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ORIGINAL ARTICLE **OPEN ACCESS**

Isolation of Industrial Enzymes Producing Microbes from Environmental Sample

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ABSTRACT
Microbial cells are selected from different groups of microorganisms for the production of amylase Industrial enzyme on a wide-scale basis. This paper aims to isolate and identify industrial valuable enzyme producing microbes from algae, eel fish and soil sample. In this study, the bacterial colonies were isolated by enumeration of microbes followed by colonies selection using the nutrient agar medium. The identified colonies were screened for amylase enzyme production using milk and starch agar selection medium. Furthermore, IMViC test helps in the identification of organisms in the coliform group followed by the protein confirmation using SDS-PAGE analysis. Upon investigation, the enzymatic activity was observed only on starch agar selection medium. Therefore, the identified bacteria can be an amylase enzyme producing bacteria. Methyl red test showed positive reaction based on all the IMViC reactions performed. Moreover, the dark protein bands were observed by SDS-PAGE analysis. Taken together these results showed that, the identified microbes were able to produce significant amounts of intracellular amylase enzyme. The bacteria were isolated from the extracts which had the ability to produce industrially amylase enzyme in an efficient manner.
Keywords: *Microbial-enzymes, amylase, bacteria, IMVIC, SDS-PAGE.*

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INTRODUCTION

Enzymes are biological macromolecules produced by a living organism that acts as a catalyst to bring about a specific biochemical reaction either through intracellular or extracellular process which is eventually termed as "Biocatalyst." As enzyme mediated processes are eco-friendly and non-toxic they are expeditiously gaining interest. As per the need for industrial applications, enzymes of microbial origin are cultured largely by genetic manipulations. The microbial enzymes are used for the treatment of industrial effluents containing aromatic compounds by bioconversion of toxic chemical compounds to innocuous products which have gained recognition worldwide for their widespread use in various sectors of industries. [3]

Amylases are the second type of enzymes used in the formulation of enzymatic detergent, and 90% of all liquid detergents contain these enzymes [11]. Detergent industries are the primary consumers of enzymes, in terms of both volume and value. The use of enzymes in detergents formulations enhances the detergents ability to remove tough stains and making the detergent environmentally safe. Nattokinase (EC 3.4.21.62), a potent fibrinolytic enzyme, is a promising agent for thrombosis therapy which is the most prominent medical use of microbial enzymes for the removal of dead skin and burns by proteolytic enzymes, and clot-busting by fibrinolytic enzymes[2].

The α-Amylase production can be obtained from different microorganisms species especially from fungal and bacterial sources as they have dominated applications in several industrial sectors. But for commercial applications they are mainly derived from the genus *Bacillus* such as *Bacillus licheniformis*, *Bacillus stearothermophilus*, and *Bacillus amyloliquefaciens* which find a greater potential in a number of industrial processes [9]. Among bacteria, α-amylase obtained from thermoresistant bacteria like *Bacillus licheniformis* or from engineered strains of *Escherichia coli* or *Bacillus subtilis* is used during the first step of hydrolysis of starch suspensions as they have the ability to survive in all sorts of inhospitable environments above 70°C [8]. The thermophilic fungus *Thermomyceslanuginosus* is also an excellent producer of amylase and its thermostability was proven by [7].

Feed enzymes are used in animal diet formulation as it can increase the digestibility of nutrients leading to greater efficiency in feed utilization. They are added to degrade specific feed components that have no nutritional value. Commercially available feed enzymes used for poultry includes phytases, proteases, α-galactosidases, glucanases, xylanases, α-amylases, and polygalacturonases [4-7]. Thus, the applications of microbial enzymes in food, pharmaceutical, textile, paper, leather, and other industries are increasing rapidly over conventional methods due to less harm to the environment [3]. Consequently, the present study was to isolate industrial enzyme producing microbes from environmental samples.

MATERIAL AND METHODS

SAMPLE COLLECTION

The algal, eel fish and soil samples were collected from Vayalur, Trichy.

CHEMICAL REAGENTS

The composition of nutrient broth for 100ml is 0.5 gm of peptone, SRL (India), 0.5 gm of Nacl SRL (India),0.1 gm of Beef Extract, SRL (India),0.2 gm of Yeast Extract, SRL (India). The composition of nutrient medium for the inoculation of taken sample in Petri plates of 0.5 gm of peptone, SRL(India),0.1 gm of Beef Extract, SRL (India),0.2 gm of Yeast Extract, SRL (India),0.5 gm of NaCl, SRL (India), 1.75 gm of Agar powder, SRL (India) for 100 ml.

Identification of amylase enzyme in starch Agar medium was prepared by the composition of 0.2gm of Yeast extract, SRL (India), 0.1 gm of beef extract,1.75gm of Agar powder, SRL (India),0.1 of milk solids, SRL (India).

ISOLATION OF MICROBES

The extract of the sample was taken and dissolved into 10 ml of nutrition broth. After inoculation, to sterile tube was kept into the incubator at 37 degree Celsius for 24 hr in control incremental condition. The nutrient agar was prepared and then the sample was pour into the plate. The L-rod was used to spread the sample in the plate. After incubation of plate at 37 °C at 24 hr. The plate was observed and the colony was viewed in the plate.

CHARACTERIZATION OF MICROORGANISM

GRAM STAINING

A loop full of sample was spread in the slide, which was taken from plate. The slide was smeared in the flame. The crystal violet dye was added kept it for 1 min and wash the slide in the water. Gram iodine was added and kept it for 1 minute then it washed out. The decolourising agent was added kept it 1 min and then it was out finally the saffron in strain was added after a minute it was washed in the water. It was observed under microscope the purple colours indicate gram positive and the pink colour was indicated gram negative.

MOTILITY

The motility test was performed by hanging drop method. The cover slip was taken also then its edge was coated with Vaseline. The loop full of sample was transferred into the cover slip and placed it over the cavity slide. The slide was viewed under microscope and observed the organism, whether it was motile or non- motile.

SCREENING AND SELECTION OF INDUSTRIALLY IMPORTANT MICROBES

Milk agar medium and potato dextrose agar medium was prepared and screened for industrial enzyme producing microbes. Petri plates divided into the different small boxes, a loop full of culture was taken and placed a single streak on the Milk Agar Plates and PDA agar plates. After Incubation, the zone of clearance was examined in both milk and PDA agar medium.

a. Potato Dextrose Agar Medium

The potato dextrose agar medium was prepared by dissolving 20 gm of potato influsion, 2 gm of dextrose and 1.5 gm of agar in 100ml of distilled water. The dissolved medium was autoclaved at 15 lbs pressure at 121°C for 15 minutes. The autoclaved medium was mixed well and poured onto 100mm petri plates (25-30 ml/plate) while still molten.

b. Milk Agar Medium

The milk agar medium was prepared with the composition of 0.3 gm of Yeast extract, 0.5 gm of beef extract, 1.5 gm of Agar powder, 0.1 of milk solids in 100 ml of distilled water. The dissolved medium was autoclaved at 15 lbs pressure at 121°C for 15 minutes. The autoclaved medium was mixed well and poured onto 100mm petri plates (25-30 ml/plate) while still molten.

SELECTION OF MUTATION COLONY

The isolated pure culture was spread over the nutrient agar medium in the plate. The plate was separated into two sections; the half of the petri plate portion was covered with aluminium foils which protect the sample from the UV exposure. The remaining half portion was exposed under UV for different time period (10, 20, 30 minutes). After exposure, the plate was incubated at 37 degree Celsius for 24 hours in

bacteriological incubator. The plate was observed, the mutant colonies were isolated from the UV exposed area after 24 hours.

SDS-PAGE ANALYSIS

The determination of molecular weight of the enzyme was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the Laemmli method. The protein bands were determined through the Coomassie Brilliant blue staining method.

STATISTICAL ANALYSIS

The difference in estimated parameters between the groups was analyzed using one-way ANOVAwithBonferroni's test. Data expressed as mean ± SD. All parameters were analyzed at 95% confidence intervals and a P-value of <0.05 was considered to be statistically significant. Statistical analysis of the data was performed using Graphpad Prism version 6.00 for Windows, GraphPad Software, San Diego California USA.

RESULTS

Screening, isolation and identification of industrial important enzyme producing microorganism from the algal sample. From the algal samples, ten different bacterial colonies were isolated which produced very minimum enzyme activity. Those colonies were isolated and stained with gram staining method. The shape of the bacterial isolate was rod shaped.

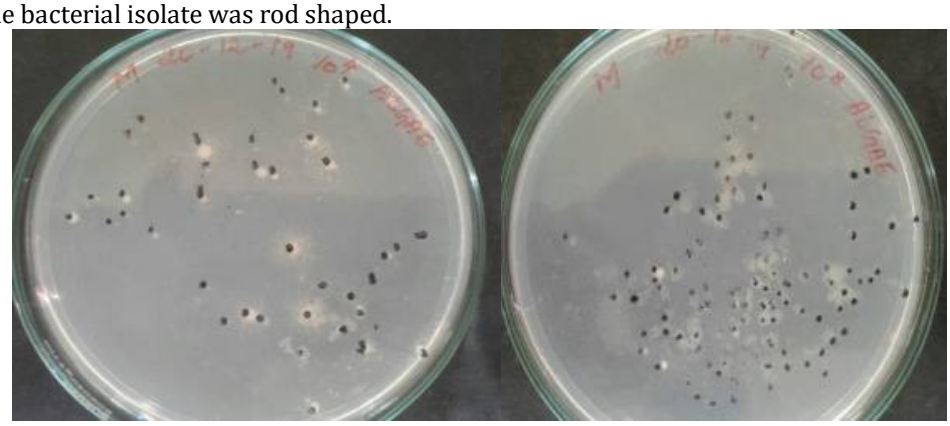


Fig .1 Colony formations of 10^4 and 10^8 algal samples

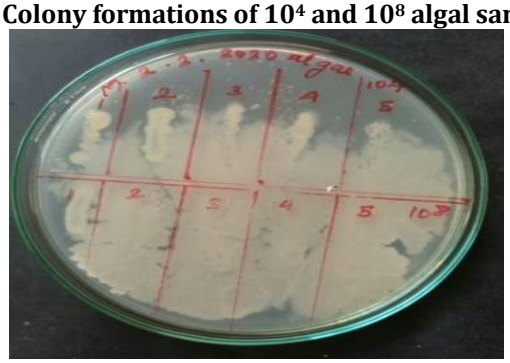


Fig.2 Isolation of individual colonies from 10^4 and 10^8 plates

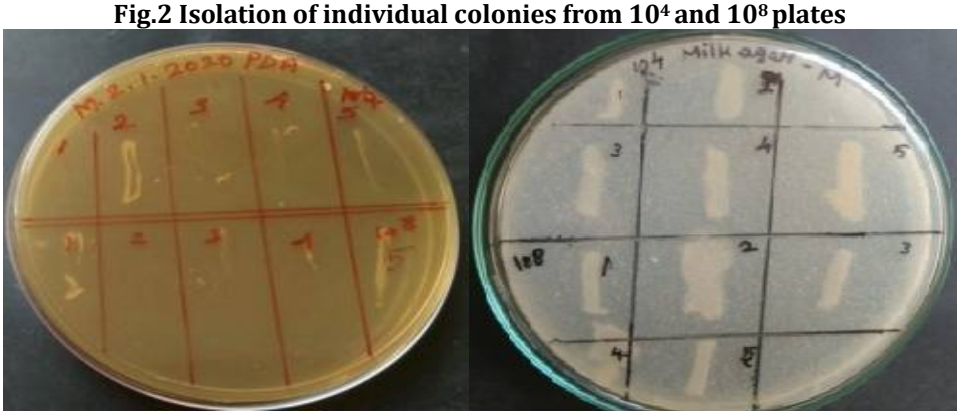


Fig .3 Screening of protease and amylase enzyme producing bacterial isolates by PDA and milk agar

Screening, isolation and identification of industrial important enzyme producing microorganism from the Eel fish sample

In the Eel fish samples, several colonies were isolated by spread plate technique. Those colonies were isolated and stained with gram staining method in which the isolate shape was cocci as well as rod shaped.

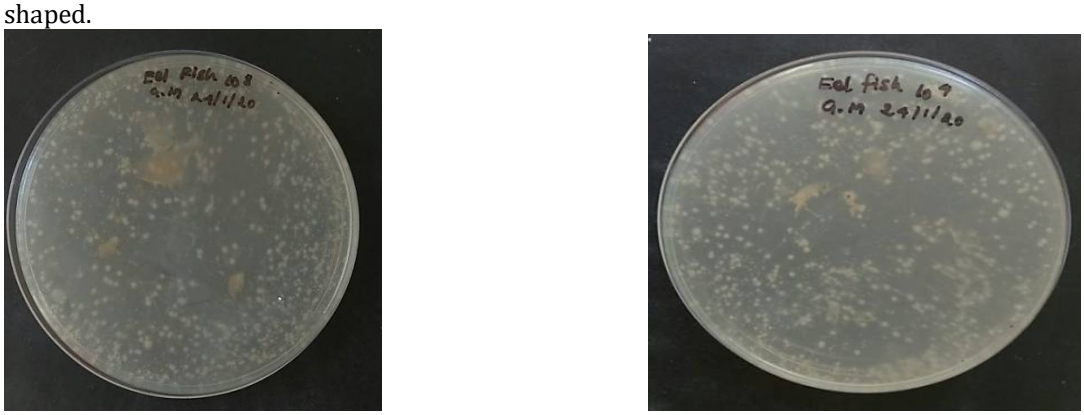


Fig .6 Colonies formation of 10^4 and 10^8 dilution of eel fish samples

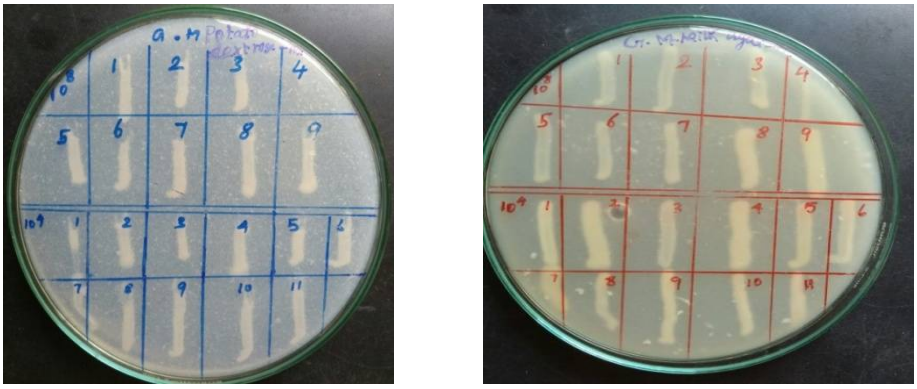


Fig.7 Screening of enzyme producing microbes by PDA and milk agar medium

Screening, isolation and identification of industrial important enzyme producing microorganism from soil sample

The bacterium isolated from the soil was capable of producing amylase enzyme as the zone of clearance was observed around colonies on the starch agar plate. Those colonies positive were isolated and stained with gram staining method. The shape of the bacterial isolate was rod shaped gram-positive bacteria.

Fig.10 Colonies observed at serial dilution of 10^4in soil sample

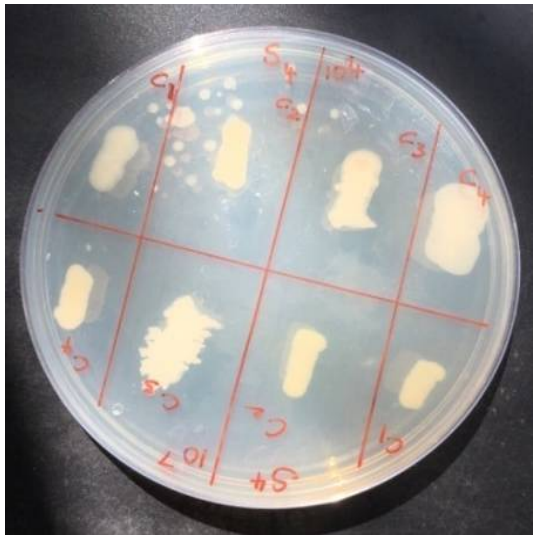


Fig.11 Isolation of colonies from the 10^4and10^7 dilution plate

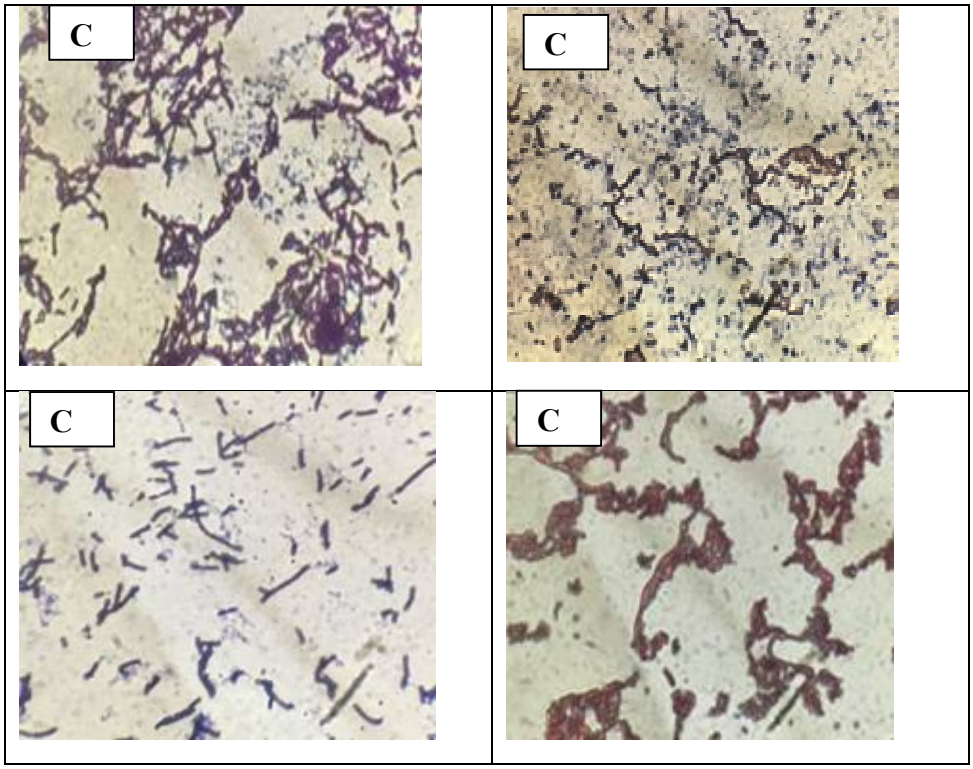
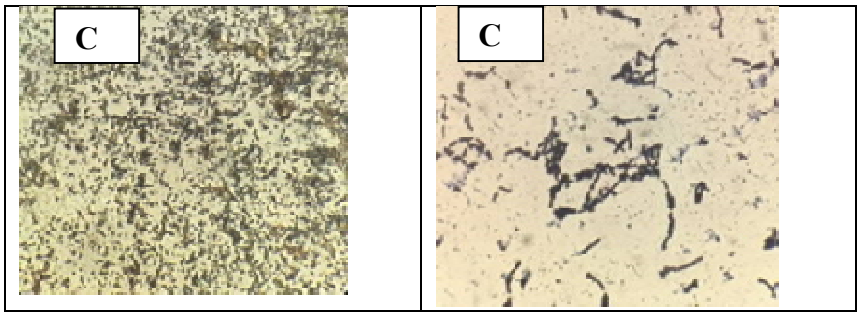


Fig. 12 Gram staining of soil sample colonies at 10^4 serial dilution



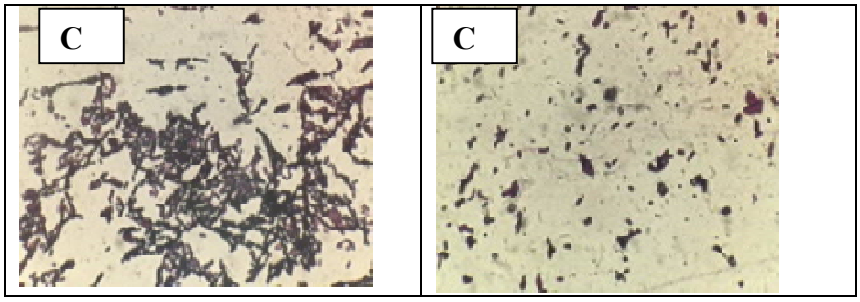


Fig.13 Gram staining of soil sample colonies at 10^7 serial dilution

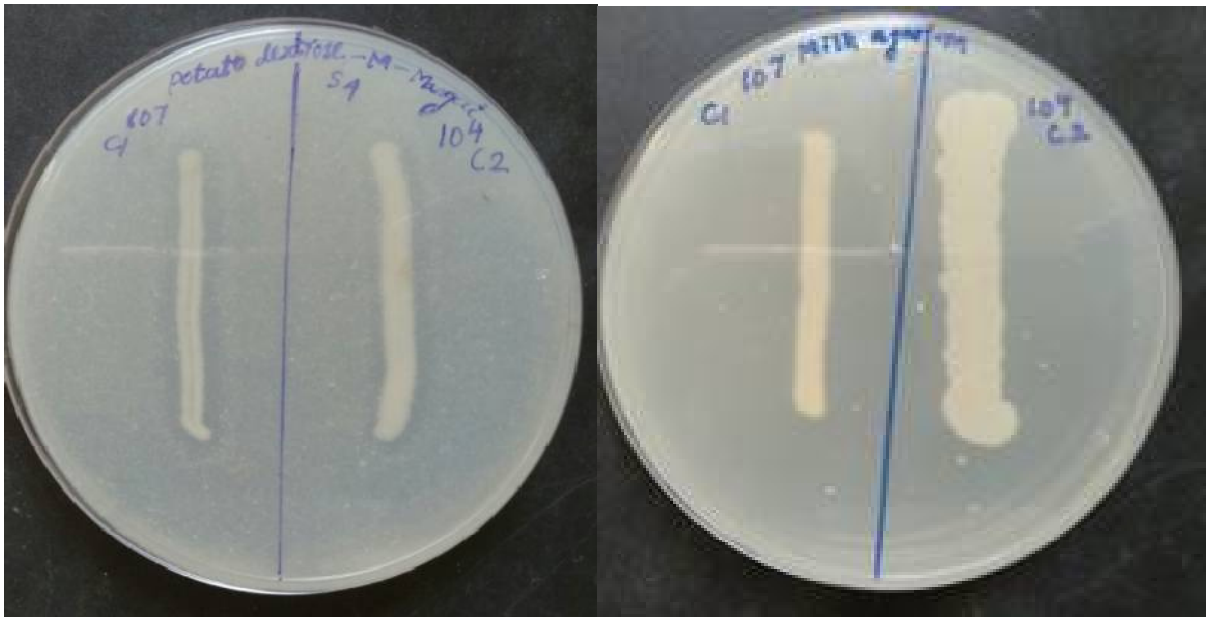


Fig. 14 Enzymatic activity of 10^7 and 10^4dilution soil sample colonies in PDA and milk agar

Biochemical screening and identification of industrial enzyme producing microbes

There are 38 bacterial colonies were isolated from the three different environmental samples. All the bacterial colonies were subjected to gram staining and unique colonies were shortlisted. There colonies were further identified by IMViC biochemical assays.IMVIC tests showed the colonies were methyl red positive result. The methyl red positive bacteria producing sufficient amount of acid during fermentation of glucose.

Table 1.Identification of unique bacterial colonies from the three different samples

S NO.	TEST SAMPLE & SERIAL DILUTION	SHAPE	GRAM STAINING
1	S 1 10^4C 1	Long chain rods	Positive
2	S 1 10^4C 2	Rods	Positive
3	S 1 10^4C 3	Long chain rods	Positive
4	S 1 10^4C 4	Long chain rods	Negative
5	S 1 10^7C 1	Short rods	Positive
6	S 1 10^7C 2	Long chain rods	Positive
7	S 1 10^7C 3	Long chain rods	Positive
8	S 1 10^7C 4	Short rods	Positive
9	S 2 10^8C 1	Short rods	Positive
10	S 2 10^8C 2	Rods	Negative
11	S 2 10^8C 3	Rods	Negative
12	S 2 10^8C 4	Rods	Negative

Table 2 Biochemical assays for amylase enzyme producing microbes

S.NO	BIO CHEMICAL TEST	SAMPLE	RESULT
1.	Indole test	10^7	-
		10^4	-
2.	Methyl red test	10^7	+
		10^4	+
3.	Vogesproskauer test	10^7	-
		10^4	-

Amylase enzymatic activity of bacterial isolate from the soil sample

The amylase enzymatic activity was performed using bacterial lysate and their supernatant. The results showed that the bacterial isolate produces intracellular amylase enzyme production as shown in fig 15.

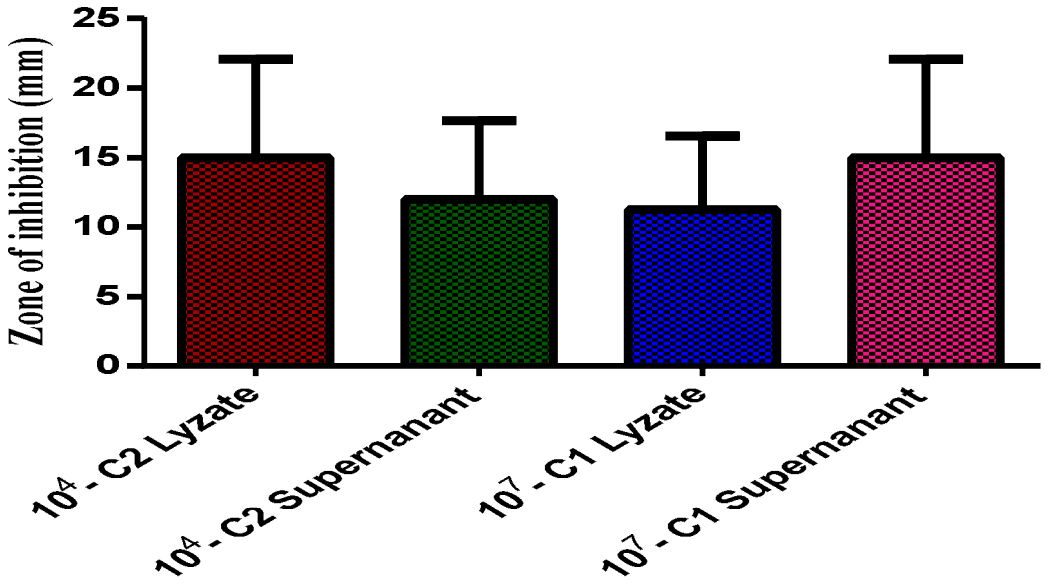


Fig.15 Amylase enzymatic activity of 10^4 C2 colonies of soil sample in PDA agar medium

SDS PAGE analysis of amylase enzyme

The SDS-PAGE analysis was performed for amylase enzyme producing bacterial lysate. The results showed the presence of amylase enzyme band at 68 kDa.

Fig.16 SDS-PAGE analysis of amylase enzyme

DISCUSSION

Amylase is one of those enzymes that have high specificity and catalytic characteristics which have enabled them to be used in various industrial sectors for the production of a wide range of products. In the present study we isolated an industrial enzyme producing microbes from the environmental sample and their biochemical characterization by IMViC tests and their enzymatic activity. Isolation of bacteria was carried out using algal, eel fish and soil samples. From the algal samples, ten different bacterial colonies were isolated which produced very minimum enzyme activity.

Those colonies were isolated and stained with gram staining method. The shape of the bacterial isolate was rod shaped. In the Eel fish samples, several colonies were isolated by spread plate technique. Those colonies were isolated and stained with gram staining method in which the isolate shape was cocci as well as rod shaped. The bacteria isolated from the soil were capable of producing amylase enzyme as the zone of clearance was observed around colonies on the starch agar plate. Those colonies positive were isolated and stained with gram staining method. The shape of the bacterial isolate was rod shaped gram-positive bacteria.

There are 38 bacterial colonies were isolated from the three different environmental samples. All the bacterial colonies were subjected to gram staining and unique colonies were shortlisted. There colonies were further identified by IMViC biochemical assays.IMVIC tests showed the colonies were methyl red positive result. The methyl red positive bacteria producing sufficient amount of acid during fermentation of glucose.

The amylase enzymatic activity was performed using bacterial lysate and their supernatant. The results showed that the bacterial isolate produces intracellular amylase enzyme production as shown in fig 15.The SDS-PAGE analysis was performed for amylase enzyme producing bacterial lysate. The results showed the presence of amylase enzyme band at 68 kDa.

The important step of enzyme production is isolation and screening of the bacterial strains. On primary screening, the bacterial strains showed amylase positive with a maximum zone formation. The mechanism of a clear zone observed was due to the fact that the amylase produced during the growth of the organisms has hydrolysed the starch around the colony. Selection of suitable fermentation medium is very essential for the growth of microorganisms as well as for the production of amylase.

The composition and concentration of media greatly affect the growth and production of extracellular amylase by bacteria. Alpha amylase production is induced by the presence of starch in the production medium.

The result in this investigation suggests that the zone of clearance with the amylase activity was observed clearly in the soil sample. The isolate displaying maximum amylase activity on quantitation was selected and confirmed the protein band by SDS PAGE analysis.

CONCLUSION

Amylases are significant enzymes for their specific use in the industrial starch conversion process. The present study attempts to explore the potential of indigenously isolated bacterial strains to produce amylases efficiently. The strains isolated from algal, eel fish and soil samples found to be amylase positive. Among all the three samples, the soil sample showed a clearer and sharp zone of clearance. The results are significant as they indicate that the enzyme will be active even under the presence of high temperatures and could be used under harsh conditions of textile wet processing.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest

REFERENCES

1. Adamczak M., Sajja K.H. Strategies for improving enzymes for efficient biocatalysis. Food Technol. Biotechnol. (2004);42:251–264.
2. Bharathiraja S., Suriya J., Krishnan M., Manivasagan P., Kim S.-K. Production of Enzymes From Agricultural Wastes and Their Potential Industrial Applications. Adv. Food Nutr. Res. 2017; 80:125–148.
3. Bommarius A.S., Riebel-Bommarius B.R. Biocatalysis: Fundamentals and Applications. Wiley-VCH; Weinheim, Germany: (2004) p. 611.
4. Bornscheuer U.T., Huisman G.W., Kazlauskas R.J., Lutz S., Moore J.C., Robins K. Engineering the third wave of biocatalysis. Nature. (2012); 485:185–194.
5. Daniel R. M., Danson M. J., Hough D. W., Lee C. K., Peterson M. E., Cowan D. A. Enzyme stability and activity at high temperatures. In: Siddiqui K. S., Thomas T., editors. *Protein Adaptation in Extremophiles*. New York, NY, USA: Nova Science Publishers; (2008). pp. 1–34.

6. Drepper T., Funken H., Heck A., Kovacic F., Krauss U., and Pietruszka J. (2014) Special issue dedicated to Karl-Erich Jaeger on the occasion of his 60th birthday. J Biotechnol 191: 1–2.
7. Giddings G., Allison G., Brooks D. and Carter A. (2000) Transgenic plants as factories for biopharmaceuticals. Nat. Biotechnology. 18, 1151–1155.
8. Gurung N., Ray S., Bose S., Rai V. A broader view: Microbial enzymes and their relevance in industries, medicine, and beyond. Biomed. Res. Int. (2013); 2013:1–18.
9. Guy JE, Isupov MN, Little child JA. The structure of an alcohol dehydrogenase from the hyper thermophilic archaeon *Aeropyrumpernix*. J Mol Biol. (2003); 331:1041–1051
10. Hassan S.S., Williams G.A., Jaiswal A.K. Lignocellulosic Bio refineries in Europe: Current State and Prospects. Trends Biotechnol. (2018) doi: 10.1016/j.tibtech.2018.07.002.
11. Isupov M. N., Fleming T. M., Dalby A. R., Crowhurst G. S., Bourne P. C., Little child J. A. Crystal structure of the glyceraldehyde-3-phosphate dehydrogenase from the hyper thermophilica rchaeon *Sulfolobussol fataricus* . *Journal of Molecular Biology*. (1999); 291(3):651–660.
12. Jaenicke R., Böhm G. The stability of proteins in extreme environments. *Current Opinion in Structural Biology*. (1998);8(6):738–748.
13. Katchalski-Katzir E., Kraemer D.M. Eupergit C, a carrier for immobilization of enzymes of industrial potential. J. Mol. Catal. B. (2000); 10:157–176.
14. Kuhad R.C., Gupta R. and Singh A. (2011) Microbial cellulases and their industrial applications. Enzyme Res. (2011), 10.
15. Lamble H. J., Heyer N. I., Bull S. D., Hough D. W., Danson M. J. Metabolic pathway promiscuity in the Archaeon *Sulfolobussol fataricus* revealed by studies on glucose dehydrogenase and 2-Keto-3-deoxygluconate aldolase. *Journal of Biological Chemistry*. (2003);278(36):34066–34072.
16. Littlechild J., James P., Novak H., Sayer C. *Thermophilic Microbes in Environmental and Industrial Biotechnology, Biotechnology of Thermophiles*. 2nd. Springer; 2013.
17. Martinez-Martinez M., Coscolin C., Santiago G., Chow J., Stogios P.J., Bargiela R., Gertler C., Navarro-Fernandez J., Bollinger A., Thies S., et al. Determinants and Prediction of Esterase Substrate Promiscuity Patterns. ACS Chem. Biol. (2018);13:225–234. doi: 10.1021/acschembio.7b00996. [PubMed] [CrossRef] [Google Scholar]
18. Polizzi K.M., Bommarius A.S., Broering J.M., Chaparro-Riggers J.F. Stability of biocatalysts. Curr.Opin. Chem. Biol. (2007);11:220–225.
19. Raveendran S., Parameswaran B., Ummalyma S.B., Abraham A., Mathew A.K., Madhavan A., Rebello S., Pandey A. Applications of Microbial Enzymes in Food Industry. Food Technol. Biotechnol. (2018);56:16–30.
20. Ravindran R., Jaiswal A.K. Exploitation of Food Industry Waste for High-Value Products. Trends Biotechnol. (2016);34:58–69.
21. Reetz M.T., Carballeira J.D., Vogel A. Iterative saturation mutagenesis on the basis of B factors as a strategy for increasing protein thermostability. Angew. Chem. Int. Ed. (2006); 45:7745–775.
22. Russell N. J. (1998). Molecular adaptations in psychrophilic bacteria: potential for biotechnological applications. Adv. Biochem. Eng. Biotechnol. 61, 1–21.
23. Sangeetha R., Arulpandi I., Geetha A. Bacterial lipases as potential industrial biocatalysts: An overview. Res. J. Microbiol. (2011);6:1–24. doi: 10.3923/jm.2011.1.24
24. Siebers B, Schönheit P. Unusual pathways and enzymes of central carbohydrate metabolism in Archaea . CurrOpinMicrobiol. (2005);8:695–705.
25. Siebers B., Schönheit P. Unusual pathways and enzymes of central carbohydrate metabolism in Archaea. *Current Opinion in Microbiology*. (2005);8(6):695–705.
26. Straath of A.J.J., Panke S., Schmid A. The production of fine chemicals by biotransformations. Curr. Opin. Biotechnol. (2002);13:548–556.
27. Toogood H. S., Hollingsworth E. J., Brown R. C., et al. A thermostable L-aminoacylase from *Thermococcuslitoralis*: cloning, overexpression, characterization, and applications in biotransformations. *Extremophiles*. (2002);6(2):111–122
28. Tyler J., Simurdiak M.R., Zhao H. Biocatalysis. In: Lee S., editor. Encyclopedia of Chemical Processing. Volume 1. CRC Press; London, UK: (2006). pp. 101–110.
29. Vihinen M., Mantasala P. (1989). Microbial amylolytic enzymes. Crit. Rev. Biochem. Mol. Biol. 24, 329–418.
30. Zhang J., Wang X., Huo D., Li W., Hu Q., Xu C., et al. (2016). Metagenomic approach reveals microbial diversity and predictive microbial metabolic pathways in Yucha, a traditional Li fermented food. *Sci. Rep.* 6:32524. 10.1038/srep32524

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