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Original Research Article

Amylase Production & Characterization by using Bacillus Cereus Isolated from Coal Mines

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Abstract:

Background: Amylase was initially described in the early 1800s and is one of the pioneering enzymes to undergo scientific investigation. Although this enzyme was originally termed diastase, it was later renamed "amylase" in the early 20th century. The primary role of amylases is to break down the glycosidic bonds within starch molecules, transforming complex carbohydrates into simpler sugars. Amylase enzymes are categorized into 3 main classes—alpha-, beta-, and gamma amylases—each targeting distinct segments of the carbohydrate molecule. Alpha amylase is present in humans, animals, plants, and microbes, whereas beta amylase is primarily found in microbes and plants. Gamma amylase, on the other hand, can be located in both animals and plants.

In 1908, a study by Wohlgemuth identified the presence of amylase in urine, paving the way for its application as a diagnostic laboratory test. Amylase is a frequently ordered standard diagnostic test, often combined with lipase, particularly when acute pancreatitis is suspected in patients. Amylases are among the most important industrial enzymes and also have great significance in biotechnological studies. In this study, the bacterial strain Bacillus cereus (JX125391) was identified by microscopic, biochemical characterization and screened for the amylolytic activity by starch agar plate method. The maximum enzyme activity was observed at pH 7.5 and temperature 35°C (2.78 U/ml). Partial purification of amylase enzyme was performed by Ammonium sulphate precipitation followed by dialysis. Partially purified amylase exhibited specific activity of 0.053 U/ml/mg which corresponds to 6.62 purification fold and 44.0 % Yield.

Materials and Methods: Bacillus cereus was propagated on nutrient agar medium. The pure culture was streaked on starch agar plates containing peptone 0.05%, KCl 0.01%, MgSO4. 7H2O 0.05%, (NH4)2SO4 0.01%, NaH2PO4 0.01%, starch 2% [15], agar 1.7 g and NaCl 10% and incubated for 3 days at 37°C.

Discussion and Results: Amylase is a heterogeneous calcium-dependent metalloenzyme with a diverse molecular weight range, typically between 54 and 62 kDa. The compact size of amylase facilitates efficient filtration through the glomeruli. Amylase is eliminated through both the renal system and the reticuloendothelial system. This enzyme exists as 2 isoenzymes—pancreatic (P-type) and nonpancreatic (S-type)—which are products of 2 closely linked loci on chromosome 1. Additional amylase heterogeneity results from allelic variation, with 12 alleles for the S-type and 6 alleles for the P-type. Production of amylase by the Bacillus cereus was optimum(2.78 U/ml) at 35°C temperature and as the temperature increased or decreased, there was gradual decrease in the protein content and enzyme activity. At 50°C, the production of amylase and protein content was low (1.39 U/ml).

Conclusion: Amylase is one of the most important enzymes known and is of great significance having approximately 25 % of enzyme market. They find potential applications in food, pharmaceutical and fine chemical industries. Earlier severalstudies reported the biological production of amylase from microorganisms.

Keywords: Amaylase, Organisim, Enzyme, Purification, Bacillus.

Introduction

Enzyme, the most important products obtained for the needs of humans through plants, animals and several microbial sources. Presently, enzymes are being used in the industrial area especially in the food, paper, textile and leather industries. Amylase is an enzyme which breakdown the starch or glycogen. The term amylase was actually used to designate enzymes which are capable of hydrolyzing α- 1, 4- glucosidic bonds of amylose, amylopectin, glycogen and their degradation products [1-3]. For the production of amylase in bulk quantity, microorganisms are being used. The

microbes are easy to manipulate to obtain enzyme of desired characteristics [4]. Amylases have been derived from several fungi, yeast, actinomycetes and bacteria. Some members of the genus Bacillus are heterogeneous and they are highly versatile to their adaptability to the environment [5]. Amylase has been purified from various Bacillus species such as Bacillus megaterium [6] from Bacillus subtilis [7], from Bacillus licheniformis SPT 27[8]. Amylase is classified into two types' endoamylase and exoamylase. The cultivation of microbial amylase from bacteria is depend on the types of strain, composition of medium, temperature, pH, methods of cultivation, cell growth, requirements of nutrients. Metal ions, time of incubation and thermo stability [9]. Application of amylase widely spread in many sectors like detergents, baking, food, textile industries.

The use of amylase in saccharification and liquefaction of starch, the clarification of haze formed in beer or fruit juices and for pretreatment of animal feed to improve digestibility [10]. Recently interest and demand for enzymes with novel properties are very high in various industries and it leads to the discovery of various types of the amylases with unique properties [11]. All amylases are glycoside hydrolyser and act on alpha-1, 4-glycosidic bonds [12]. Alpha amylase (E.C 3.2.1.1) for industrial purpose used in starch liquefaction, brewing, textile, pharmaceuticals, paper, drug, detergent, toxic wastes removal and oil drilling[13]. Amylases are among the most important enzymes and are of great significance in present day biotechnology taking approximately 25% of the enzyme market [14].

Materials and Methods

Bacillus cereus was propagated on nutrient agar medium. The pure cultures were streaked on starch agar plates containing peptone 0.05%, KCl 0.01%, MgSO4. 7H2O 0.05%, (NH4)2SO4 0.01%, NaH2PO4 0.01%, starch 2% [15], agar 1.7 g and NaCl 10% and incubated for 3 days at 37°C.

Screening of amylase producing bacteria: The amylolytic activity of the isolated organism was determined according to the method of [16]. Briefly, a loop full of isolate was streaked aseptically on starch medium. The plate was then incubated at 37°C for 24 hours. After incubation petriod, lugol's iodine solution was flooded over the plate, and allowed to stand for 20 minutes. A clear zone surrounding the bacterial growth indicates starch hydrolysis (+) by the organism due to its production of the extracellular enzymes. This represents the amylolytic activity of the bacterial species.

Amylase production: Amylase production was carried out by submerged fermentation. 500 ml of the production medium was inoculated with 10 ml of bacterial inoculum. The flask was loaded on a rotary shaker incubator at a speed of 2000 rpm at 37°C for 24 hours. After incubation, fermented broth was centrifuged at 7000 rpm for 15 min in a cooling centrifuge. Supernatant was collected and used for the estimation of amylase.

Amylase enzyme assay: Amylase activity was measured by the 3, 5-dinitrosalicylic acid (DNS) method [17-18] by monitoring the amount of reducing sugar liberated from starch. Amylase was assayed by adding 1ml of enzyme (fermented broth supernatant) to 0.5 ml of 1% soluble starch and incubated for 30 min at 37°C. The reaction was stopped by adding 1 ml of 3, 5 dinitrosalicylic acid, followed by boiling for 10 min. The final volume was made to 5 ml with distilled water and the absorbance due to the produced 3-amino, 5-nitrosalicyclic acid measured at 540 nm with a spectrophotometer. One amylase unit (U) was defined as the amount of enzyme per ml culture filtrate that released 1microgram glucose per minute.

Effect of temperature and pH on enzyme production: Effect of temperature on enzyme production and enzyme activity was studied by adjusting the incubation temperature at 20, 35, 45 and 50°C and production medium pH 6.5, 7.5, 8.5 and 9.5 respectively.

Partial purification of amylase enzyme: Partial purification of amylase enzyme was achieved by ammonium sulphate precipitation followed by dialysis. 100 ml of cell free extract was saturated with ammonium sulphate up to 80%. The content was incubated overnight and centrifuged at 5000 rpm for 20 min. Supernatant was collected and saturated up to 90% with ammonium sulphate. Then the content was centrifuged at 5000 rpm for 20 min and pellet was collected for further analysis. The enzyme mixture was transferred in a dialysis bag and immersed in phosphate buffer at 4°C for 24 hr. buffer was continuously stirred using a magnetic stirrer throughout the process. Buffer was changed three times during the process in order to obtain proper purification.

Discussion and Results

Amylase is a heterogeneous calcium-dependent metalloenzyme with a diverse molecular weight range, typically between 54 and 62 kDa. The compact size of amylase facilitates efficient filtration through the glomeruli. [19]. Amylase is eliminated through both the renal system and the reticuloendothelial system.[20]. This enzyme exists as 2 isoenzymes—pancreatic (P-type) and nonpancreatic (S-type)—which are products of 2 closely linked loci on chromosome 1. Additional amylase heterogeneity results from allelic variation, with 12 alleles for the S-type and 6 alleles for the P-type.[21]. Both types of amylase also undergo

post-translational modifications involving deamidation, glycosylation, and deglycosylation, giving rise to various isoforms. Amylase exhibits a broad tissue distribution, with the most significant levels of P- and S-type activities being found in the exocrine pancreas and salivary glands, respectively.[22].

P-type amylase is synthesized by pancreatic acinar cells and released into the intestinal tract through the pancreatic duct system. The enzymatic activity of P-type amylase is optimized under the slightly alkaline conditions of the duodenum.[23]. Meanwhile, the salivary glands host the highest S-type amylase activity, initiating starch hydrolysis during mastication in the mouth and the passage through the esophagus. However, this action is terminated upon exposure to stomach acid.

S-type amylase is also detectable in extracts from testes, ovaries, fallopian tubes, Mullerian ducts, striated muscle, lungs, and adipose tissue, as well as in bodily fluids such as semen, colostrum, tears, and milk. Approximately 25% of plasma amylase is excreted by the kidneys, with the majority being reabsorbed within the proximal tubules.[24]. The liver is believed to be the primary organ responsible for amylase elimination, leading to a half-life of approximately 10 hours. Serum amylase is intricately controlled within the body,[25], with a delicate balance between its production and clearance rates. Elevated amylase levels can result from heightened production, whether originating in the pancreas or outside it, or from a diminished clearance rate.

Genetic regulation is likely to play a crucial role in the preliminary determination of salivary amylase. In newborns, the predominant amylase isozymes detected in urine originate from saliva, and as development progresses, both salivary and pancreatic amylase isozymes become more prominent. The functional integrity of amylase is entirely dependent on the presence of calcium.[26]. However, complete functionality is achieved only in the presence of specific anions, including chloride, bromide, nitrate, or monohydrogen phosphate. Chloride and bromide are the most effective activators. The pH optimum for amylase activity falls within the range of 6.9 to 7.0.[27].

The analyte amylase is an endoglycosidase enzyme belonging to the hydrolase class, and it catalyzes the hydrolysis of 1,4-α-glucosidic linkages between adjacent glucose units in complex carbohydrates.[28]. Notably, straight-chain (linear) polyglucans, such as amylose, and branched-chain polyglucans, such as amylopectin and glycogen, are hydrolyzed at different rates.[29]. In the case of amylose, the enzyme cleaves the chains at alternate α-1,4-hemiacetal (-C-O-C-) links, thereby forming maltose and some residual glucose. In the case of branched polyglucans, the enzyme generates maltose, glucose, and a residue of limit dextrins. Notably, the enzyme does not target the α-1,6-linkages at the branch points.[30].

Thermostability is a desired characteristic of most of the industrial enzymes. Thermostable enzymes isolated from thermophilic organisms have found a number of commercial applications because of their stability. As enzymatic liquefaction and saccharification of starch are performed at high temperatures (100–110°C), thermostable amylolytic enzymes have been currently investigated to improve industrial processes of starch degradation and are of great interest for the production of valuable products like glucose, crystalline dextrose, dextrose syrup, maltose and maltodextrins [31]. Bacillus subtilis, Bacillus stearothermophilus, Bacillus licheniformis, and Bacillus amyloliquefaciens are known to be good producers of thermostable α-amylase, and these have been widely used for commercial production of the enzyme for various applications. Thermostable α-amylases have been reported from several bacterial strains and have been produced using SmF as well as SSF. However, the use of SSF has been found to be more advantageous than SmF and allows a cheaper production of enzymes. The production of α-amylase by SSF is limited to the genus Bacillus, and B. subtilis, B. polymyxia, B.mesentericus, B. vulgarus, B. megaterium and B.licheniformis have been used for α-amylase production in SSF. Currently, thermostable amylases of Bacillus stearothermophilus or Bacillus licheniformis are being used in starch processing industries [32].

Industrial enzymes produced in bulk generally require little downstream processing and hence are relatively crude preparations. The commercial use of α-amylase generally does not require purification of the enzyme, but enzyme applications in pharmaceutical and clinical sectors require high purity amylases. The enzyme in the purified form is also a prerequisite in studies of structure-function relationships and biochemical properties. Different strategies for purification of enzymes have been investigated, exploiting specific characteristics of the target biomolecule. Laboratory scale purification for α-amylase includes various combinations of ion exchange, gel filtration, hydrophobicity interactions and reverses phase chromatography. Alternatively, α-amylase extraction protocols using organic solvents such as ethanol, acetone and ammonium sulfate precipitation and ultrafiltration have been proposed. These conventional multi-step methods requires expensive equipments at each step, making them laborious, time consuming, barely reproducible and may result in increasing loss of the desired product. However, liquid–liquid extractions consist of an

interesting purification alternative since several features of the early processing steps can be combined into a single operation. Liquid–liquid extraction is the transfer of certain components from one phase to another when immiscible or partially soluble liquid phases are brought into contact with each other. This process is widely employed in the chemical industry due to its simplicity, low costs, and ease of scale up. Purification of biomolecules using liquid–liquid extraction has been successfully carried out on a large scale for more than a decade. Advantages of using this system are lower viscosity, lower cost of chemicals and shorter phase separation time. The dynamic behavior of these systems has to be investigated and understood to enhance plant-wide control of continuous liquid–liquid extraction and to assess safety and environmental risks at the earliest possible design stage.

Identification of amylase producing bacteria: The bacterial isolates were characterized on the basis of colony characteristics, microscopic appearance and biochemical tests. Considering the colony characteristics (Table 1), microscopic appearance and biochemical tests the isolate was identified as Bacillus cereus (Figure 1).

Table 1:

	Characterization of Bacteria	Result
Cultural characters	**Colony morphology on nutrient agar**	**Small, round, mucoid, yellow, fast growing colonies**
Microscopic characters	**Spore staining**	**Spore forming**
Biochemical characters	**Indole**	+
	Methyl Red	+
	Catalase	+
	Citrate utilization	-
	Oxidase	-
	Nitrate reduction	+
	Gelatin liquefaction	+
	Hydrogen sulphide	+

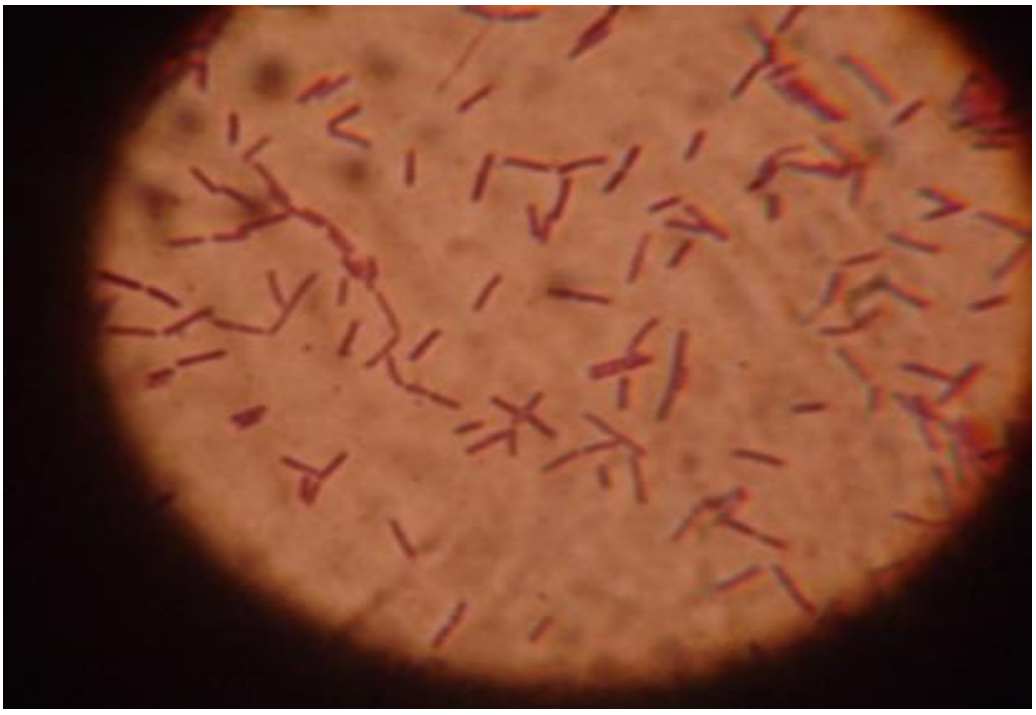

Figure 1:

Screening of bacterial strain: Growth of Bacillus cereus on starch agar plate after the addition of iodine the clearing surrounding the bacterial growth indicates starch hydrolysis (+) Fig 2.

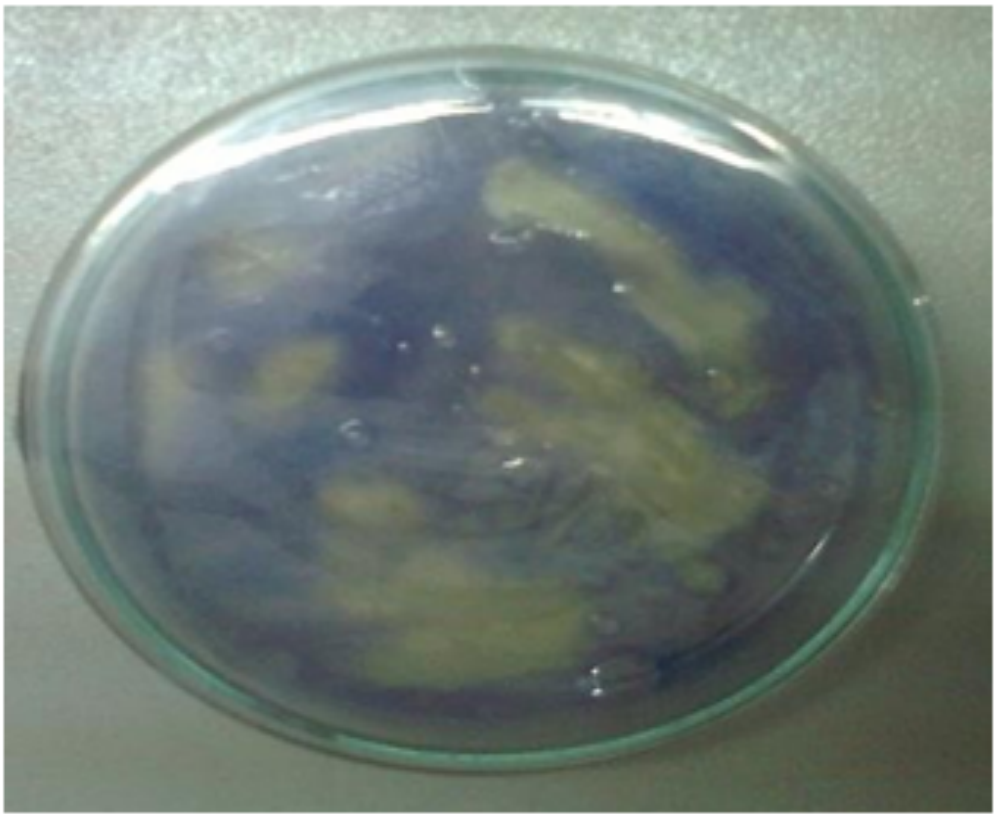

Figure 2:

Effect of temperature on enzyme activity and protein content: Production of amylase by the Bacillus cereus was optimum(2.78 U/ml) at 35°C temperature and as the temperature increased or decreased, there was gradual decrease in the protein content and enzyme activity (Fig 3). At 50°C, the production of amylase and protein content was low (1.39 U/ml).

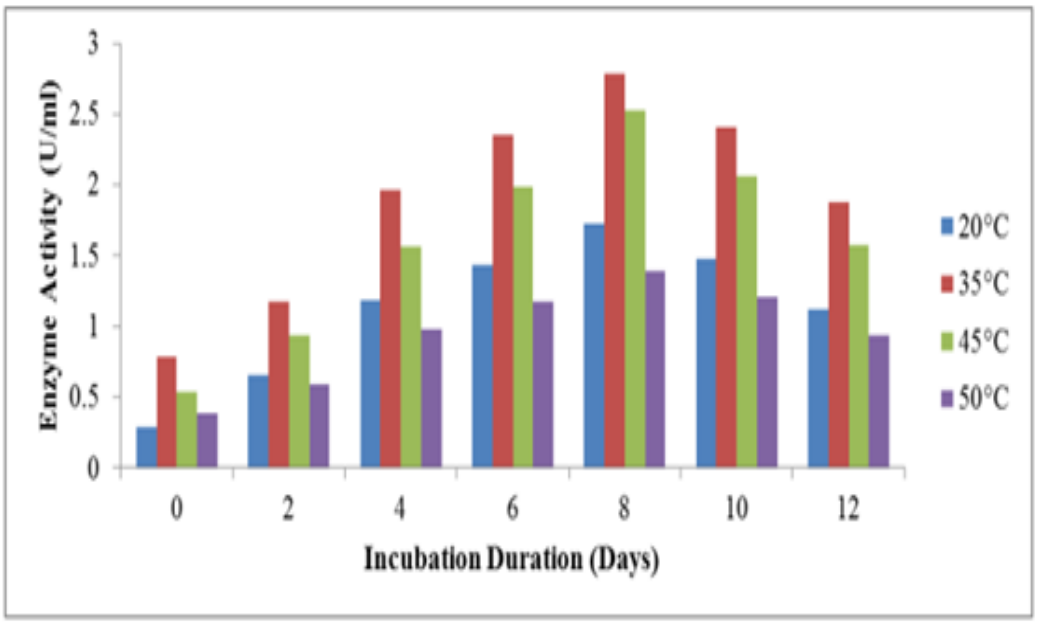

Figure 3:

Effect of pH on enzyme activity:
Optimum pH for the amylase production was found at 35°C, as the pH was increased, there was gradual decrease in growth of the organism, protein content and enzymeactivity (Fig 4). The organism did not grow in

production medium adjusted to pH below 5 and above 10.

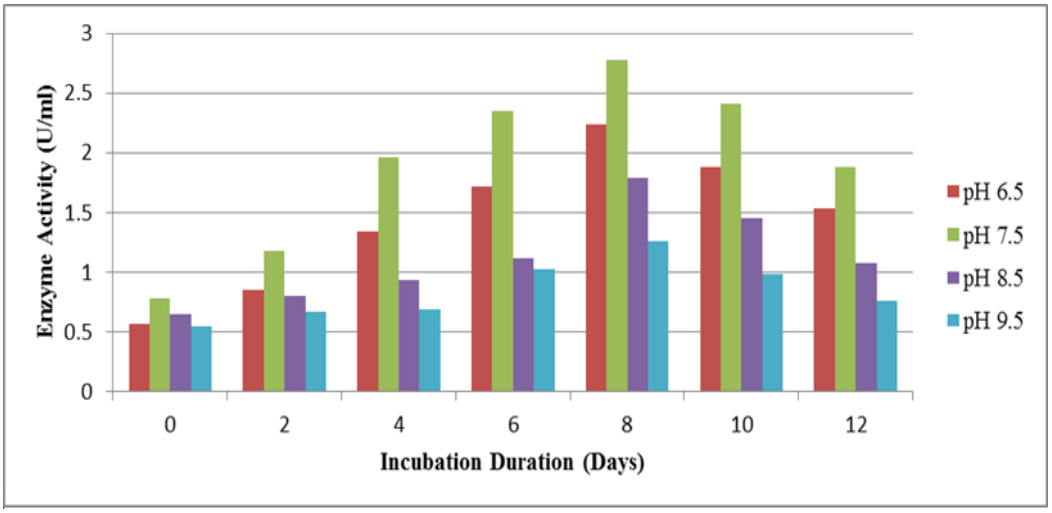

Figure 4:

Partial purification of amylase: Partial purification of amylase enzyme was performed by Ammonium sulphate precipitation followed by dialysis. Partially purified amylase exhibited specific activity of 0.053 U/ml/mg which corresponds to 6.62 purification fold and 44.0 % Yield (Table 2).

Table 2:

PurificationSteps	Enzyme Activity (mg)	Protein Activity (U/ml/mg)	Specific Activity (U/ml/mg)	Yield %
Crude Extract	0.608	70	0.008	100
(NH4)2SO4	0.409	20	0.02	67.2
Dialysis	0.268	5	0.053	44

Conclusion:

Amylase is one of the most important enzymes known and is of great significance having approximately 25 % of enzyme market. They find potential applications in food, pharmaceutical and fine chemical industries.

Earlier several studies reported the biological production of amylase from microorganisms. In this study Bacillus sp. cereus which was isolated from the soil of coal mines screened on starch agar plates which showed positive hydrolysis of starch.

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