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Isolation, Biochemical Characterization and Production of Immobilised β-Amylase Chitosan Beads Using Bacteria from Waste Water Effluents for its Industrial Production Aspect

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Authors' contributions

This work was carried out in collaboration among all authors. Author JC designed the study, wrote the protocol and performed the analysis. Authors PS and VS managed the analyses of the study. Author NS managed the literature searches and guided in the paper writing. All authors read and approved the final manuscript.

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ABSTRACT

Aims: An amylase, the enzyme that catalyses the hydrolysis of starch into sugars are produced in both animals and plants and as well as in some bacteria also. Nowadays, the use of amylase enzyme in different industrial sectors and particularly in controlling the industrial water pollution has been increased as this enzyme is effective against different types of industrial effluents such as wastes from dairies, confectionaries, municipal wastes, bakery and so on which are expelled out in water without giving any proper treatment. As, the production of synthetic amylase enzyme for this purpose is quite costly, in this study, the waste water effluent was used to isolate the amylase producing bacteria, hence, decreasing the cost.
Methodology: The samples were taken from drains coming from bakery, municipal waste, etc, and five bacterias were isolated at dilution 10^{-6} which were named accordingly as Tan1, Tan2, Tan3, Tan4 and Tan5 further followed by Gram staining and biochemical characterization tests for further confirmation of amylase producing bacteria followed by the immobilisation of the β-amylase enzyme produced by the bacteria.

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Results and Conclusion: Two bacterias were identified as amylase producing, Tan1 and Tan2, in which Gram positive bacteria showed higher amylase production at 30°C(Tan1). Compared to the free β-amylase, the immobilised β-amylase enzyme showed broader pH and temperature ranges, enhanced thermal stability, better storage stability, reusability and higher accessibility of the substrate to the immobilised β-amylase. Improved activity recovery and enzymatic properties of immobilised β-amylase chitosan beads in present study holds a promising future in industrial applications.

Keywords: Amylase; chitosan beads; immobilisation; waste water effluents; high production; high stability.

1. INTRODUCTION

Enzymes are proteins that act as biological catalysts (biocatalysts). Catalysts act as accelerators in chemical reactions. The molecules upon which enzymes may act are called substrate, and the enzyme converts the substrates into different molecules known as products. Almost all metabolic processes in the cell need enzyme catalysis in order to occur at rates fast enough to sustain life [1]. Metabolic pathways depend upon enzymes to catalyze steps. Like all catalysts, enzymes increases the reaction rate by lowering its activation energy. Some enzymes can make their conversion of substrate to product occur many millions of times faster and an extreme example is orotidine 5'-phosphate decarboxylase, which allows a reaction that would otherwise take millions of years to occur in milliseconds [2,3]. Enzymes are used in many industrial applications especially when extremely specific catalysts are required.Enzymes in general are limited in the number of reactions they have evolved to catalyze and also by their lack of stability in organic solvents and at high temperatures. As a consequences, protein engineering is an active area of research and involves attempts to create new enzymes with novel properties, either through rational design or in vitro evolution [4,5].

These efforts have begun to be successful and a few enzymes have now been designed "from scratch" to catalyze reactions that do not occur in nature [6]. An amylase is such an enzyme, that catalyses the hydrolysis of starch into sugars. It is present in both animals and plants and in some bacteria also. Alpha and Beta amylases are important in brewing beer and liquor made from sugars derived from starch. β-amylase produces maltose from starch by hydrolyzing the α-1,4-glucan linkages. β-amylase has been used for various research and industrial applications [7]. β-amylase is arguably the most important enzyme as it cleaves two bound glucose molecules(maltose) from the reducing end of the chain [8]. However, thermostable forms have been identified [9,10], which allows additional activity at higher temperatures [11].

In this study, the immobilised β-amylase was formed by using the amylase producing bacteria in a chitosan beads.

2. MATERIALS AND METHODS

2.1 Sample Collection

The sample were collected from the drains of dairy, bakery, municipal waste, in sterile plastic bags and bottles located near Vidyavihar Phase-1, Pathribagh Chowk, Dehradun, Uttarakhand, India.

2.2 Isolation of Bacterial Microbiota Effluent

Isolation of Bacteria from the Effluent was done with the help of the glasswares that washed by chromic acid prepared by the protocol given by Fillmore Freeman [12].

2.3 Isolation of Mixed Colony

After that the isolation of mixed colony was done in the Nutrient Agar Media(NAM) that was prepared by dissolving its entire component in 250 ml distilled water in 1000 ml Erlrnmeyer flask followed by autoclave at 15 lb pressure for 15 mins. Thereafter, pouring of the media was done on sterile autoclaved petriplates inside the laminar air flow cabinet and the plates were kept for few minutes for solidifying without disturbing it. Now, the spent wash was spread over NAM plates by spread plate method followed by serial dilution at 10^{-6} by serial dilution method. After this, the plates were incubated for 48 hrs. at 30°C.

2.4 Obtaining of Pure Cultures

The pure cultures were obtained after incubation, the mixed cultures from the NAM plates were

subcultured separately by using picking off technique, picking the single discrete colonies from the mixed culture with the help of sterile loop and then streaking these colonies on different NAM plates by sector plate method.

2.5 Colony Morphology of Pure Cultures

The colony morphology of the discrete colonies was made by plate's observation of the single colony in the NAM plate. The same method was used for different strains isolates.

2.6 Gram Staining of the Isolates

The isolates were further stained by Gram Staining method, the protocol given by Hans Christian, Gram [13] and slides were then observed under microscope.

2.7 Biochemical Tests

Different Biochemical Tests were performed for confirmation of the bacteria and they were performed accordingly. Oxidase Test: The oxidase test was performed according to the protocol given in American Society for Microbiology Manual [14].Amylase Test: The ability to degrade starch is used as a criterion for the determination of amylase production by a microbe. Starch in the presence of iodine produces a dark-blue coloration of the medium and a yellow zone around a colony in an otherwise blue medium indicates the amylolytic activity. It was done at 30°C temperature. Catalase Test: The test was performed according to the protocol given in American Society for Microbiology Manual [15]. Indole Test: The test was performed according to the protocol given in American Society for Microbiology Manual [16]. Citrate Utilization Test: The test was performed according to the protocol given in Bailey and Scott's Diagnostic Microbiology 10th Ed. [17]. Methyl Red and Voges-Proskauer Test: The test was performed according to the protocol given in American Society for Microbiology Manual [18].

2.8 Measurement of the Growth of the Isolates

The measurement of growth of the isolates was done in a nutrient broth which was prepared and then 0.5 ml of the 24 hrs old bacterial culture was inoculated in it. Thereafter, the bacterial growth measurement was carried out at an interval of 2 hrs. starting from the zero hrs., against uninoculated nutrient broth using spectrophotometric method at a wavelength of 600 nm. The growth was taken in terms of the absorbance.

2.9 Enzyme Assay

The estimation of enzyme assay was carried out at an interval of 2 hrs. 1ml of sample was taken in a test tube followed by its centrifugation to 10,000 rpm for 5 mins and the extracted supernatant was taken in a test tube followed by an addition of 0.5 ml of starch and then it was incubated for 3 mins. Thereafter, 1 ml of di-nitrosalicylic acid was added followed by the heating at 100°C for 5 mins. The tube was then allowed to cool down and measured at the wavelength of 540 nm.

2.10 Partial Purification of the Enzyme

The partial purification of enzyme was done after 48 hrs. growth of the culture, it was centrifuged at 10,000 rpm for 20 mins followed by the collection in a flask and then the addition of ammonium sulphate. Thereafter, the supernatant was kept on the stirrer overnight and next day, it was again centrifuged at 10,000 rpm for 15-20 mins. After this, the pellet was moved out in a separate eppendorf tube followed by the pouring in the column of silica gel. The phosphate buffer was then poured and the enzyme was isolated at the bottom of the column. At last, it was put on the hot plate for a while which allowed the formation of crystals of partially purified enzyme.

2.11 Formation of Immobilised β-amylase Chitosan Beads

Formation of Immobilised β-amylase Chitosan Beads in which the pre-treated chitosan beads were prepared based on the reported method with minor modifications [19]. 2 gm of chitosan was dissolved in 100 ml of acetic acid solution(5.0%,w/v). The solution was added to 200 ml of sodium hydroxide solution(2.0 M) containing 40 ml of ethanol through a needle(0.7 mm) at room temperature resulting in the instant formation of chitosan gelled spheres.The immobilisation of β-amylase in chitosan beads was done sccording to the protocol given by Cetinus and Oztop (Senay Akkus Cetinus, Hesna Nursevin Oztop, Dursun Saraydin).

3. RESULTS

3.1 Partial Purification and Immobilisation of β-Amylase Enzyme in Chitosan Beads

Partially purified enzyme formed as powdery crystal further immobilised in chitosan beads. After hardening, the beads with the immobilised amylase enzyme were separated and washed with water. The diameter of the beads was approximately 1.5-1.8 mm.

4. DISCUSSION AND CONCLUSION

In Table 1, we could observe the different morphology of the isolates which were isolated from the waste water effluent. After that only two strains, that is, Tan1 and Tan2 were taken for Gram staining in which Tan1 was found to be Gram positive and Tan2 as Gram negative. Thereafter, only Tan1 was taken for further study. The growth of the strain Tan1 showed sigmoid curve on the graph and this growth kept on increasing till it reached the mid log phase after 20 hrs and continuously kept on increasing till 40 hrs. of incubation to late log phase. The early stationary phase was achieved after 45 hrs of incubation, the groeth was studied in starch broth media. Maximum bacterial growth was found on 40 hrs and can be seen in Fig. 1. In the amylase activity test we detect that the particular microorganism is capable of utilizing starch. Amylase enzyme was produced maximum at 28 hrs. which could be seen in Fig. 2. Waste water could be used to produce β-amylase enzyme, work done in β-amylase production from packaging-industry wastewater using a novel strain *Paenibacillus chitinolyticus CKS 1* [20].

Table 1. The observed morphology of 5 strains Tan1, Tan2, Tan3, Tan4 and Tan5 bacteria

Strain	Shape	Chromogenesis	Opacity	Texture	Surface
Tan 1	Irregular	White	Opaque	Mucoid	Smooth
Tan 2	Irregular	Pale white	Opaque	Mucoid	Smooth
Tan 3	Round	Yellow	Opaque	Butyrous	Smooth
Tan4	Irregular	White	Translucent	Mucoid	Smooth
Tan 5	Round	Yellow	Opaque	Viscoid	Smooth

After this result, only strain Tan1 and Tan2 were taken for Gram staining

Table 2. The result of Gram staining of two strains Tan1 and Tan2

Strain	Shape	Colour	Conclusion
Tan1	Rod	Purple	Gram positive, bacilli
Tan2	Round	Pink	Gram negative, cocci

Only Tan1 strain was taken for further tests

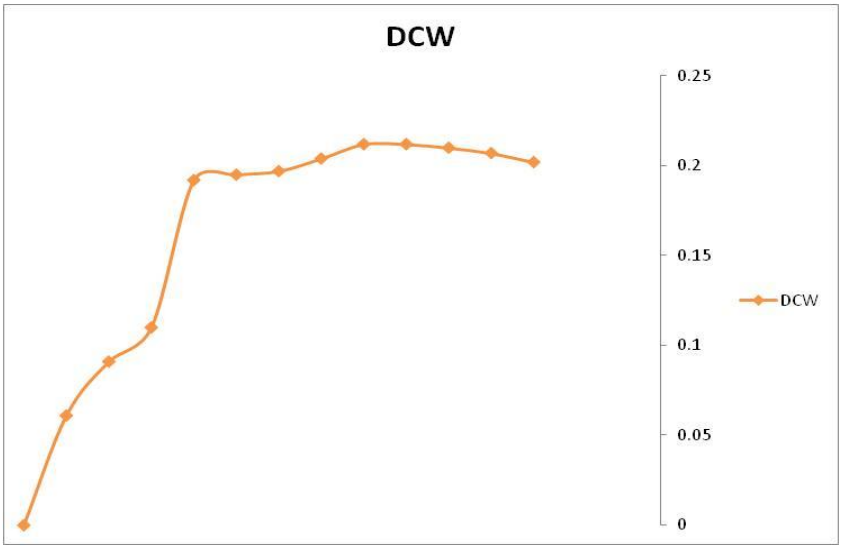

Fig. 1. Measurement of growth curve of strain Tan1

Table 3. The result of biochemical test for Tan1 strain of bacteria

S.No.	Biochemical Test	Result
1.	Amylase Test	Positive
2.	Oxidase Test	Positive
3.	Catalase Test	Positive
4.	Indole Test	Negative
5.	Citrate Utilization Test	Positive
6.	Methyl Red and Voges-Proskauer Test	Positive

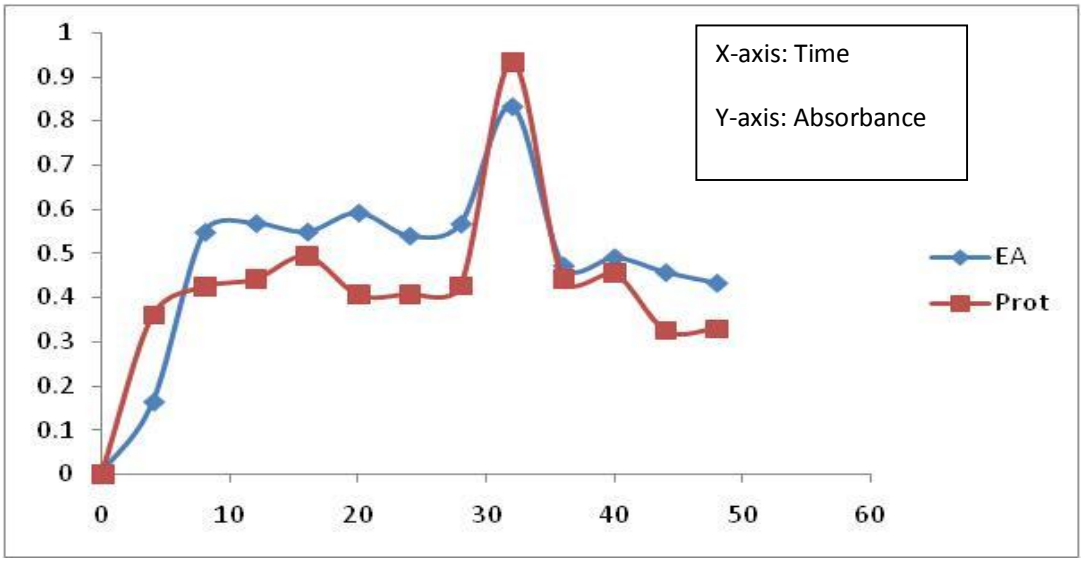

Fig. 2. Enzyme assay estimation

It could be concluded from this experiment that the amylase producing bacteria isolated from the waste water effluents showed high amount of amylase enzyme production, i.e., Tan1 strain, which was found more efficient and was highly stable under wide range of conditions. The result suggested that the enzyme isolated from the isolate could have a promising application in waste water treatment of various industrial effluents. Compared to the free β-amylase, the immobilised enzyme exhibited broader pH and temperature ranges, enhanced thermal stability, better storage stability and reusability and higher accessibility of the substrate to the immobilised β-amylase. Improved activity recovery and enzymatic properties of immobilisedβ-amylase in present study holds a promising future in various industrial applications.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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