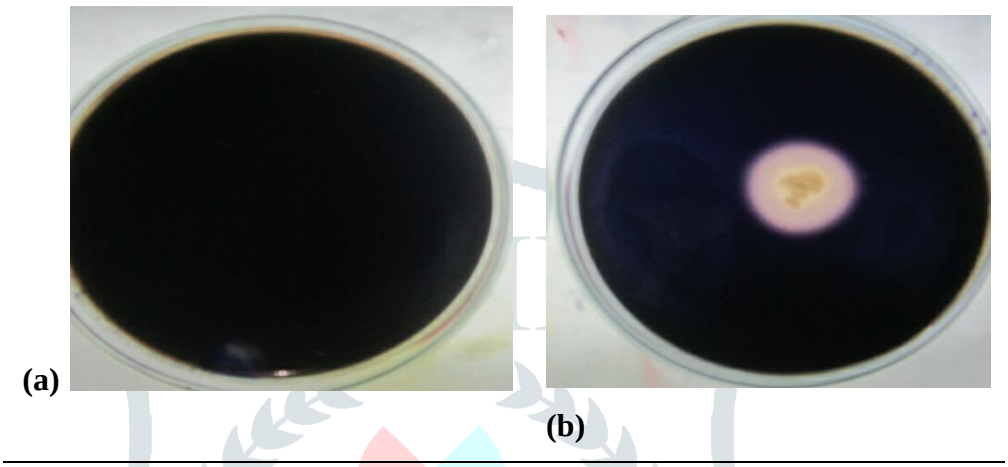

Figure 1: The zone of hydrolysis of DJCP 07 (b) when compared with control (a)

Identification of selected strain

Depending on the 16s rRNA sequencing, the bacterial isolates were identified as the species *Bacillus cereus***.**

Strain improvement of the selected strain

Strain improvement of the selected strain was done by UV radiation and EtBr (Ethidium Bromide) treatment to cause the mutation in their genome and found to be of some beneficial character, EtBr (5µg/ml) concentration was found to be a good strain improvement career.

Growth medium and culture condition

On the basis of media optimization, the following medium was found to be good for the production of alkaline amylase they are (beef extract 1%, maltose 1%, MgSO4 0.1%, NaCl 0.08%, KCl 0.08%, and Na2HPO4 0.2% with pH 11).

Effect of carbon source

Maximum growth of bacteria was achieved when glucose was the carbon supplement. Growth was increased when the maltose 0.8% (1.96 IU/ml) was used as carbon source (Figure 2).

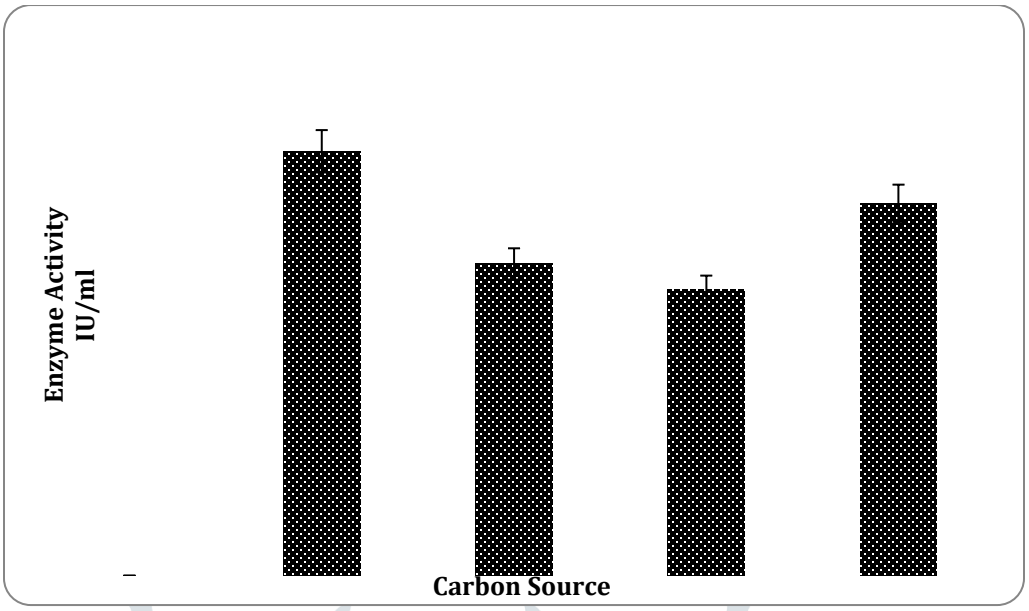

Figure 2: Enzyme activity according to carbon source

Effect of nitrogen source

Maximum growth and enzyme production 2.92 IU/ml was achieved when beef extract was used as the nitrogen source (Figure 3).

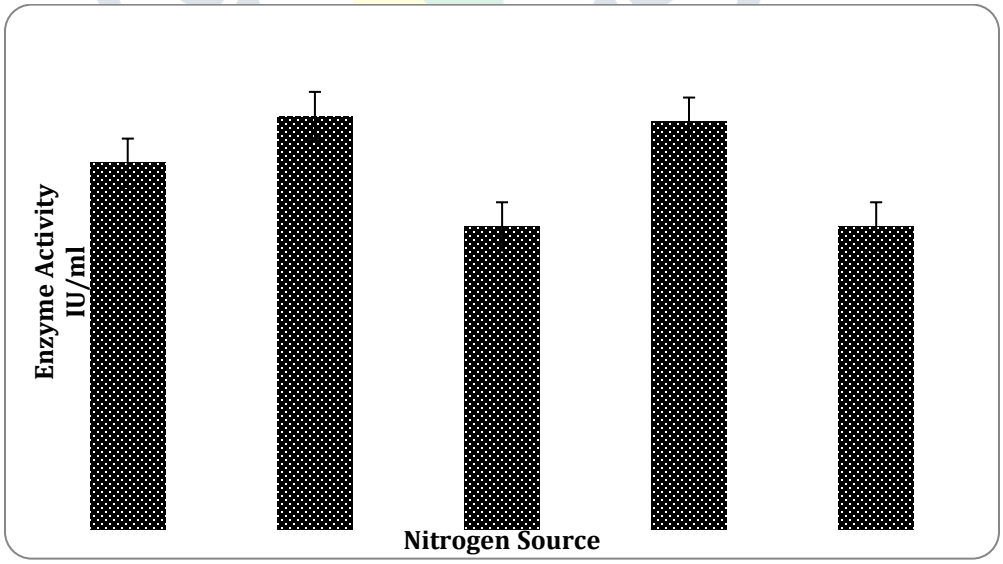

Figure 3: Enzyme activity according to Nitrogen source

Enzyme production

The production of enzymes is achieved by solid-state fermentation using wheat bran as a substrate. The fermented PDB broth (100ml) was inoculated with 24 hrs fresh bacteria culture and left for room temperature for 5 days. After 5 days the fermented media was spun with 0.1M tris buffer with pH 11 at 10000 rpm for 10 minutes at 4°C. After centrifugation, the supernatant was collected (75ml) and it was known to be crude enzyme (Figure 4).

Figure 4: Solid-state fermentation using Wheat Bran

Enzyme purification

The crude enzyme is purified by the ammonium sulfate precipitation method. The 35.415 ammonium sulfate was mixed in a 75 ml crude extract of amylase and mix vigorously in a stirrer at 4°C, then spin at 10000 rpm for 10 minutes at 4°C in a refrigerated centrifuge, the pellet was collected and it was known to be a partially purified amylase.

Effect of pH

The majority of bacteria can grow in a wide pH range. The influence of pH 7 and 11 is optimal, as seen in the graph. The pH has a significant impact on enzyme activity (Figure 5). This is because the charge distribution on both the substrate and enzyme molecules affects substrate binding and catalysis. (Shsh and Madamwar, 2005).

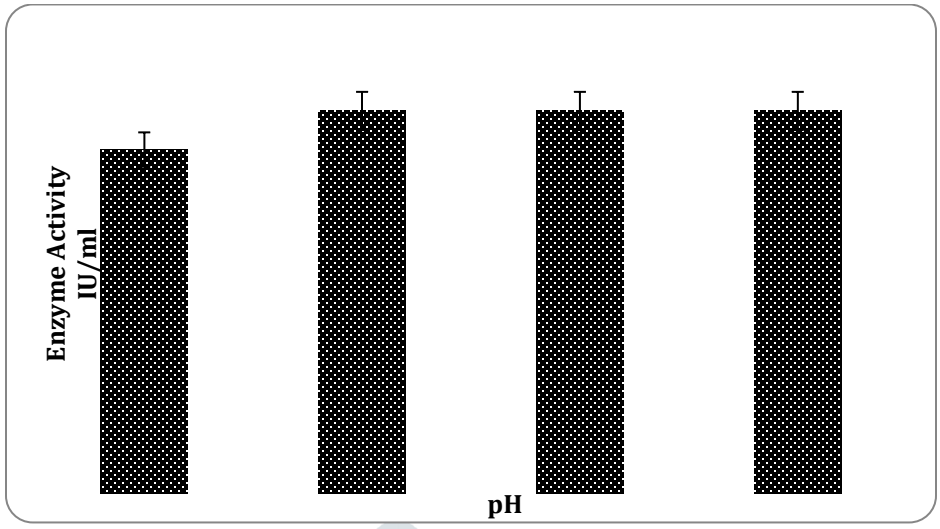

Figure 5: Effect of pH on enzyme activity

Effect of temperature

The effect of temperature on enzyme activity was studied in the range of 22-50°C, the optimum alkaline amylase activity was found at 28°C i.e. 0.8 IU/ml other temperature lesser enzyme activity is observed as shown in Figure 6.

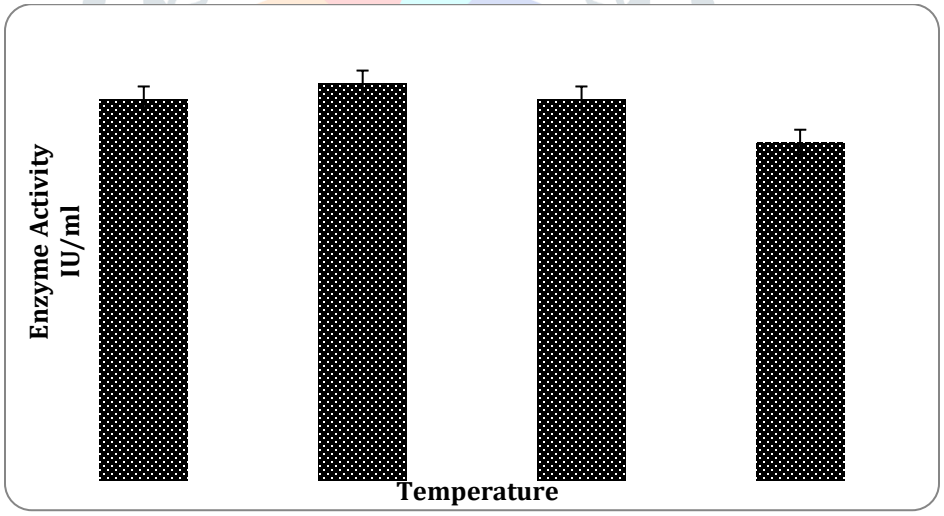

Figure 6: Effect of temperature on enzyme activity

Effect of chemical Activators

The activators used such as $MgCl_2$ and $CaCl_2$ increased the enzyme activity to 1.016 IU/ml, 0.872 IU/ml in comparison with without activator, 0.824 IU/ml, as shown in Figure 6.

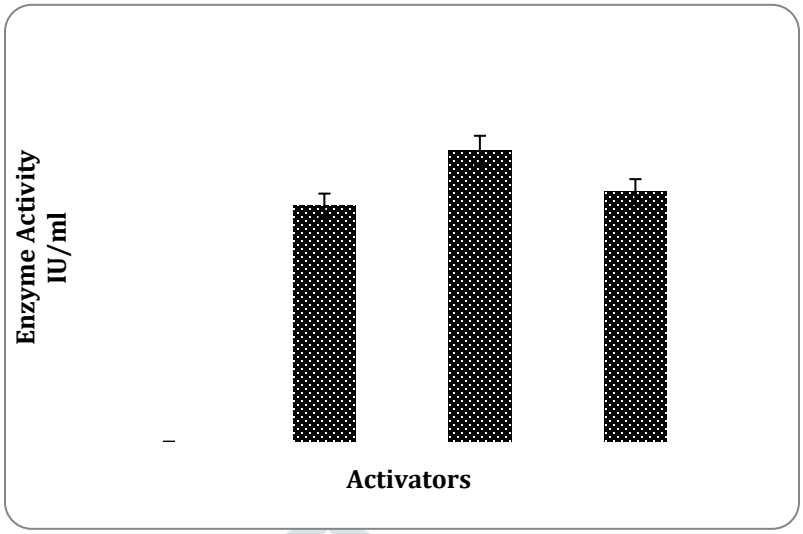

Figure 6: Effect of chemical Activators on enzyme activity

Effect of chemical Inhibitors

The inhibitors used such as SDS and EDTA decreased the enzyme activity by 0.776 IU/ml, 0.816 IU/ ml in comparison with without an activator, 0.852 IU/ml, as shown in Figure 7.

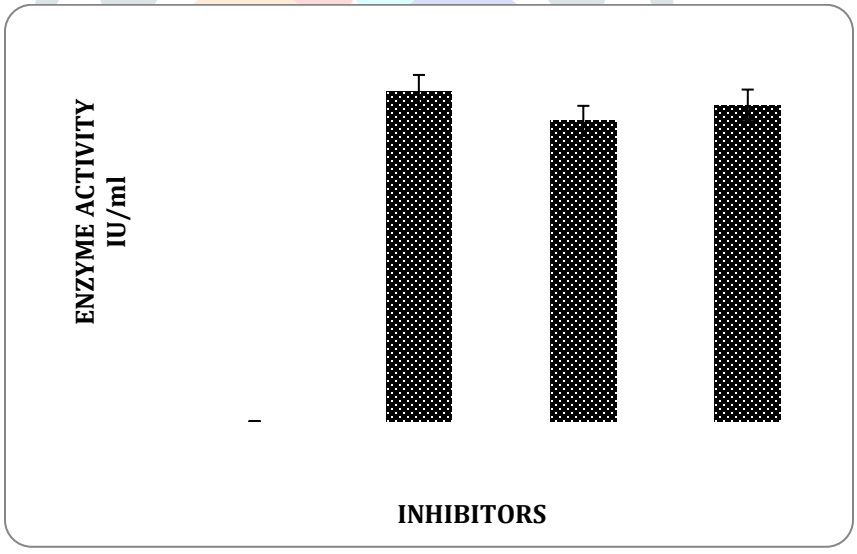

Figure 7: Effect of chemical Inhibitors on enzyme activity

Conclusion

From the fish gut samples collected, seven bacterial cultures were recovered. The primary screening revealed that DJCP 07 isolates can produce alkaline amylase enzyme at pH 11 and have a clear hydrolysis zone. After parametric optimization maximum, amylotic activity was observed when pH was 7-11 and the temperature was 28°C. After use of various chemical agents that is used as activators and inhibitors to increase or decrease the activity of the enzyme was found at activators (WA 0.824 IU/ml and with activators 1.016 and 0.872 IU/ml) and inhibitors (WI 0.852 IU/ml and with inhibitors 0.776 and 0.816 IU/ml) respectively.

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