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FABRICATION OF 1,4-ALPHA-D-GLUCAN GLUCANOHYDROLASE HOLDING GEL-SCAFFOLDS USING AGAR-AGAR, A NATURAL POLYSACCHARIDE AND POLYACRYLAMIDE, A SYNTHETIC ORGANIC POLYMER FOR CONTINUOUS LIQUEFACTION OF STARCH

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Abstract

1,4-alpha-D-glucan glucanohydrolase is among the most widely used commercial hydrolytic enzymes acting randomly on the glycosidic linkages of starch resulting in its saccharification and liquefaction. Its applicability in different industries can be improved by enhancing its stability and reusability. Therefore, in the present study attempts have been made to enhance the industrial applicability of 1,4-alpha-D-glucan glucanohydrolase from *Bacillus subtilis* KIBGE-HAR by adapting immobilization technology. The study developed mechanically stable, enzyme containing gel-frameworks using two support matrices including agar-agar, a natural polysaccharide and polyacrylamide gel, a synthetic organic polymer. These catalytic gel-scaffolds were compared with each other in terms of kinetics and stability of entrapped 1,4-α-D-glucan glucanohydrolase. In case of polyacrylamide gel, K_m value for immobilized enzyme increased to 7.95 mg/mL, while immobilization in agar-agar resulted in decreased K_m value i.e 0.277 mg/mL as compared to free enzyme. It was found that immobilized enzyme showed maximum activity at 70 °C in both the supports as compared to free enzyme having maximum activity at 60 °C. Immobilized 1,4-α-D-glucan glucanohydrolase exhibited no change in optimal pH 7.0 before and after entrapment in polyacrylamide gel and agar-agar. The enzyme containing gel-scaffold was found suitable for repeated batches of starch liquefaction in industrial processes. Agar-agar entrapped 1,4-α-D-glucanglucanohydrolase was capable to degrade starch up to seven repeated operational cycles whereas polyacrylamide entrapped enzyme conserved its activity up to sixth operational cycle.

Keywords: Amylase. Entrapment. Kinetics. Polymers.

1. Introduction

1,4-α-D-glucan glucanohydrolase (EC 3.2.1.1) belongs to the class of hydrolases. It is an endo-acting amylase and catalyzes the breakdown of α 1, 4 glycosidic bond usually present in amylose and amylopectin of starch. The resultant products are maltotriose and maltose from cleavage of amylose, and limit dextrin and glucose from the breakdown of amylopectin (Raghu and Rajeshwara 2015). It is one of the ubiquitous enzymes found in a variety of creatures from microbes to higher plants and animals. Microbial 1,4-α-D-

glucan glucanohydrolase are of great importance because they are easy and economical to produce, manipulate and optimize (Bueno et al. 2016). This enzyme comprises 65% of the global enzyme manufacturing market and utilized in variety of applications which include starch saccharification and liquefaction giving rise to valuable metabolites like bioethanol and sugar syrups, textile desizing, paper recycling and treatment of starch processing wastewater (Pervez et al. 2014; Simair et al. 2017). In spite of the great applicability of enzymes in industrial processes, their practical implementation is quite limited due to their susceptibilities to the inflexible conditions of different industrial processes. Increased usage of green methodologies on commercial scale derives the breakthrough of using immobilization technology for the application of enzymes in bioprocessing and bioanalysis (Dwevedi 2016). Immobilized enzyme not only offers the retention of catalytic efficiency but also its reusability over extended time and temperature is increased (Brenaet al. 2013). Immobilization is actually the confinement of soluble biocatalyst in a limited area of space in order to prevent its structural modifications which can be induced by the harsh environmental conditions, thus this technology plays an important role in reducing the cost of the biocatalyst and improves its industrial lifespan (Dwevedi 2016). Immobilization can be achieved by ionic or covalent binding with the support or by physical confinement via entrapment or cross linking. An efficiently immobilized enzyme requires a good choice of mechanically stable, inert support ranging from agar to magnetic nanoparticles (Vaghari et al. 2016). Entrapment, being less traumatic to enzyme, is advantageous over covalent binding. In entrapment, enzyme is trapped within the lattice structure of support material or in polymer membranes. Entrapment lessens the enzyme outflow and increases the mechanical strength. The technique allows the modification of supports and other parameters to create a suitable microenvironment for enzyme by optimization.

Common polymers used for entrapment are alginate, agar, gelatin, carrageenan, and polyacrylamide. Agar is a naturally occurring heterogeneous polysaccharide consisting of agarose and agaropectin. Polyacrylamide is a synthetic linear polymer of acrylamide and acrylic acid. These matrices are economical, easy to handle, provide inert environment and give the potential catalytic output. The present study was therefore designed to compare the immobilization efficiencies of agar and polyacrylamide for 1,4-α-D-glucan glucanohydrolase and thus to put an effort in the green technology to meet the industrial demand of this promising biocatalyst.

2. Material and Methods

Materials and Strain

The strain used was *Bacillus subtilis* KIBGE-HAR, isolated and identified in Dr. A.Q. Khan Institute of Biotechnology and Genetic Engineering (KIBGE), University of Karachi. Starch and other fermentation medium components were purchased from Merck (Germany).

All the analytical grade chemicals were used and purchased from the recognized vendors. $(NH_4)_2SO_4$ and agar-agar from Scharlau (Cambodia), Acrylamide from Sigma (Germany) and Bis-acrylamide from Acros (US) were used for partial purification and immobilization studies.

An automatic autoclave machine (Astell, UK), High speed refrigerated centrifuge (Sigma 3K 30), Spectrophotometer (Optizen 1412, Korea) and incubators (Memmert, Germany) were also used in the study.

Production and partial purification of 1,4-Alpha-D-Glucan glucanohydrolase from *Bacillus subtilis* KIBGE-HAR

Pure culture of *Bacillus subtilis* KIBGE-HAR was used to produce 1,4-alpha-D-glucan glucanohydrolase by submerged batch fermentation technology. Growth medium contained (g/L): starch 15.0, peptone 5.0, yeast extract 2.0, NaCl 0.5, $MgSO_4.7H_2O$ 0.5 and $CaCl_2$ 0.002. The pH of the fermentation medium was kept at 7.0 before sterilization. The thermophilic strain of *Bacillus subtilis* KIBGE-HAR was grown in the medium at 50 °C for 24 hours. After 24 hours the fermented medium was centrifuged at 10,000✕g for 10 minutes to collect the extracellular enzyme. The crude enzyme was then

partially purified by salt precipitation method using 40 g% saturation with $(NH_4)_2SO_4$. The precipitates of 1,4-alpha-D-glucan glucanohydrolase were collected in 2 mL of Tris-HCl buffer (pH 7.0) and dialyzed against the same buffer for one hour at 4 °C. The dialyzed enzyme was used for immobilization studies.

Assay of soluble enzyme activity

1,4-alpha-D-glucan glucanohydrolase activity was determined by quantification of the reducing sugar released by hydrolysis of starch using 40 mg/ml maltose as a standard (Miller 1959). One Unit of soluble 1,4-alpha-D-glucan glucanohydrolase is defined as "*the amount of enzyme required to produce 1 µmol of reducing sugar from 15 mg/ml starch per unit time under the assay conditions*".

Immobilization of 1,4-Alpha-D-Glucan Glucanohydrolase in agar-agar gel

Immobilization of enzyme in agar-agar was achieved by dissolving 1.5 g% agar-agar (5.0 mL) in deionized water using boiling water bath. The solution was then cooled until the temperature dropped to 45 °C, then 5.0 mL partially purified enzyme was added and stirred for 30 seconds on a magnetic stirred plate until a homogenous solution was formed which was immediately poured into a petri plate of known weight. The enzyme-agar mixture was allowed to solidify at 4 °C for 30 minutes. After solidification, agar gel containing entrapped 1,4-alpha-D-glucan glucanohydrolase was cut into small blocks of equivalent size of 2.0 x 2.0 sq mm. The gel blocks were washed thoroughly with deionized water before further analysis.

Immobilization of 1,4-Alpha-D-Glucan Glucanohydrolase in Polyacrylamide gel

For this purpose, 2.5 mL solution A, containing 9.5 % acrylamide and 0.5 % N,N'-methylene bisacrylamide was prepared and mixed with 2.5 mL partially purified enzyme and 4.95 mL deionized water under constant stirring on a magnetic stirrer plate for one minute at low speed. Thereafter, 50 µL APS (10 %) was added and stirred for one minute followed by the addition of 50 µL 14.4 M TEMED, with constant stirring for 30 seconds. The mixture was then poured into preassembled glass plates. After solidification, gel was cut into small blocks of 2.0 x 2.0 sq mm size, washed with deionized water and stored in Tris-HCl buffer (50 mM, pH 7.0) at 4 °C.

Assay of immobilized 1,4-Alpha-D-Glucan Glucanohydrolase activity

Immobilized enzyme activity was determined by taking 1.0 g enzyme gel blocks in a test tube and then 1.0 mL substrate was added. Reaction was allowed to proceed for specified time and temperature. Thereafter, 0.5 mL reaction mixture was taken out and quantified by DNS method described above. An immobilized enzyme unit is defined as "*the amount of 1,4-alpha-D-glucan glucanohydrolase that liberates 1 µmol of reducing sugar from the substrate per gram gel blocks under the assay conditions*". The calculations for percent immobilization efficiency in Polyacrylamide and Agar-agar are represented in Table 1.

Kinetics of immobilized 1,4-Alpha-D-Glucan Glucanohydrolase

Effect of time on immobilized enzyme and substrate reaction

The assay time for the maximum activity of immobilized enzyme with 15 mg/mL starch was determined by performing the enzyme assay for different time intervals ranging from 5.0 to 20.0 minutes.

Effect of substrate on entrapped enzyme activity

A substrate maximum for immobilized enzyme was obtained by reacting 1.0 g enzyme gel blocks with 1.0 mL substrate of different concentrations (10-40 mg/mL) under the standard conditions of

reaction. The Michaelis-Menten constant, K_m and V_{max} have been calculated using the Lineweaver Burk plot.

Table 1. Immobilization efficiency.

Specifications	Polyacrylamide entrapped enzyme	Agar-agar entrapped enzyme
Weight of the gel blocks	5.21 g	4.87
Volume of enzyme trapped in gel blocks	6.5 mL	5.21
Volume of enzyme trapped/g gel	1.25 mL/g	1.07
Units of enzyme before entrapment	780 U/mL/min	938
Units of enzyme after entrapment	492 U/g/min	664
Immobilization efficiency	63 %	71 %

Effect of temperature on entrapped enzyme activity

In order to evaluate the effect of temperature on enzyme entrapped in the supports, 1.0 ml substrate and 1.0 g enzyme gel blocks were taken in test tubes and kept at different temperatures including 40, 50, 60, 70 and 80 °C.

Effect of pH on entrapped enzyme activity

Immobilized enzyme activity was estimated using 1.0 mL substrate, prepared in Tris-HCl buffer of different pH ranging from pH 5.0 to pH 9.0, at specific temperature.

Reusability of entrapped enzyme

The reusability of 1,4-alpha-D-glucan glucanohydrolase entrapped in agar-agar and Polyacrylamide gel blocks was also determined. For this purpose, the enzyme gel blocks were removed from the reaction mixture after the first reaction with substrate, washed with deionized water, dried and used again for the next reaction. The second reaction was followed by series of repeated reactions using the same procedure for gel blocks recovery until the entrapped enzyme activity was significantly decreased.

3. Results

Effect of time on immobilized enzyme and substrate reaction

Time for reaction of immobilized enzyme with substrate was noted at different intervals and it was found that when 1,4-α-D-glucan glucanohydrolase was immobilized in agar-agar and polyacrylamide gel, maximum activity had been found at 10.0 and 15.0 minutes respectively showing increase in reaction time by 5.0 minutes and 10.0 minutes from the free enzyme respectively (Figure 1).

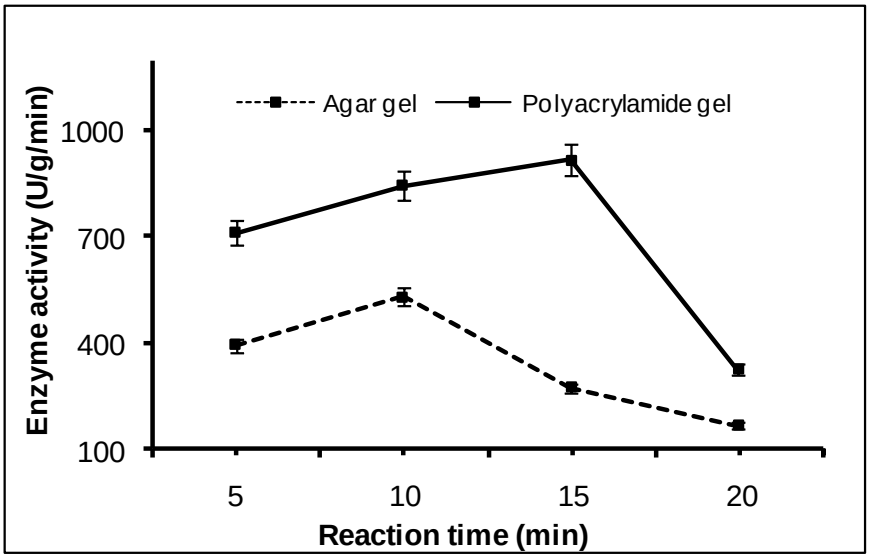

Figure 1. Effect of reaction time with substrate (starch) on entrapped 1,4-alpha-D-glucan glucanohydrolase activity.

Substrate maxima of immobilized 1,4-α-D-glucan glucanohydrolasein polyacrylamide and agar-agar

In case of polyacrylamide immobilization, K_m value for immobilized 1,4-α-D-glucan glucanohydrolase was increased from 2.737 mg/mL to 7.952 mg/mL as compared to free enzyme (Figure 2). V_{max} of entrapped enzyme in polyacrylamide was affected with ~1.877-fold decrease from 1398 U (for soluble enzyme) to 744.8 U. In case of agar entrapped enzyme, a decrease in K_m value to 0.2764 mg/mL was noticed in comparison with free enzyme (Figure 3). Entrapment in agar led to decrease of V_{max} to 335.2 U, that is, ~4.17-fold, compared to free enzyme.

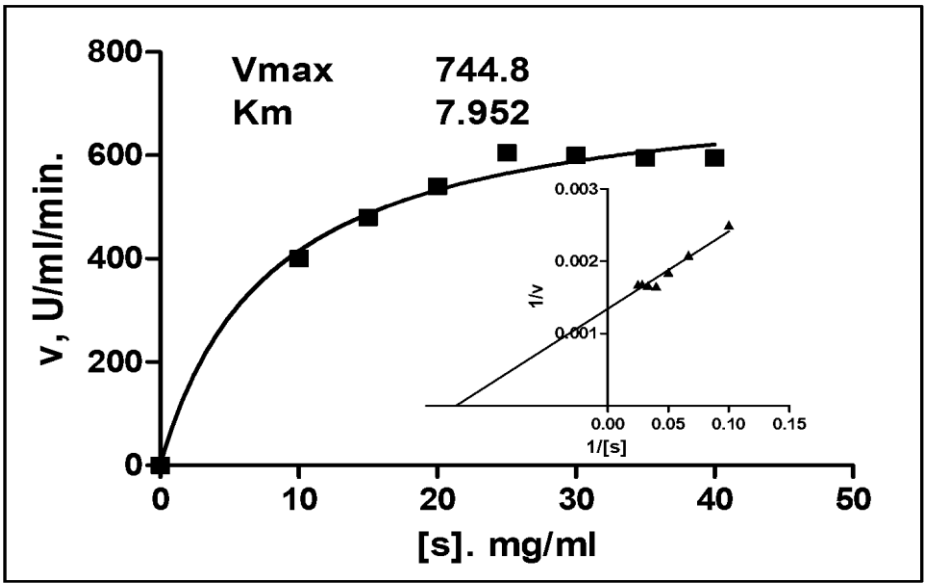

Figure 2. Michaelis-Menten and Lineweaver-Burk plot of 1,4-alpha-D-glucan glucanohydrolase entrapped in polyacrylamide gel.

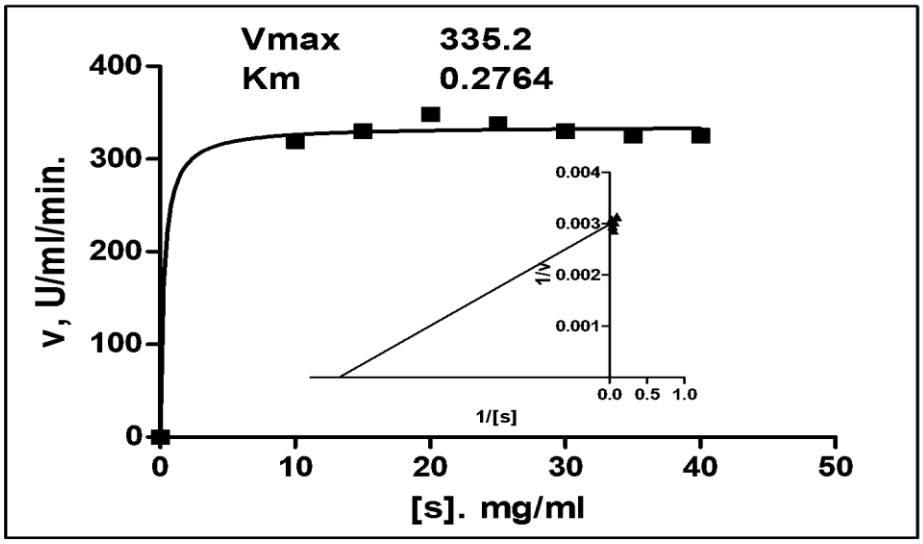

Figure 3. Michaelis-Menten and Lineweaver-Burk plot of 1,4-alpha-D-glucan glucanohydrolase entrapped in agar gel.

Effect of immobilization on optimum temperature of 1,4-α-D-glucan glucanohydrolase activity

Different temperatures ranging from 50°C to 80°C were used for the assay of immobilized 1,4-α-D-glucan glucanohydrolase. The optimum temperature of 1,4-α-D-glucan glucanohydrolase activity in both the supports was raised by 10°C as compared to the optimal temperature of soluble enzyme that was 60°C (Figure 4).

Effect of immobilization on optimum pH of 1,4-α-D-glucan glucanohydrolaseactivity

Effect of pH optima on different supports were performed and compared with free enzyme. When 1,4-α-D-glucan glucanohydrolase was entrapped in agar-agar and polyacrylamide gel, no change was observed in optimum pH before and after entrapment i.e. at pH 7.0, agar-agar entrapped enzyme and polyacrylamide-entrapped enzyme, both showed the maximum activity (Figure 5).

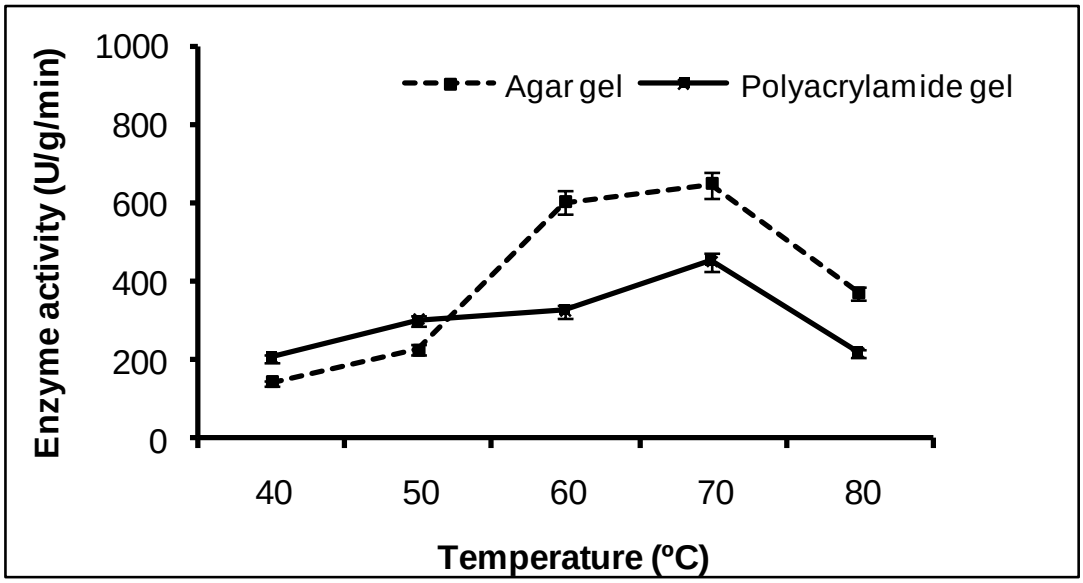

Figure 4. Effect of temperature on activity of 1,4-alpha-D-glucan glucanohydrolase immobilized in Agar-agar and Polyacrylamide gel.

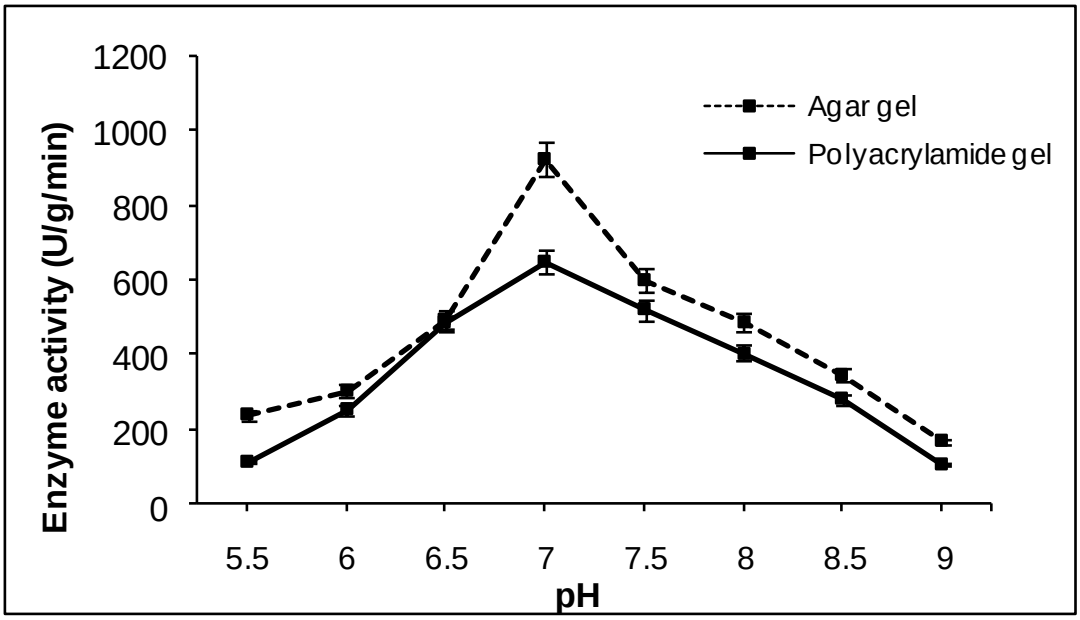

Figure 5. Effect of pH on activity of 1,4-alpha-D-glucan glucanohydrolase immobilized in Agar-agar and Polyacrylamide gel.

Reusability of immobilized enzyme

In the present study, polyacrylamide entrapped enzyme retained 87 % catalytic activity after second reuse and 65 % activity was noticed after third reuse. Immobilized enzyme activity decreased after each cycle and complete loss of activity was seen during seventh cycle (Figure 6). In case of agar-agar immobilization 22 % of the initial activity was retained after seventh cycle.

4. Discussion

Reaction time of substrate and enzyme is an important parameter for maximum enzyme activity. 1,4-α-D-glucan glucanohydrolase utilized more time for optimal starch hydrolysis after immobilization in both the supports. Enzyme entrapped in polyacrylamide required more time as compared to that entrapped in agar-agar which may be due to cross linking network of acrylamide and bisacrylamide. Protease entrapped in polyacrylamide microspheres also exhibited a rise in reaction time (Sattar et al. 2018).

In case of polyacrylamide immobilization, Km value for immobilized 1,4-α-D-glucan glucanohydrolase was increased as compared to free enzyme. This finding suggests that starch being the substrate of high molecular weight, faced resistance while entering into the microenvironment of gel blocks and interacting with the binding sites of enzymes resulting in the increased requirement of substrate for optimal enzyme activity. Similar finding was reported previously for maltase entrapped in polyacrylamide gel (Nawaz et al. 2016). Vmax of entrapped enzyme in polyacrylamide was also found to decrease by 1.877 fold. Decrease in Vmax following immobilization has also been reported by Pramaniket al. (2013) who reported that increased ΔG° in immobilized enzyme system was associated with slower interaction of enzyme and substrate. The decreased number of binding sites for substrate upon immobilization might also be the reason why Km is increased and Vmax is decreased upon immobilization.

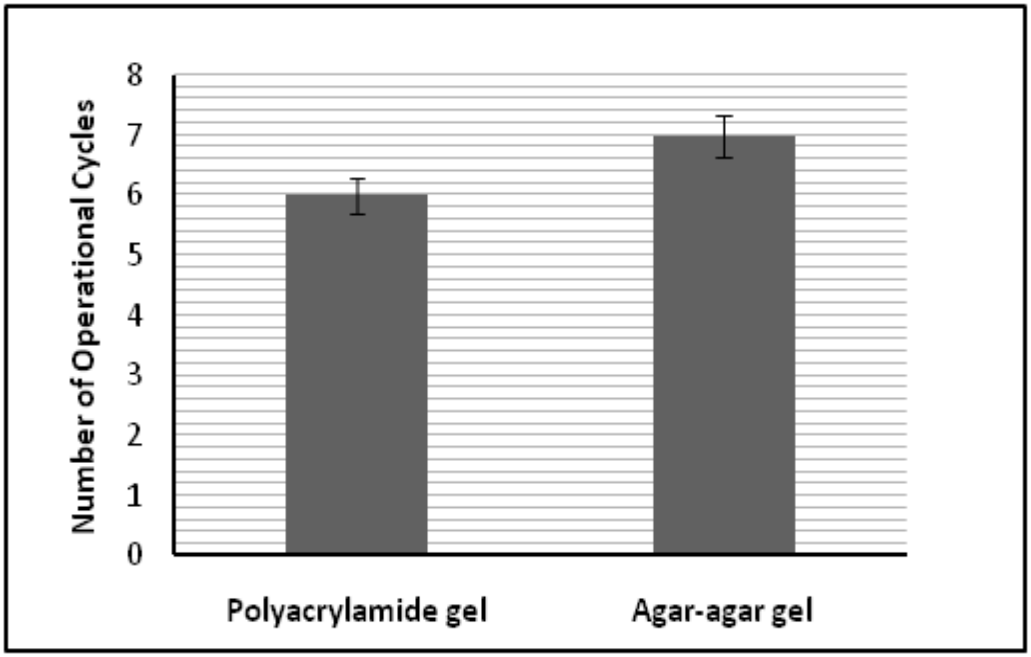

Figure 6. Reusability of 1,4-alpha-D-glucan glucanohydrolase entrapped in Agar-agar and Polyacrylamide gel.

In case of agar-agar entrapped enzyme, a decrease in Km was observed as compared to free enzyme. This suggests that the active site of immobilized enzyme in agar-agar gel-scaffold were oriented to the exterior surface and accommodated with the substrate freely. In contrast to the result of present study, elevated value of Km was reported by Mesbahand Wiegel (2018) for amylase AmyD8 after entrapment in agar-agar. Vmax of agar agar entrapped 1,4-α-D-glucanglucanohydrolase was 4.17-fold reduced as compared to free enzyme whereas Sharma et al. (2014) reported a decrease in Vmax of α-amylase in agar beads to about 8-fold as compared to free enzyme.

Immobilized enzyme exhibited improved temperature stability in both agar-agar and polyacrylamide gel-scaffolds. Similarly, rise in optimum temperature has also been reported for α-amylase immobilized in L-asparagine modified chitosan beads (Yazganet al. 2018). An extracellular laccase also exhibited an increase in its optimum temperature by 5 °C when entrapped in agar-agar and polyacrylamide gel (Reda et al. 2018). This finding suggests that both the support matrices were suitable to stabilize the enzyme for industrial operations conducting at higher temperatures.

Optimum pH of 1,4-α-D-glucan glucanohydrolase was not affected following immobilization in both agar-agar and polyacrylamide gel-scaffolds. The result was deviated from the results of Ahmed et al. (2020) who indicated that the optimum pH was shifted from 5 to 6.5 following immobilization of amylase in sodium alginate.

The reusability of immobilized enzyme is the most influential factor that made immobilized enzyme more valuable than free enzyme in industrial applications. 1,4-α-D-glucan glucanohydrolase enclosed in the protective shield of Agar-agar and polyacrylamide gel-scaffolds were found suitable for seven and six repeated batches of starch liquefaction respectively. Mahajan et al. (2010) reported that when 1,4-α-D-glucan glucanohydrolase was entrapped in polyacrylamide gel, the beads were fragile which could not show any operational stability. Sharma et al. (2014) worked on agar and calcium agar as entrapment materials for amylase and reported that agar entrapped enzyme activity was declined to 20 % after sixth cycle of repeated use whereas enzyme enclosed in calcium agar was found to be more stable.

5. Conclusions

It is concluded that both Polyacrylamide and Agar-agar are promising immobilization matrices for enzymes and showed comparable results regarding their catalytic properties and stability. Immobilization in both Polyacrylamide and Agar-agar gel scaffolds contributed to the enhanced thermal and operational

stability of 1,4-α-D-glucan glucanohydrolase making the enzyme more suitable for continuous liquefaction of starch in food, textile and detergent industries.

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Ethics Approval: Not applicable.

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References

AHMED, N.E., et al. Optimization and immobilization of amylase produced by Aspergillus terreus using pomegranate peel waste. *Bulletin of the National Research Centre*. 2020, **44**, 1-12. https://doi.org/10.1186/s42269-020-00363-3

BRENA, B., GONZÁLEZ-POMBO, P. and BATISTA-VIEIRA, F. Immobilization of enzymes: a literature survey. *Immobilization of enzymes and cells*. 2013, **2013**, 15-31.https://doi.org/10.1007/978-1-62703-550-7_2

BUENO, et al. Microbial enzymes as substitutes of chemical additives in baking wheat flour-Part II: combined effects of nine enzymes on dough rheology. *Food and bioprocess technology*. 2016, **9**, 1598-1611. https://doi.org/10.1007/s11947-016-1744-8

DWEVEDI, A. Enzyme immobilization: an important link between agriculture and industries. *Enzyme Immobilization. Springer, Cham*. 2016, **2016**, 45-64. https://doi.org/10.1007/978-3-319-41418-8_3

MAHAJAN, R., et al. Comparison and suitability of gel matrix for entrapping higher content of enzymes for commercial applications. *Indian journal of pharmaceutical sciences*. 2010, **72**, 223.https://doi.org/10.4103/0250-474X.65010

MESBAH, N.M., and WIEGEL, J. Improvement of activity and thermostability of agar-entrapped, thermophilic, haloalkaliphilic amylase AmyD8. *Catalysis Letters*. 2018, **148**, 2665-2674. https://doi.org/10.1007/s10562-018-2493-2

MILLER, G.L. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical chemistry*. 1959, **31**, 426 428. https://doi.org/10.1021/ac60147a030

NAWAZ, M.A., et al. Polyacrylamide gel-entrapped maltase: an excellent design of using maltase in continuous industrial processes. *Applied biochemistry and biotechnology*. 2016, **179**, 383-397. https://doi.org/10.1007/s12010-016-2001-3

PERVEZ, S., et al. Saccharification and liquefaction of cassava starch: an alternative source for the production of bioethanol using amylolytic enzymes by double fermentation process. *BMC biotechnology*. 2014, **14**, 1-10.https://doi.org/10.1186/1472-6750-14-49

PRAMANIK, S., et al. Kinetic and Thermodynamic Studies of Free and Alginate/Agar-Agar Immobilized a-Amylase Catalyzed Reaction. *Asian Journal of Chemistry*. 2013, **25**, 6557.https://doi.org/10.14233/ajchem.2013.14361

RAGHU, H.S., and RAJESHWARA, N.A. Immobilization of [alpha]-Amylase (1, 4-[alpha]-D-Glucanglucanohydralase) by calcium alginate encapsulation. *International Food Research Journal*. 2015, **22**, 869.

REDA, F.M., et al. Decolorization of synthetic dyes by free and immobilized laccases from newly isolated strain Brevibacteriumhalotolerans N11 (KY883983). *Biocatalysis and agricultural biotechnology*. 2018, **15**, 138-145. https://doi.org/10.1016/j.bcab.2018.05.020

SATTAR, H., et al. Agar-agar immobilization: An alternative approach for the entrapment of protease to improve the catalytic efficiency, thermal stability and recycling efficiency. *International journal of biological macromolecules*. 2018, **111**, 917-922. https://doi.org/10.1016/j.ijbiomac.2018.01.105

SHARMA, M., et al. Entrapment of-amylase in agar beads for biocatalysis of macromolecular substrate. *International scholarly research notice*. 2014, **2014**. https://doi.org/10.1155/2014/936129

SIMAIR, A.A., et al. Production and partial characterization of α-amylase enzyme from Bacillus sp. BCC 01-50 and potential applications. *BioMed research international*. 2017, **2017**. https://doi.org/10.1155/2017/9173040

VAGHARI, H., et al. Application of magnetic nanoparticles in smart enzyme immobilization. *Biotechnology letters*. 2016, **38**, 223-233. https://doi.org/10.1007/s10529-015-1977-z

YAZGAN, I., et al. Modification of chitosan-bead support materials with l-lysine and l-asparagine for α-amylase immobilization. *Bioprocess and biosystems engineering*. 2018, **41**, 423-434. https://doi.org/10.1007/s00449-017-1876-x

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