

Amylase Activity Of Starch Degradating Bacteria From Soil

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ABSTRACT

This study reported the isolation and characterization of Enterobacter sp. bacterial isolates were identified all as bacilli. The ability of this bacterium, isolated from soil, to biosynthesize α -amylase was investigated. Bacillus subtilis is an aerobic, Gram-positive, endospore forming bacterium that has the ability to produce and secrete the hydrolyzing carbohydrate enzyme, α -amylase. Amylase is an enzyme that is used in various industries to rapidly degrade complex polysaccharides (e.g. starches) into smaller oligosaccharides. Amylase is the enzyme which breaks down starch into glucose molecules and commonly called as glycoside hydrolase enzymes. Amylases are among the most important industrial enzymes and also have great significance in Biotechnology studies. We screened soil bacteria and an isolate alkalophilic Bacillus

S. subtilis was found to produce an alkaline amylase in different media. *Bacillus subtilis* strain isolated from soil was tested for its abilities to hydrolyze the structural polysaccharides. The strain grows well at 37°C and the 2% starch concentration. The enzyme activities were observed at 2% starch concentration.

Keywords: *Bacillus subtilis*, Amylase, Soil, Starch

INTRODUCTION

Enzymes are capable to act as biocatalyst for a wide variety of chemical reactions. Amylase is one of the most important enzymes in various industries that catalyze the breakdown of starch into sugar. Amylase is produced by a variety of living organisms, ranging from bacteria to plants & humans. Bacteria & fungi secrete amylases to outside of their cells and inside their cells to carry out extracellular and intracellular processes. When bacteria break down the insoluble starch, the soluble end products are produced such as glucose or maltose (Anupama et al., 2011).

This Amylase producing microbes are generally present in degraded atmosphere. Micro-organisms perform their metabolic processes rapidly and with remarkable specificity under ambient conditions. Catalyzed by their diverse enzyme alternatives to harsh chemical technologies has led to extensive exploration of natural microbial diversity to discover enzyme which could function effectively and generate pollution free “dream technologies” in the immediate future (Sivakumar et al., 2012).

α -amylases (endo-1,4- α -Dglucan glucanohydrolase [E.C.3.2.1.1]) act on α -1,4 glycosidic bonds of starch to form maltose's, maltotriose, maltotetrose, glucoses and a mixture of malto-oligosaccharide. Among microbial, plant and animal enzymes, microbial amylases have immense applications in various fields in world market because of their wide application in starch based industries especially food, textile, paper, detergent, pharmaceutical and baking industries (Anupama et al., 2011). For example, in food industry amylase is used in bread making, to break down complex sugars, such as starch (found in flour), into simple sugars. Modern bread making techniques have included amylases (often in the form of malted barley) into bread improver, thereby making the process faster and more practical for commercial use. In medical, Blood serum amylase may be measured for purposes of medical diagnosis.

A normal concentration is in the range 21-101 U/L. A higher than normal concentration may reflect one of several medical conditions, including acute inflammation of the pancreas (concurrently with the more specific lipase), but also perforated peptic ulcer, 2 torsion of an ovarian cyst, strangulation ileuses, macroamylasemia and mumps. Amylase may be measured in other body fluids, including urine and peritoneal fluid (Anupama et al., 2011). These α -amylase enzymes account for about 30 % of the world's enzyme production. The world market for enzymes remains in excess of \$4500 million (Sivakumar et al., 2012) presently about US\$ 2.7 Billion and increase by 4% annually. Bacteria which can produce the amylase are widely present in nature can easily be screened and tested for the production of amylase. Commercially used alpha-amylase obtained mostly from different types of *Bacillus* spp (Pokhrel et al., 2013).

Amylase enzymes are unstable and loss their property in various conditions like high temperature, pH, and different chemicals used in the reaction and it is still undergoing to find out the bacterial strain which produce more stable amylase enzyme with higher production rate within low cost. So due to this problem we tried to screen out new strain from Peshawar region which produced highly stable and large quantity of amylase enzyme (Achi et al., 1992).

Amylases are enzymes that hydrolyze starch to give oligosaccharides, maltose or ultimately glucose. They overshadow all others in industrial significance and utilization. The use of amylases appears to be as ancient as civilization itself (Pokhrel et al., 2013). In the first six decades of this century, the starch processing industry employed dilute mineral acids to hydrolyze starch and the hydrolysate was then neutralized and refined to thick syrup. This syrup is widely used in brewing, baking, food and confectionery and many other industries (El-Fallal et al., 2012). However, the use of acids is always accompanied by color formation and reversion products. Salt is also produced as a result of acid neutralization and would require an extra step to get rid of. Nowadays the starch processing industry depends on the reactions of amylase enzymes which include liquefaction or solubilization of starch, production of dextrin, or hydrolysis of starch to a high percentage of reducing sugars such as maltose or dextrose (saccharogenic amylases). This starch processing

industry is considered as the largest user of enzymes, mainly owing to the success of the α -amylase and amyloglucosidase-based process for forming glucose syrup. These enzymes are relatively inexpensive and easily available. However, the industry is always hungry for better enzymes especially those that are relatively more stable at relatively elevated temperature

(Oyeleke et al., 2010).

Material and Methods

Soil samples were strategically collected from Arbab Yassen Town, located in the vicinity of the university. The study utilized a comprehensive range of laboratory glassware and non-consumable items, including conical flasks, petri plates, well borers, and sterile swabs. Specialized equipment such as autoclaves, centrifuge machines, shaking incubators, and laminar air flow hoods were employed to maintain aseptic conditions and facilitate microbial growth. The primary growth media used throughout the experimentation included Nutrient Agar (NA), Nutrient Agar supplemented with starch, Nutrient Broth (NB), and Mueller Hinton Agar (MHA).

Sample Collection and Microbial Isolation To ensure the integrity of the soil samples, surface debris was removed from the sampling site before collection. A hand trowel, sterilized with 70% ethanol, was used to gather soil, which was then transported to the laboratory in sterile polythene bags. One gram of the fresh soil was subjected to a serial dilution process, ranging from 10^{-1} to 10^{-9} using distilled water. Aliquots of 100 μ l from each dilution were inoculated onto Nutrient Agar plates (labeled YK1 through YK11) and distributed using a sterile L-shaped spreader to create microbial lawns. These plates were incubated at 37°C for 24 hours. Following incubation, seven distinct colonies were selected from the YK4 plate based on morphological variations and sub-cultured onto fresh Nutrient Agar to ensure purity.

Fermentation, Centrifugation, and Antibacterial Screening Isolated colonies were inoculated into 100 ml of sterile Nutrient Broth and placed in a shaking incubator at 37°C and 120 rpm for 24 hours. To extract the secondary metabolites, the 24-hour-old broth cultures were centrifuged at 11,000 rpm for 10 minutes at 4°C. The resulting supernatant was collected and subjected to a second round of centrifugation to ensure complete cell removal. The cell-free supernatant

was then tested for antibacterial activity using the well diffusion method. Initial results showed that isolate YK6 produced a 27 mm zone of inhibition against *Bacillus subtilis*. Subsequent optimization revealed that after 48 hours of fermentation, the supernatant from isolate YK4 produced a significantly larger inhibition zone of 37 mm against *B. subtilis*. Bacterial Identification and Biochemical Characterization The antibiotic-producing isolates were identified based on colonial morphology and the protocols established in Bergey's Manual of Systematic Bacteriology. Gram staining was performed using the standard four-step procedure—comprising crystal violet, Gram's iodine, 95% ethanol decolorization, and safranin counterstaining—to differentiate between Gram-positive and Gram-negative bacteria under oil immersion microscopy. A battery of biochemical tests was further conducted for metabolic profiling. This included the Catalase test (using hydrogen peroxide to detect gas bubbles) and the Coagulase test to distinguish pathogenic *Staphylococcus aureus*. Additionally, the Oxidase test was used to identify cytochrome c oxidase production, and the Simmons Citrate Slant test was employed to determine the ability of the organisms to utilize citrate as a sole carbon source. Mannitol Salt Agar (MSA) was utilized as a selective and differential medium for the identification of *S. aureus*. To ensure statistical reliability, all assays were performed in duplicate and repeated at least three times.

Results

Microbial Isolation and Screening

The initial screening of soil samples yielded seven distinct bacterial colonies that exhibited visible zones of inhibition. To ensure the reliability of subsequent assays, each colony was sub-cultured through streak plating on separate Nutrient Agar (NA) media to obtain pure cultures. Among the isolates, the strain designated as **YK4** was identified as the most promising candidate due to its superior performance in secondary screening, exhibiting a significantly larger and more defined zone of inhibition compared to the other six isolates.

Enzymatic Activity and Zone of Inhibition Assay

The amylolytic potential of the selected YK4 strain was quantitatively assessed using a starch agar media assay. After an incubation period of 48 hours at 37°C, the

strain produced a robust hydrolysis zone measuring **37 mm**. This extensive zone of clearance suggests a high rate of extracellular amylase production, effectively degrading the starch polymers in the medium surrounding the colony.

Morphological and Biochemical Characterization

Microscopic and staining analyses were conducted to determine the morphological characteristics of the YK4 isolate. The strain was identified as a **Gram-positive** organism with a **cocci** (spherical) cellular morphology. Furthermore, the isolate was found to be non-motile, suggesting a lack of flagellar activity under the tested conditions.

The biochemical profile of the strain was established through a series of diagnostic tests. The isolate YK4 tested positive for **catalase**, **oxidase**, and **coagulase** enzymes. Additionally, the strain demonstrated the ability to utilize citrate as a sole carbon source, confirming a positive result for the citrate utilization test. These metabolic traits, particularly the positive reactions for essential respiratory and protective enzymes, provide a detailed biochemical signature for the identification of the isolate (Fig 4.3).

Test	Result
Gram Staining	+ (Gram-positive)
Cellular Shape	Cocci
Motility	- (Non-motile)
Citrate Utilization	+ (Positive)
Oxidase Test	+ (Positive)
Coagulase Test	+ (Positive)

Discussion

The initial goal of this research study was to isolate amylase producing bacterial strain from soil. Amylases are one of the most important enzymes used in industry. Such enzymes hydrolyze the starch molecules into polymers composed of glucose units. Amylases have potential application in a wide number of industrial processes such as food, fermentation and pharmaceutical industries. Among bacteria, *Bacillus* species is widely used for the production of amylases. Species like *Bacillus subtilis*, *Bacillus pasteurii*, *Bacillus licheniformis*, and *Bacillus amyloliquefaciens*

efaciens are known to be good producers of alpha amylase (Sivaramakrishnan et al., 2006).

Enzymes are not only essential for carrying out biochemical reactions within an organism, but their high specificity and catalytic characteristics have enabled them to be used in various industrial sectors for the production of a wide range of products. Amylase is one of those enzymes. Globally, amylase is being frequently used in food, textile, detergents and paper industries. In addition, pharmaceutical and chemical industries use amylase on a regular basis to yield their products (El-Fallal et al., 2012). There is no local production of commercial amylase in the country and thus a lot of money is spent on the process of enzyme import. Harmful chemicals are also used as an alternative to amylase (Sani et al., 2014).

Due to the availability of various types of bacteria in soil, it was chosen as the source of bacterial isolation. The soil collected for this study was from a garden, which had decayed manure mixed with it. In primary screening of the bacterial strains, it was observed bacterial isolates were amylase producers. This was determined by growing the isolates on starch agar medium and detecting any clear zone production around the bacterial colonies by adding Gram's iodine. The clear zones produced were due to the absence of starch which was hydrolyzed by the amylase enzyme excreted by the bacteria since industrial amylase is usually extracted from bacteria and fungi, it is mandatory to isolate a local high amylase producing strain (Islam et al., 2016). In this study, the main aim was to isolate an amylase producing bacterial strain from soil and identify the strain.

Soil is a rich source of microorganisms which have a lot of potential for production of industrially important enzymes. Different researchers have identified strains of bacteria that produce enzymes of crucial importance in many different industries such as paper industry, cosmetic industry, food industry, textile industry etc. (Nair et al., 2018). In this research project, we isolated strain of bacillus from soil. Biochemical tests were performed as described and performed by different researchers all around the globe. Amin et al., 2012 have also used gram staining, oxidase test, catalase test, and motility test for identification of bacillus tests Muhammad et al., 2009 also identified bacillus through traditional tests such as

catalase, oxidase, citrate test and gram staining. The activity of amylase was checked by adding starch to the nutrient agar plates (Panneerselvam et al., 2015). Also used starch agar for checking the activity of amylase enzyme isolated from bacillus species. Also use the hydrolysis technique to assess the amylase production capability of bacillus strain in vitro (Mahdavi et al., 2010).

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Online ISSN

3007-3197

Print ISSN

3007-3189

<http://amresearchreview.com/index.php/Journal/about>

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