iJRASET
International Journal For Research in Applied Science and Engineering Technology

INTERNATIONAL JOURNAL FOR RESEARCH

IN APPLIED SCIENCE & ENGINEERING TECHNOLOGY

Volume: 5 **Issue:** XII **Month of publication:** December 2017

DOI:

www.ijraset.com

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International Journal for Research in Applied Science & Engineering Technology (IJRASET)
ISSN: 2321-9653; IC Value: 45.98; SJ Impact Factor :6.887
Volume 5 Issue XII December 2017- Available at www.ijraset.com

Improvement of the Stability and Activity of Aspergillus oryzae α-Amylase Enzyme using Natural Matrices

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Abstract: ***The aim of the present study is to improve the stability and the activity of some industrial enzymes using natural matrices. It is clearly seen that the activity of the free amylase enzyme is much lesser than the immobilized enzyme. Among the immobilized matrices, enzyme immobilized coconut fiber shows higher activity compared to the other two matrices. The study also proved that the enzyme immobilized natural matrices shows much higher activity than the calcium alginate matrice that is most frequently used for enzyme immobilization in various industries. When compared with the other natural matrices used in the study the protein micro particle immobilized amylase shows lesser activity but the study has shown that protein micro particles do stably adsorb amylase and therefore can be used for enzyme immobilization. The optimum pH for amylase immobilized on coconut fiber and egg shells was found to be pH 8.2 ,which clearly shows that after immobilization the optimum pH has shifted towards the alkaline range at pH 8.2 from acidic range of pH 4.0-5.0 of free α- amylase from A. oryzae. The optimum temperature for amylase immobilized on coconut fiber and egg shells was found to be 100 ℃ which was much higher than the free amylase enzyme (50-55℃). The enzyme immobilized on coconut fiber is much more stable at 100℃ than that on the egg shells. This shows that coconut fiber is much more stabilizing matrice than egg shells. The higher activity of the immobilized enzyme after glutaraldehyde treatment on both coconut fiber as well as egg shells in this study supported the fact that glutaraldehyde is used in enzyme immobilization as a cross linker which increases enzyme activity by increasing bonding between enzyme molecules as well as the enzyme and the matrix. When used for clarification of apple juice the glutaraldehyde treated enzyme immobilized coconut fibermatrice showed maximum clarification. These matrices can therefore prove to be eco-friendly, cost efficient matrices for many industrial processes.***

Keywords: α-amylase, Immobilization, coconut fiber, egg shells, enzyme activity

I. INTRODUCTION

Enzymes are being used in numerous new applications in the food, feed, agriculture, paper, leather, and textiles industries, resulting in significant cost reductions. At the same time, rapid technological developments are now stimulating the chemistry and pharma industries to embrace enzyme technology, a trend strengthened by concerns regarding health, energy, raw materials, and the environment [1].The growing use of industrial enzymes is dependent on constant innovation to improve performance and reduce cost. This innovation is driven by a rapidly increasing database of natural enzyme [2].Enzyme immobilization provides an excellent base for increasing availability of enzyme to the substrate with greater turnover over a considerable period of time. Several natural and synthetic supports have been assessed for their efficiency for enzyme immobilization. Nowadays, immobilized enzymes are preferred over their free counterpart due to their prolonged availability that curtails redundant downstream and purification processes[3].

Although there are several studies on immobilization of amylase enzymes, very little progress has been made in the use of immobilized enzyme processes in industry because of the high cost of carriers used for enzyme immobilization. To overcome this drawback, natural matrices such as egg shells, coconut fibers and protein microparticles were investigated in this study for use in enzyme immobilization. Immobilization of laccase in green coconut fiber and its subsequent use in clarification of apple juice and textile dyes decolourization has been reported [4, 5]. Egg shell as a carrier for enzyme immobilization has also been reported previously [6]. However, the use of protein microparticles for enzyme immobilization has been investigated for the first time in this study.

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II. MATERIALS AND METHODOLOGY

A. Sample Collection

Eggshells were collected from the local market of Chennai and coconut fibers were collected from kitchen garden.

B. Sample Preparation:

Eggshells were washed and the egg shell membrane was removed manually, then was broken down into small pieces. Coconut fibers were separated into single strand from the bunch and cut into small pieces of approximately 2 cm. The samples were sterilized at 120°C for 45 min at 15lbs.Protein microparticles were prepared using desolvation technique [7]. 400 mg of BSA (Bovine Serum Albumin) was dissolved in 4 ml of 10 mMNaCl solution, then titrated to pH 7.5-9.0 and under constant stirring desolvation of BSA solution was achieved by drop wise addition of ethanol. Ethanol was added drop wise until the solution became turbid. After the desolvation process, 470 µl of 8% glutaraldehyde in water was added to induce particle cross linking. Cross linking process was performed under stirring of the suspension over a time period of 24 h. The resulting microparticles were purified by 3 cycles of centrifugation and redispersion of the pellet to the original volume in 10 mMNaCl at pH 7.5-9.0 respectively. The particle size was measured using Zetasizer Nano Z instrument.

C. Enzyme Preparation

Commercially available α-amylase enzyme (*Aspergillusoryzae)* from Himedia was used for enzyme preparation. 1mg of the enzyme was dissolved in 1ml of the phosphate buffer. This was the concentration of enzyme used throughout the experiment.

D. Enzyme Immobilization

The matrices and the enzyme solution were prepared. 1 g of the matrices wasweighed and 10 ml enzyme solution was added for the matrices to be fully immersed in the enzyme. The matrix-enzyme complex was then kept for overnight incubation at 37°C. Next day the unbound enzyme solution was poured off and the immobilized enzyme matrices were ready to use.

E. Monitoring The Progress Of The Free And Immobilized Amylase Enzyme Reaction Over A Time Period

Enzyme assays were performed according to according to Miller [8]. 1% Corn starch solution which acts as the substrate for the amylase enzyme was prepared and added to the immobilized matrices and the sample was collected at a regular intervals of 20 minutes for 8 cycles. 1ml of distilled water and 1ml of DNSA (Dinitrosalicylic acid) was added to the product and was kept in boiling water bath for 20 min. Absorbance of the solution was measured at 540nm.

F. Activity Of Free And Immobilized Enzymes

100 mg of the matrices (egg shells, coconut fiber, protein microparticles) was prepared in 1ml of the enzyme solution (1 mg/ ml) and was incubated overnight. The unbound enzyme solution was poured off.

The enzyme was also immobilized in calcium alginate beads. For preparing the enzyme immobilized beads, 10 mg of α- amylase enzyme powder was added to 10 ml of 3% sodium alginate solution. 50 ml of 0.2M calcium chloride solution was prepared and kept in deep freezer . Calcium alginate beads were prepared by adding sodium alginate, drop wise into ice cold 0.2M calcium chloride solution. The beads were incubated in ice cold calcium chloride solution to harden. 100 mg of the beads were weighed and transferred to a sterile test tube.5 ml of 1% Corn starch solution was added to all the immobilized matrices and 1 ml of free enzyme (1 mg / ml). The tubes were incubated for 15 min at room temperature and 1 ml of distilled water and 1ml of DNSA (Dinitrosalicylic acid) was added to the product and was kept in boiling water bath for 20 min. Absorbance of the solution was measured at 540nm.

The activity of the free and immobilized enzyme was calculated using the formula given below :

$$\text{Free enzyme (U)} = \frac{\text{Value from standard maltose graph}}{\text{mg of amylase X incubation time}}$$

$$\text{Immobilized enzyme(U)} = \frac{\text{Value from standard maltose graph}}{\text{mg of support X incubation time}}$$

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U is the unit of enzyme activity. It is the amount of enzyme that catalyses the reaction of 1µmol of substrate per minute.

G. Activity Of Immobilized Enzymes At Different Ph And Temperature

100 mg of the immobilized matrices was prepared. Phosphate buffers with different pH (5.8,6.4,7.8,and 8.2) were added along with 1% Corn starch and incubated for 20 min at room temperature. Colorimetric assay was performed with DNSA as previously. Absorbance was measured at 540nm.100 mg of immobilized matrices was prepared in phosphate buffer pH 8.2. The matrices along with 1% Corn starch solution was kept in three different temperatures (-4°C,37°C,100°C), and were incubated for 20 min. Colorimetric assay was performed with DNSA as previously. Absorbance was measured at 540nm.

H. Activity Of Immobilized Enzyme After Glutaraldehyde Treatment

100 mg of the immobilized matrices were prepared. 1 ml of 8% glutaraldehyde was added to the matrices and was incubated for 2 h at room temperature. Glutaraldehyde was used as a cross linking agent. After incubation the glutaraldehyde solution was poured off. 1% Corn starch and incubated for 20 min at room temperature. Colorimetric assay was performed with DNSA as previously. Absorbance was measured at 540nm.

I. Starch Agar Assay

Starch agar was prepared by using commercially available starch agar medium from Himedia. 3 g of starch agar was dissolved in 100 ml distilled water and autoclaved. The agar was poured into the sterile petriplates and was allowed to solidify. Enzyme immobilized matrices were prepared and was placed over the starch agar and was incubated over night at 37°C. Sterile coconut fiber and egg shells were used as control. Sterile disc loaded with enzyme was also placed on the plate. After overnight incubation Lugol's iodine solution was poured onto the starch plates and was kept for some time and the plates were observed.

J. Clarification Of Fruit Juices Using Immobilized Enzyme

100 mg of the matrices (coconut fiber egg shell and glutaraldehyde treated coconut fiber and egg shell) were prepared in a sterile test tube. 5ml of apple juice was added to the immobilized matrices and was incubated for 2 h at 50°C in water bath. At the end of incubation the temperature was increased to 100°C and maintained for 5 min and was cooled to normal temperature. Then it was centrifuged at 5000 rpm for 20 min and the supernatant was collected. The supernatant was analyzed for clarity (%T 660nm) , colour (absorbance at 400 nm). Free enzyme was used as the control. The control was prepared by adding 1 ml of the free enzyme to 5 ml of the apple juice and proceeded as mentioned above for the other samples.

III. RESULT AND DISCUSSION

A. Sample preparation

Eggshells and coconut fibers were prepared as mentioned previously and protein microparticles prepared using desolation technique is shown in Fig.1. The microparticle size statistics intensity report is shown in Fig.2 and the size distribution by intensity report is shown in Fig.3. As per these reports the average protein microparticles size 1202nm.

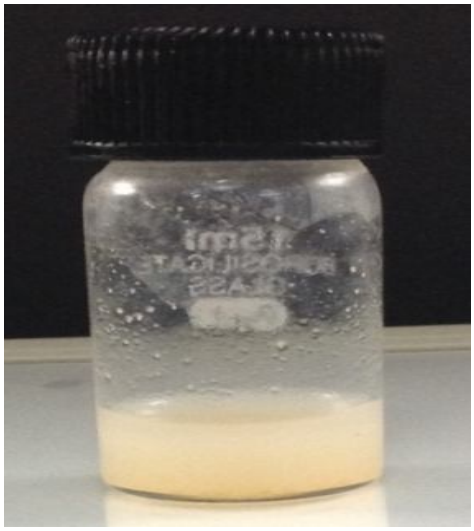

Fig.1 Protein microparticles

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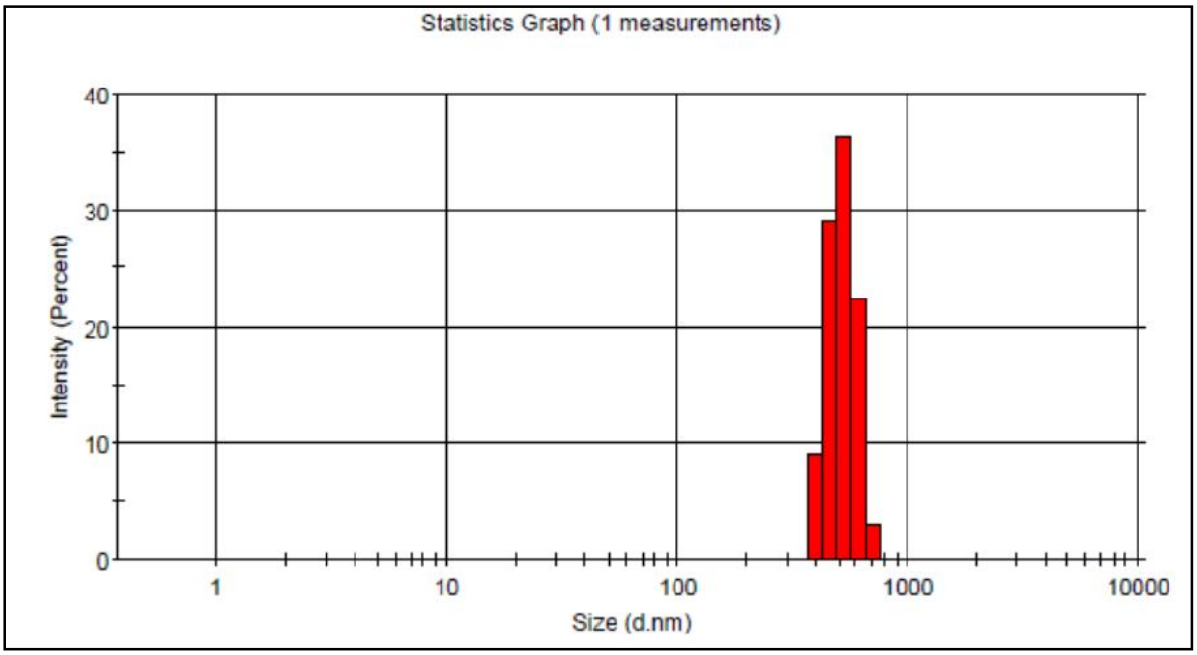

Fig.2 Size statistics intensity

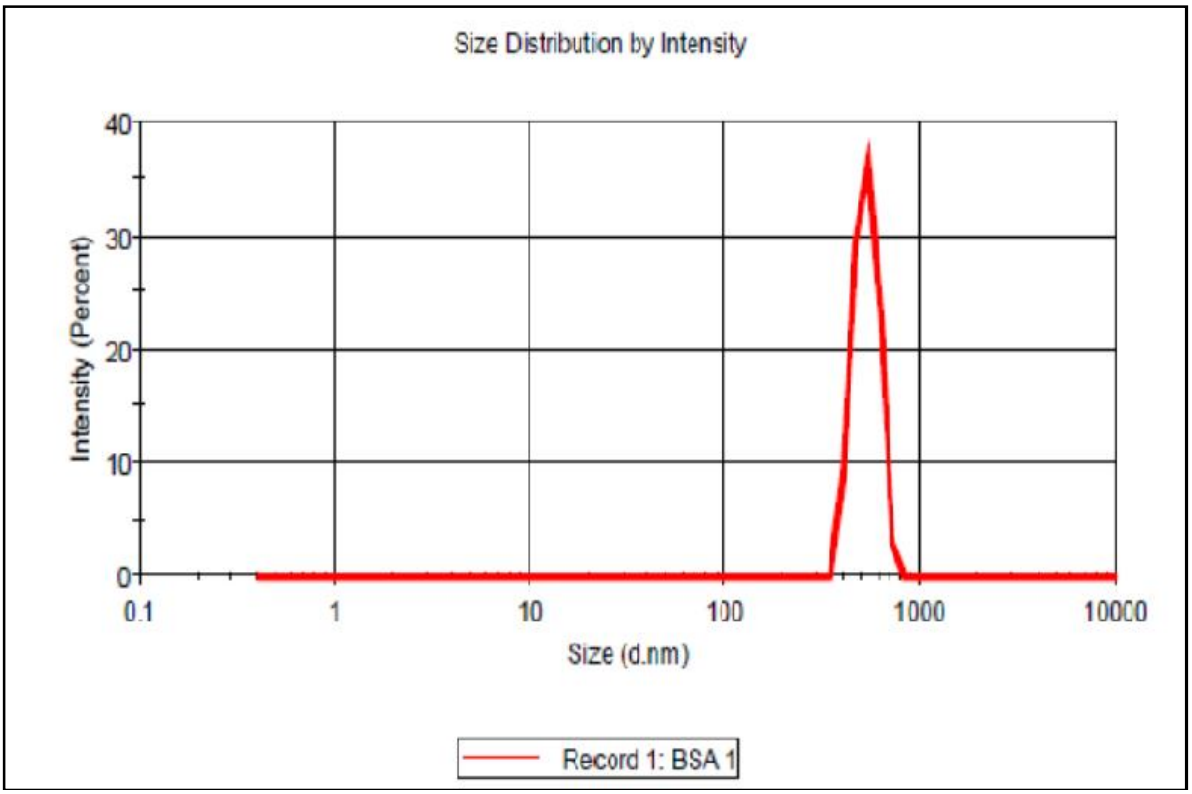

Fig.3 Size distribution by intensity

B. Monitoring The Progress Of The Free And Immobilized Amylase Enzyme Reaction Over A Time Period

Figs.4, 5, and 6 shows the progress of the enzyme reaction over a time period of 2 h and 40 min. In the case of the free enzyme as well as the immobilized matrices enzyme activity reached a constant after 80 min, indicating saturation of the enzyme.

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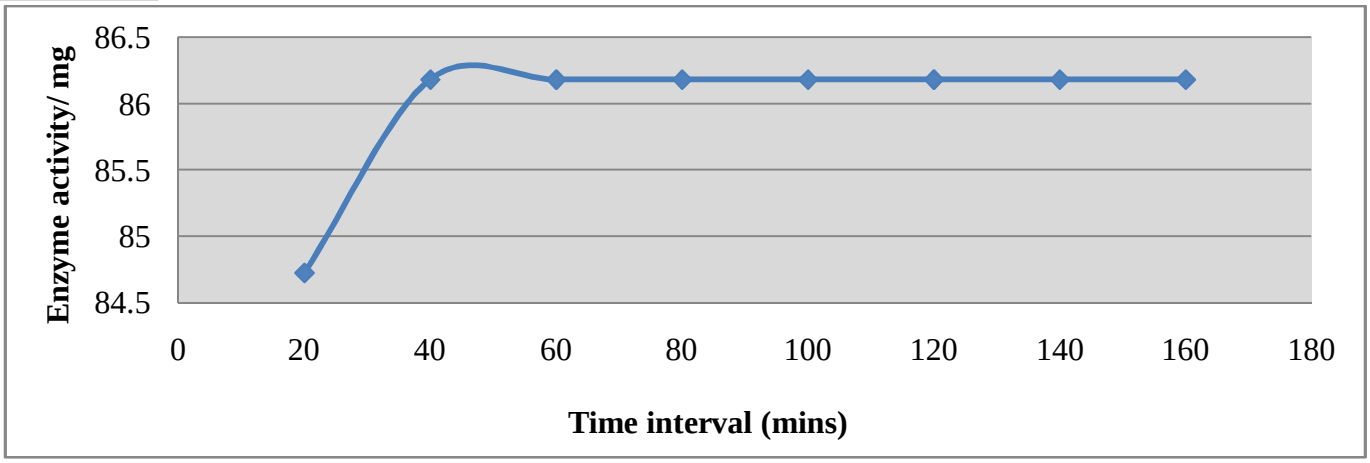

Fig. 4 Free amylase enzyme

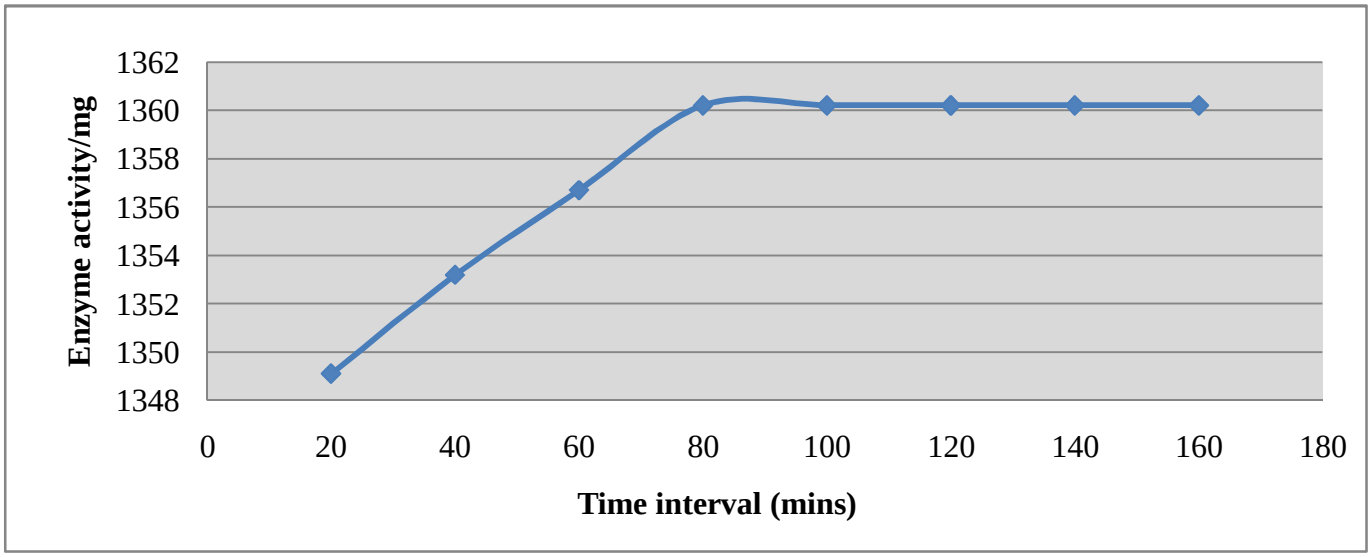

Fig. 5 Amylase immobilized coconut fiber

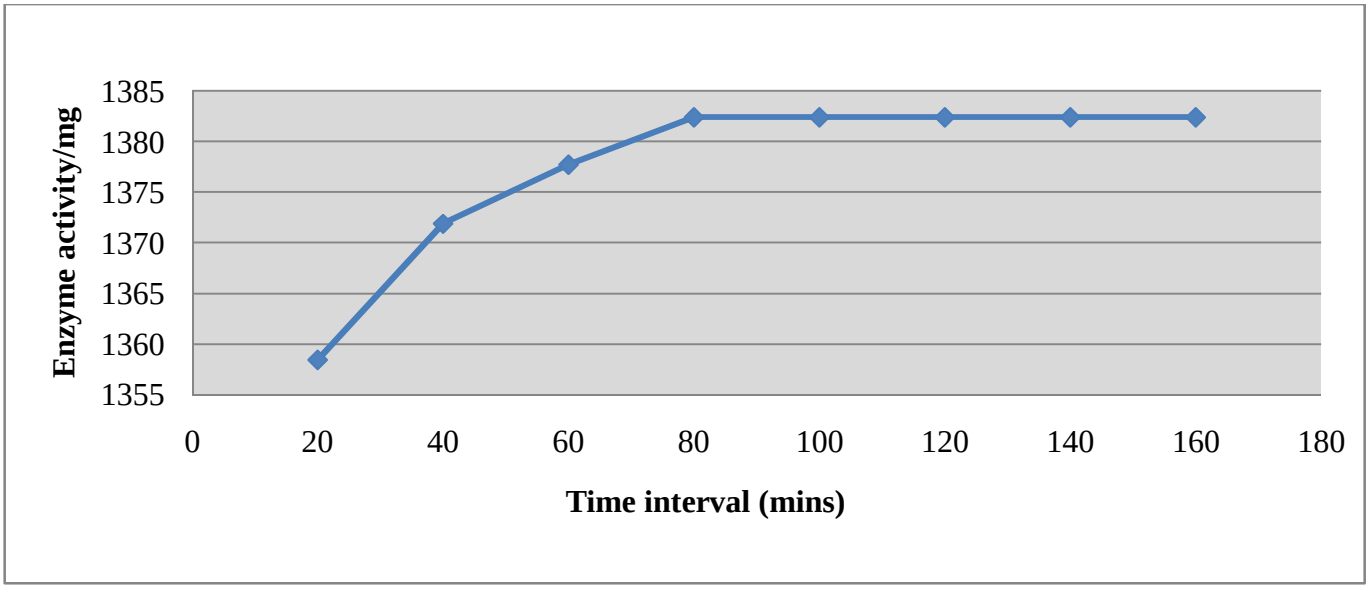

Fig. 6 Amylase immobilized egg shells

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C. Activity of free and immobilized enzyme

The enzyme activities expressed as enzyme units per mg of matrice for free as well as immobilized enzymes (egg shells, coconut fiber and calcium alginate) is represented in Fig. 7. It is clearly seen that the activity of the free amylase enzyme is much lesser than the immobilized enzyme. Among the immobilized matrices, enzyme immobilized coconut fiber shows higher activity compared to the other two matrices. Calcium alginate is a matrice that is most frequently used for enzyme immobilization in various industries and therefore it has been used as a comparative reference for other matrices[9]. As per the results obtained enzyme immobilized coconut fiber and immobilized egg shells is much better matrice than calcium alginate, as there is a 132.048 and 50.25 units (per mg of matrice) increase in enzyme activity when it is immobilized on coconut fiber and immobilized egg shells as compared to calcium alginate.

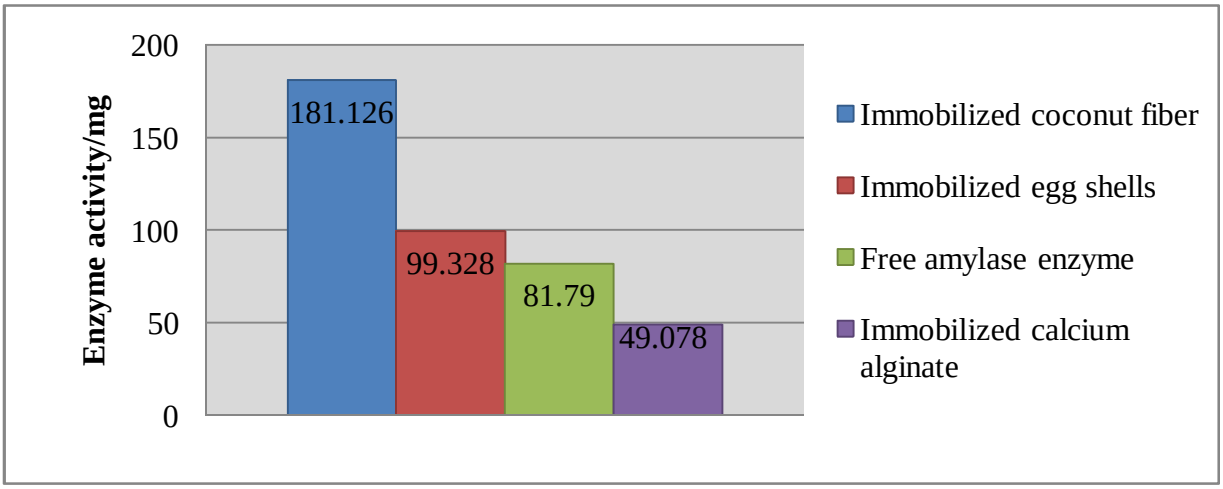

Fig. 7 Activity of free and immobilized enzymes

The enzyme activity of the enzyme immobilized protein microparticles was much lower than that of the free enzyme (Fig. 8). When compared with the other natural matrices used in the study the protein microparticle immobilized amylase shows lesser activity. However since the concentration of the protein microparticles may not be sufficient as or equivalent to the amount of natural matrices used enzyme activity on these microparticles may be seen to be lower. For this reason the concentration of the protein microparticles must be standardised to obtain enzyme activities equal to those of the other matrices. However this study has shown that protein microparticles do stably adsorb amylase and therefore can be used for enzyme immobilization. Further studies are required to study the effect of microparticle size on enzyme stability and activity.

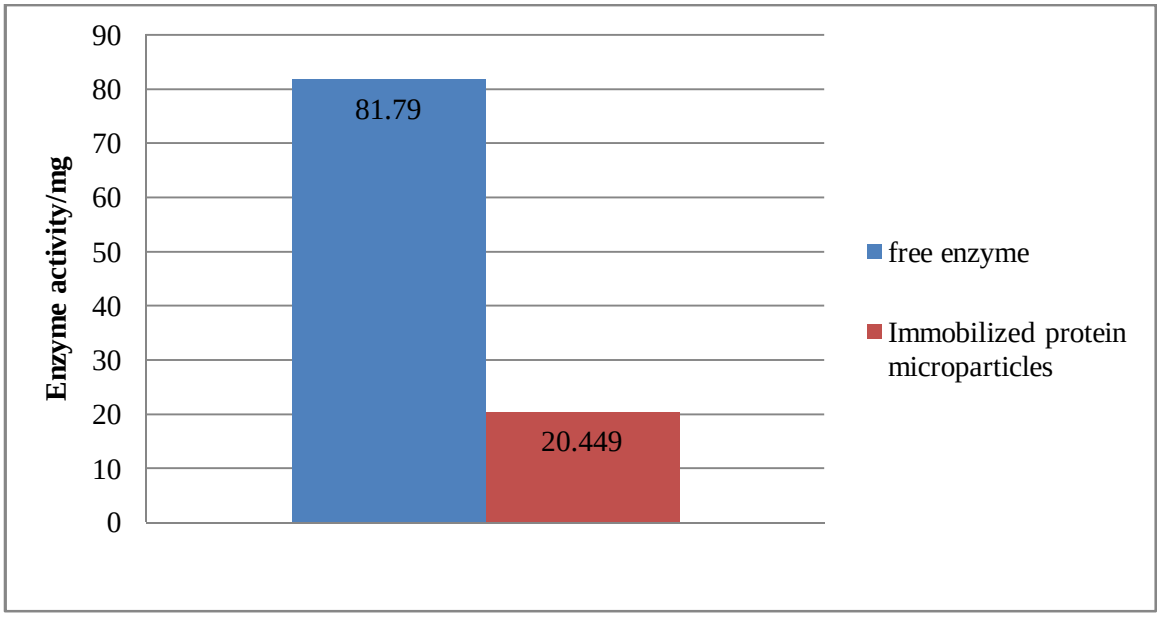

Fig. 8 Activity of free and immobilized protein microparticles

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D. Activity Of IMMOBILIZED Enzyme At Different Ph

The optimum pH for amylase immobilized on coconut fiber and egg shells was found to be pH 8.2 as shown in Fig. 9. The pH range for free α- amylase from A.oryzaeis between pH 4.0- 5.0.After immobilization, it can be clearly seen that the optimum pH has shifted towards the alkaline range at pH 8.2.

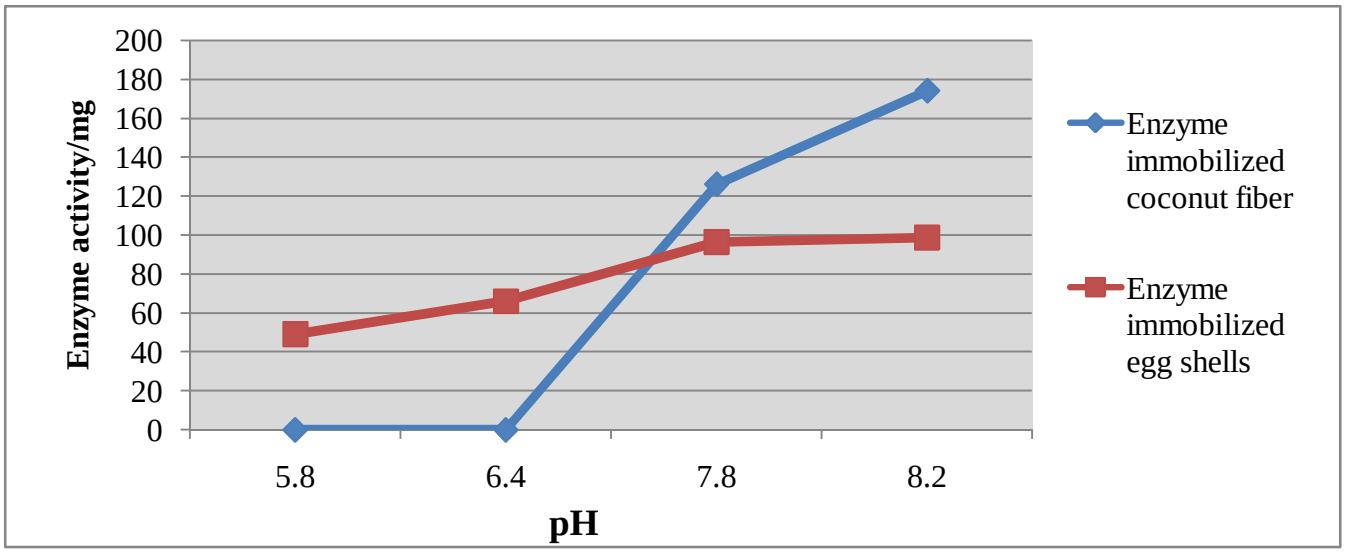

Fig. 9 Activity of immobilized enzymes at different pH

E. Activity Of Immobilized Enzymes At Different Temperature

The optimum temperature for amylase immobilized on coconut fiber and egg shells was found to be 100 °C as shown in Fig. 10.The temperature range for free α- amylase from *A.oryzae* is between 50-55°C. After immobilization, it can be clearly seen that there is a significant increase in the optimum temperatures of both the enzyme immobilized coconut fiber as well as egg shells, showing the highest enzyme activity of 838.44 and 436.45 units/mg, respectively, at a high temperature of 100°C. The enzyme immobilized on coconut fiber is much more stable at 100°C than that on the egg shells. This shows that coconut fiber is much more stabilizing matrice than egg shells. This increase in optimum temperature is much favourable for many industrial processes such as fruit juice clarification which require the use of high temperatures.

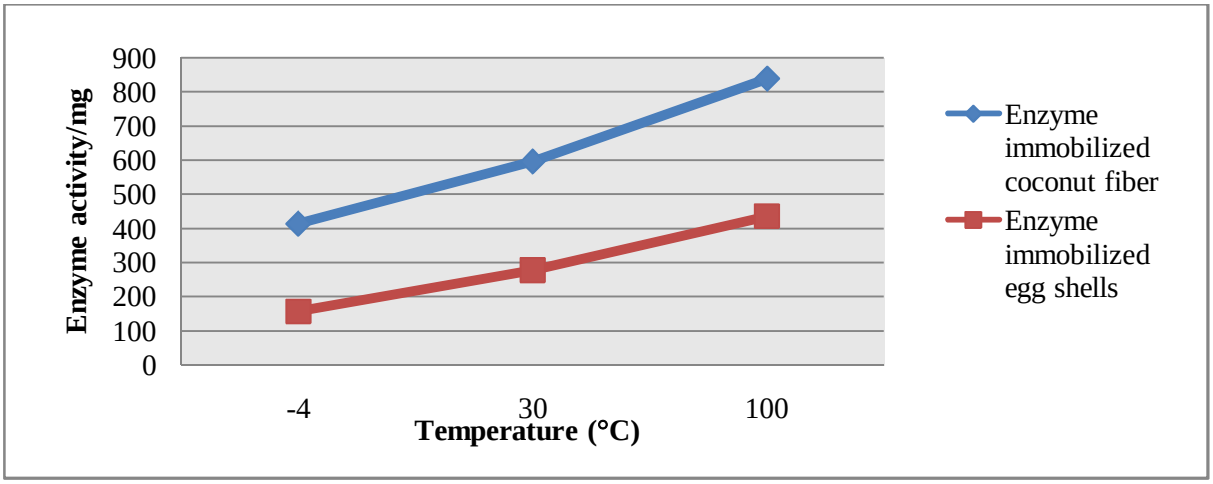

Fig. 10 Activity of immobilized enzymes at different temperature

F. Activity Of Immobilized Enzyme After Glutaraldehyde Treatment

The activity of the immobilized enzyme after glutaraldehyde treatment in the case of both coconut fiber as well as egg shells was found to be much higher than the immobilized enzymes that were not cross linked by glutaraldehyde (Fig. 11). Glutaraldehyde is often used in enzyme immobilization as a cross linker. There are several reports [10, 11] that show that the glutaraldehyde treatment

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significantly increases enzyme activity by increasing bonding between enzyme molecules as well as the matrix. The results obtained in this study also support this fact.

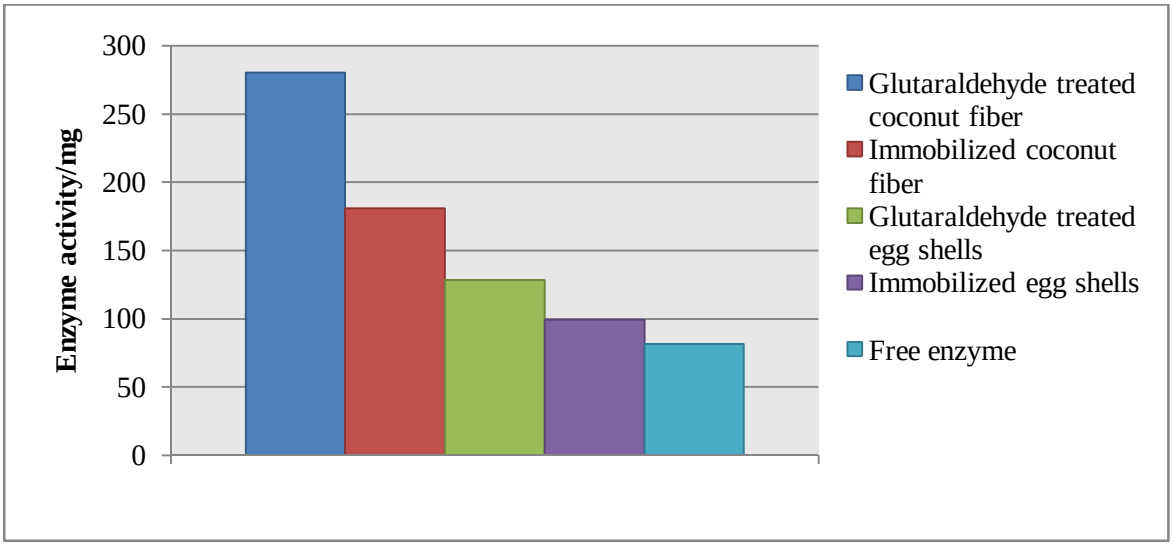

Fig.11 Activity of immobilized enzyme after glutaraldehyde treatment

G. Starch Agar Assay

As shown in Figs. 12,13 and 14, clear zones were formed around the immobilized matrices and the sterile disc loaded with enzyme.This indicates that the enzyme has been immobilized on the matrices and has retained its activity. The zones of clearance that were formed were large and visible. The maximum zone of clearance was seen for the enzyme immobilized coconut fiber. As expected the control matrices did not show any zone of clearance. These matrices can thus prove to be eco-friendly cost efficient matrices for many industrial processes as reported in similar research work earlier [12].

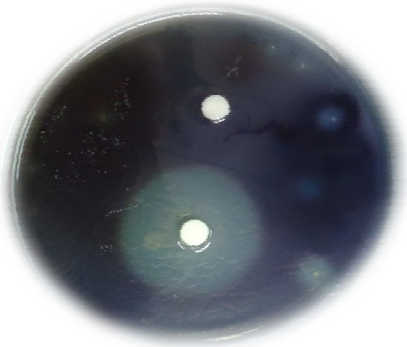

Fig. 12 Sterile disc with free enzyme

Fig. 13 Immobilized coconut fiber

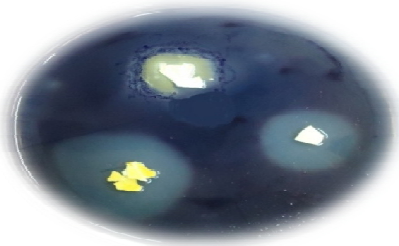

Fig. 14 Immobilized egg shells

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H. Clarification Of Fruit Juice Using Immobilized Enzymes

As seen in Table 1 the maximum clarification of apple juice was seen in the case of glutaraldehyde treated coconut fiber and egg shells which showed 51.5% and 50.8% transmittance respectively. The percentage transmittance in the case of other matrices was surprisingly lower than even the control (pure apple juice). A reason for this may be the release of some components of the fiber or the egg shells which made the apple juice more translucent. The treatment with glutaraldehyde on the other hand would have stabilized the matrice components by cross linking. The glutaraldehyde treated matrices showed almost double the percentage transmittance of the pure enzyme, which further proves that glutaraldehyde treatment stabilizes the enzyme and increases its activity. This study therefore shows that glutaraldehyde treated coconut fiber and egg shell matrices can be efficiently used for apple juice clarification. Of these two matrices the glutaraldehyde treated coconut fiber shows slightly better clarification than glutaraldehyde treated egg shells.

Sample	Transmittance (%)	Absorbance (450nm)
Control	28.2	1.068
Free enzyme	27.8	0.853
Enzyme immobilizedcoconut fiber	22.4	1.041
Enzyme immobilizedegg shells	21.4	1.000
Glutaraldehyde treatedimmobilized coconut fiber	51.5	1.772
Glutaraldehyde treatedimmobilized egg shells	50.8	1.780

Table 1: Clarification of fruit juice

IV. CONCLUSION

The study therefore proves that the enzyme immobilized natural matrices show better activity and stability than the free amylase enzyme. The natural enzyme immobilized matrices showed optimum activity at alkaline pH and temperature,which proved that these matrices can be used for many industrial purposes that require stability at high temperature and pH.Among the two natural matrices enzyme immobilized coconut fiber showed higher activity throughout the study and this shows that coconut fiber is much more stabilizing matrice than egg shells. This study also showed that the natural enzyme immobilized matrices were much effective than the calcium alginate matrice that is often used in industries for enzyme immobilization. Although the protein microparticle showed lesser activity when compared to other matrices, this study proved that they can stably adsorb amylase enzyme and can be used for enzyme immobilization. The glutaraldehyde treatment on the enzyme immobilized matrice showed a significant increase in the enzyme activity proving that glutaraldehyde increases the bonding between the enzyme molecules as well as the enzyme and the matrice. Therefore the study concludes that these natural matrices improve the activity as well as the stability of the amylase enzyme.When used for clarification of apple juice the glutaraldehyde treated enzyme immobilized coconut fibermatrice showed maximum clarification. These matrices can therefore prove to be eco-friendly, cost efficient matrices for many industrial processes.

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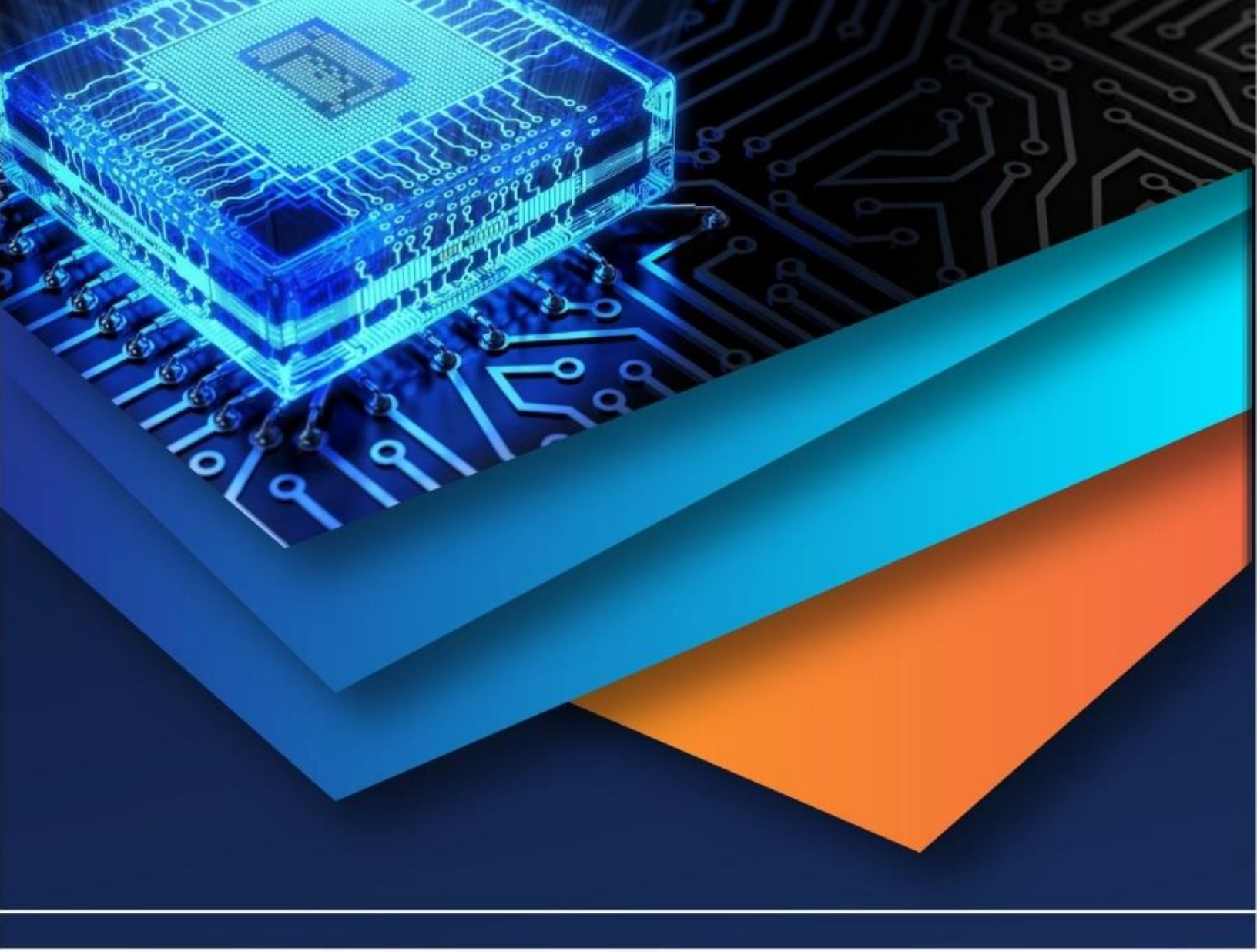

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