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Isolation, Screening And Optimization Of Amylase Producing Bacteria From Soil In Dairy Farm Sarband Peshawar, Kpk, Pakistan

Zeeshan Ahmad[1], Junaid Ahmad[1*], Salman Hussain[2], Rabia Mazhar[2], Muhammad Kamran Khan[3], Rahila Akhtar[4], Tanveer Tara[5], Haris Khan[6], Kamran Shah[7], Zakirullah Khan[8], Shubana Hayat[1]

[1]Department of Microbiology, Faculty of Biological Science, Hazara University, Mansehra, Pakistan

[2]Institute of Microbiology and Genetics, University of the Punjab Lahore

[3]Department of Biotechnology and Genetics Engineering, Hazara University Mansehra, Pakistan

[4]KEMU, MAYO Hospital Lahore

[5]Department of Medical lab Technology National Skills University

[6]Department of Microbiology, IBMS KMU Peshawar

[7]Sarhad University of Science and Information Technology

[8]Medical Technologist Department of Phlebotomy Islamabad Diagnostic Center

Abstract

Microbial enzymes have a variety of characteristics that make them valuable in a wide range of applications. Amylases are enzymes that are responsible for the breakdown of starch or glycogen. According to the findings of this study, amylase-producing bacteria isolated from soil samples of Dairy Farm Sarband Peshawar, KPK, Pakistan, were screened and characterised. As a result, ten bacterial isolates were recovered from the collected soil samples. This group selected two isolates (U5 and U6) based on the average clear zone development diameter and the time required to decolourize iodine solution, respectively. Based on their morphological and biochemical features, the isolates belonged to the genera Bacillus and Staphylococcus. The optimal amylase production time for the (U5) isolates was 72 hours, and for the (U6) isolates, it was 24 hours. This corresponded to amylase activity of 15mm zone of hydrolysis for U5 and 11mm zone of hydrolysis for U6, respectively, for the isolates. Studies on the influence of pH showed that the crude enzyme with the highest activity and stability was found at pH 9 and 7 for isolates U5 and U6, respectively. This amylase-producing bacterial strain has a lot of promise for use in Pakistan's food and agricultural industries, and it has a lot of potential.

Keywords: Amylase, Bacteria, soil, Dairy Farm Sarband Peshawar, Pakistan.

Introduction

Enzymes are biological catalysts that initiate and speed up thousands of biochemical reactions in living cells. Enzymes are specific molecules due to each enzyme's selective binding site for its respective substrate. They are applied in various fields such as food manufacturing, fermentation, animal nutrition, detergent, cosmetics, brewing and textile to paper industries, pharmaceutical (medication), and as tools for research and development (Padma & Pallavi, 2016). Among enzymes, amylase plays a significant catalytic role in breaking starch into monomeric compounds, the smallest being glucose (Gangadharan, Sivaramakrishnan, Nampoothiri, Sukumaran, & Pandey, 2008; Yassin, Jiru, & Indracanti, 2021). Although amylases can be produced biologically by plants, animals, humans, and microorganisms, enzymes derived from microorganisms are used in most industries (Islam, 2016). It is preferred over other types of amylase derived from plants and animals due to its biochemical versatility, better production rate and stability, as well as the availability of a large variety of microbial strains, all of which are readily available (Asad, Asif, & Rasool, 2011). Furthermore, the ability to generate in large quantities and the ease with which it could be engineered to get enzymes are desirable features. Currently, amylase synthesis for up to 30% of the global enzyme market is steadily rising. (Kaur, Kaur, Samyal, & Ahmed, 2012). As a source of amylase, many different types of microorganisms have been chosen because they are easy to get hold of, can grow quickly, and can secrete proteins into an extracellular medium, as well as because they have a high yield and are safe to handle (Saxena & Singh, 2011). However, amylase produced from fungi and bacteria has led to a variety of biotechnological applications (Kaur et al., 2012), including Bacillus sp., Lactobacillus sp., Proteus sp., Escherichia coli, Pseudomonas sp., Streptomyces sp., Aspergillus sp. (e.g., Aspergillus ficuum), Talaromyces emersonii, and Thermomyces lanuginosus (Pokhrel, Wanjare, Singh, Purushotham, & Kumara, 2013). Amylase's thermostability is desired for industrial applications. Thermally stable enzymes are obtained from thermophilic species (Monnet, Joly, Dole, & Bliard, 2010). Thermophilic bacteria (commonly many Bacillus species) have been economically good sources of thermostable amylases . Extremophiles are microorganisms that flourish in harsh conditions, which are common in industrial processes (Pandey et al., 2000). Although starch is scarce, hyperthermophilic bacteria have starch-hydrolyzing enzymes in their genomes. Using thermostable amylases in industrial processes reduces contamination risk and increases the diffusion rate. Thermally heated hydrothermal systems, undersea saline hot vents, and hot springs soil and water are common thermophilic ecosystems (Simair et al., 2017). Under these environments, it is hypothesised that bacteria can produce enzymes capable of functioning in extremely adverse conditions (Gangadharan et al., 2008; Pokhrel et al., 2013). Extremozymes are biocatalysts that have been molecularly modified to resist extreme environments. One of the essential industrial enzymes is thermostable amylase (Abdullah, Shaheen, Iqtedar, Naz, & Iftikhar, 2014). Amylase is used more and more worldwide, including in Pakistan. Despite this, there is a lot of interest in finding enzymes with better properties, like thermostable raw starch degrading amylase that is good for industrial applications and can be made cheaply. Getting rid of contamination and making sure that each batch of your product is the same is a big problem in a business that works at a low temperature. This means that we have to fermentation at very high temperatures, where contamination is almost non-existent (Abdullah et al., 2014). It is because this is how most starch-processing industries work. Microbes that live in mild environments can easily get denatured when they work in industrial settings where the temperature, pH, and other factors are higher or lower (Pandey et al., 2000). Research is still undergoing to find out the bacterial strain which produces more stable amylase enzyme with higher production rate within low cost. Therefore, the presence of starch-degrading enzymes that have appreciable stability at

high temperatures is crucial. We identified and characterized the bacterial strain and optimized the growth conditions. The activity and stability patterns for the crude enzyme isolated from the bacterial strain were also been reported.

Material and Method

Collection of sample

The soil sample was collected aseptically in polystyrene bags from one dairy farm Sarband, Peshawar District, KPK Province, Pakistan. Samples were labelled properly, transported to the microbiology laboratory, and kept at room temperature until further analysis.

Isolation of amylase producing bacteria

Take nine tests tubes containing 9ml of distilled water was autoclaved before use, 1 gram soil from the collected sample was added in the test tube containing 9ml of distilled water and mixed well, then subjected to serial dilution 1ml of the mixed solution was taken from each test tubes and pour dilution on the nutrient agar plates and spread it through glass spreader and incubated at 37°C for 24 hours. After incubation, different bacterial strains were isolated and subcultured for pure cultures. The pure bacterial colonies are tested for amylase enzyme production on starch agar media.

Screening for Amylase Activity (starch iodine test)

Isolated pure bacterial colonies were picked up from each plate. They were streaked in freshly prepared starch agar plates by dot method using sterile inoculating needles o obtain pure isolates as confirmed by Gram staining and 1000X light microscopic observation. The plates were then incubated are 37°C for 24 hours. After incubation, the plates were visualized by vaporizing with iodine crystals to observe the clear zone around the colonies. The isolates observed to have the largest clear zones around them were selected.

Identification of potential bacterial strain

The potential bacterial strain was biochemically identified using biochemical tests like Oxidase, indole, coagulase, citrate, motility, and catalase.

Effect of pH on Amylase production

20ml nutrient broth was prepared in three 100ml flasks, and pH was adjusted with a pH meter. Which was found to be 7. The pH was adjusted to 5,7, and 9 with 1N HCL and 1 NaOH. The flasks were labelled with specific pH of that medium and autoclaved at 121 ºC for 15minutes. The nutrient broth was incubated with test organisms. It was placed in a shaking incubator. The supernatant was taken at different flasks duration i-e 24, 48 and 72 hours. It was checked by nutrient agar plates containing 1% starch using a well diffusion method and placed in an incubator for 24 hours 37ºC. The plates were then vaporized with iodine crystals to visualize the clear zone of hydrolysis.

Optimizing Fermentation time

Nutrient agar along with 1% starch was prepared in a 250 ml flask and autoclaved at 121 ^{o}C for 15 minutes. The media was then poured into plates and placed in an incubator at 37 ^{o}C for 24 hours. The supernatants were taken from the nutrient broth culture of test organisms of different time durations, i-e

24, 48, 72 hours were checked using well diffusion methods and placed in an incubator at 37 ^{o}C for 24 hours. The plates were then treated with iodine crystals to visualize the clear zone of hydrolysis for amylolytic activity.

Results

Selection of amylase producing bacteria among selected isolates:

Only two (U5 and U6) showed strong amylase activity when inoculated on starch plates among randomly selected ten colonies from nutrient agar. The U5 and U6 strains produced 15 mm and 11 mm zone of hydrolysis, respectively, when grown on nutrient agar media supplemented with 1% starch.

(a) (b)

Figure 1. Amylase activity of strain (U5) and (U6) on casein agar plates as shown in fig.(a) and (b), respectively.

Identification and biochemical characterization of amylase producing bacteria

The identity of amylase producing bacteria was confirmed by their growth characteristics on nutrient agar medium and gram staining technique. The U5 Strain was Gram-positive, purple colour, rod-shaped appearance, arranged in single while U6 was Gram-positive, purple colour, round cocci, arranged in clusters. Further, biochemical tests confirmed that isolated strain Bacillus sp., (U5) and Staphylococcus sp., (U6).

Table No.1. Biochemical and morphological characterization of amylase producing bacteria

S.No	Biochemical Test	*Bacillus spp.*,	*Staphylococcus spp.*,
1	Catalase	Positive (+)	Positive (+)
2	Coagulase	Negative (-)	Negative (-)
3	Oxidase	Positive (+)	Negative (-)
4	Indole	Negative (-)	Negative (-)
5	Motility	Positive (+)	Positive (+)
6	Citrate	Negative (-)	Negative (-)

Determination of pH for optimum amylase activity

The effect of pH on growth and enzyme production was observed by inoculating the culture in a broth medium having different pH values, i-e 5,7 and 9, respectively. The culture was incubated at 37 ^{o}C. Two strains (U5 and U6) were determined as the best amylase producer as shown in Fig No.2., showing 16 mm zone at pH-9 by Bacillus sp., and 14 mm at pH-7 hrs Staphylococcus sp., around the culture.

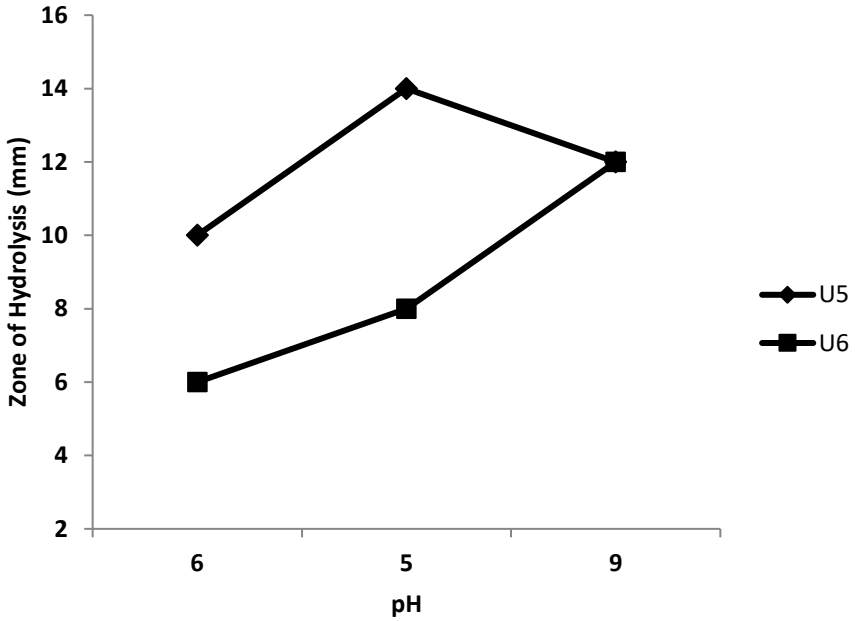

Fig 2. Amylase activity of strains Bacillus sp.,(U5) and Staphylococcus sp., (U6) at different pH values

Determination of time for optimum amylase activity:

The optimum time for maximum enzyme production was monitored by incubating the broth culture at different time intervals such as 24, 48 and 72 hrs. Only two strains (U5 and U6) were the best enzyme producers out of ten. U5 showed maximum activity after 72 hrs of incubation and produced up to 15mm zone of hydrolysis, while U6 showed maximum activity after 24 hrs of incubation and produced 11mm zone of hydrolysis.

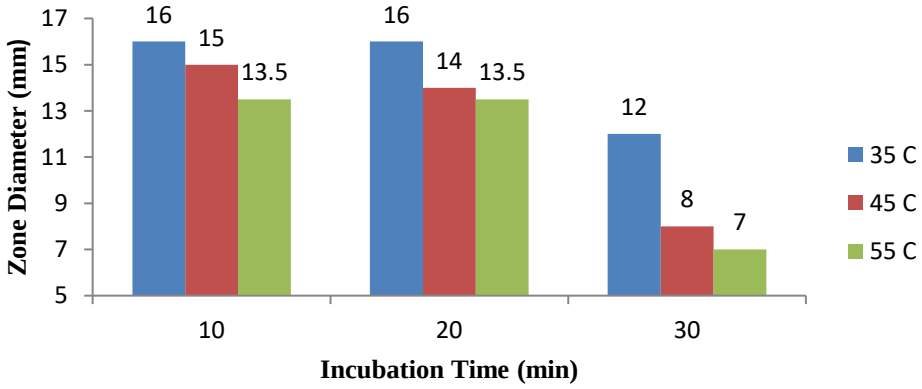

Figure.3.(a). Residual amylase activity of Bacillus sp.,(U5) in terms of zone diameter after incubation at different temperatures.

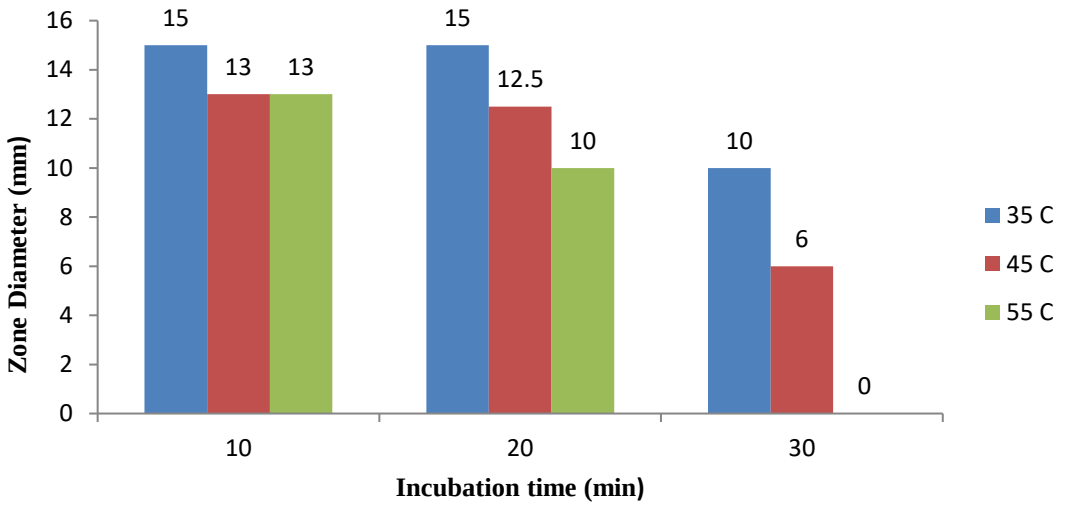

Figure 4. (b). Residual amylase activity Staphylococcus sp., (U6) in zone diameter after incubation at different temperatures.

Discussion

Enzymes are essential for carrying out biochemical reactions within an organism. Still, their high specificity and catalytic characteristics have enabled them to be used in various industrial sectors to produce a wide range of products. Amylase is one of those enzymes. Globally, amylase is frequently used in food, textile, detergents and paper industries. Amylase is regularly used in pharmaceutical and chemical industries to yield their products (El-Fallal, Dobara, El-Sayed, & Omar, 2012). Amylase is extensively used in the garments and textile industries and manufacturing food and pharmaceutical products (Budiasa, 2017). There is no local production of commercial amylase in Pakistan, and thus a lot of money is spent on the process of enzyme import. Harmful chemicals are also used as an alternative to amylase (Budiasa, 2017).

For this reason, a process must be established to generate commercial amylase in Pakistan. Since industrial amylase is usually extracted from bacteria and fungi, isolating a local high amylase producing strain is mandatory. This study aimed to isolate and amylase producing bacterial strain from soil and identify the strain through presumptive and genotypic methods (Dubois et al., 2010). The study also included characterization of the produced amylase enzyme by determining the optimum time and pH at which the enzyme works best. The soil collected for this study was from a dairy farm in Sarband, Peshawar. In a primary screening of the bacterial strains, 2 out of 10 isolates were amylase producers, producing a clear hydrolysis zone on a starch agar medium. Of this, 50% (1) strain was gram-positive, rod-shaped Bacillus sp., and 50% (1) strain was gram-positive cocci shaped Staphylococcus sp.

In this study, crude enzyme extract from 24, 48 and 72 hrs was checked on nutrient agar plates containing starch (1%) as a substrate for amylolytic activity through well plate methods. When treated with iodine crystals, two strains (U5 and U6) showed amylolytic activity on starch-containing agar plates. Strain U5 and U6 were confirmed extracellular amylase producers at 24 and 72 hrs of incubation. (Prabakaran & Hewitt, 2009) reported that the optimum time required for maximum amylolytic activity by Bacillus sp. was 48 to 72 hours. Another physical parameter that influences enzyme production is pH. In this study, the positive amylase strains were further tested at different pH from 5 to 9 on starch agar plates. The best production of amylase was observed at pH 7 and 9. Similar results were also observed by (Prabakaran & Hewitt, 2009) reported that the optimum pH for maximum amylase activity by Bacillus sp, staphylococcus and streptomyces was between 8.0 to 10.0. (Sawai et al., 1998) isolated amylase enzymes from bacillus strain KSM- 1378 having optimum activity of 8.0 to 8.5. Similarly,(Behal, Singh, Sharma, Puri, & Batra, 2006)studied thermostable amylase producing Bacillus sp that revealed an optimum enzyme activity at pH 8.0, whereas in other species, the optimum activity was at pH 7.0 reported by (Sumrin et al., 2011)

Conclusion

From the present study, it can be concluded that the bacterial isolate produces amylase, and different factors greatly regulate amylase production. Of all the isolates evaluated, the highest amylase activity was obtained from Bacillus sp. and Staphylococcus sp. Amylase produced by Bacillus species is by far the most important group of enzymes being industrially exploited.

Recommendation

- The positive amylase strains should be analyzed for other enzyme productions such as pectinase, protease, cellulase, and lipases and further be identified.
- Molecular identification of the amylase positive strains should be performed.
- The crude enzyme should be further purified and characterized.
- Different carbon and nitrogen sources should be checked for optimum growth and enzyme production.
- Site direction mutagenesis of the amylase positive strains should increase amylase thermostability and pH stability for industrial application.

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