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Original paper

Amylase Production by *Aspergillus niger* immobilized in Microporous Calcium Alginate Gel Beads

UCHENNA OYEAGU[1], CHARLES O. NWUCHE[1], CHRISTIAN N. OGBONNA[2], JAMES C. OGBONNA[1]*

[1]Department of Microbiology, University of Nigeria, Nsukka; Post Code 410,001, Enugu State, Nigeria
[2]Department of Plant Science and Biotechnology, University of Nigeria, Nsukka, Nigeria

Abstract Microporous calcium alginate gel beads were investigated as potential solutions to mass transfer limitations in immobilized cultures. The beads were prepared by gelling mixtures of sodium alginate and fillers (starch or granulated sugars) in calcium chloride solution. The resulting beads were cured in the same solution, during which the fillers leached out of the beads thereby creating micro-pores in the beads (microporous beads). The effectiveness of the microporous beads in improving amylase production by *Aspergillus niger* LC 269109 was investigated. Spores of *A. niger* were immobilized in the microporous beads and used for batch alpha amylase and gluco amylase production. Amylase production by the *A. niger* immobilized in the microporous beads were significantly higher ($p<0.01$) than the values obtained with the conventional calcium alginate gel beads. Under all the conditions investigated, gluco-amylase activities were significantly ($p<0.01$) higher than the alpha-amylase activities. Under the optimum conditions of inoculum concentration (1.0×10^5 spores/ml), pH (6), temperature (35°C), bead diameter (3 mm) and calcium chloride concentration (1.5%), the gluco-amylase and alpha amylase activities were 11.98 U/ml and 6.7 U/ml respectively, which were significantly higher ($p<0.05$) than the 7.8 U/ml and 3.2 U/ml obtained with the conventional gel beads.

Keywords Microporous beads, Immobilized cells, Mass transfer limitations, Amylases, Calcium Alginate gel beads, Cell entrapment.

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*Corresponding author: JAMES C. OGBONNA, University of Nigeria, Department of Microbiology, Nsukka; Post Code 410,001, Enugu State, Nigeria
E-mail: james.ogbonna@unn.edu.ng Telephone: +234-8037136810

Introduction

Cell immobilization has long been exploited for production of extracellular microbial products and biochemicals (OGBONNA et al, [1], DOAA and WAFAA [2]). Immobilized cell systems have wide applications in the fields of food technology, biotechnology, biomedicine and waste-water treatment (AHMAD et al, [3], MARTINS et al, [4], ELAKKIYA et al, [5]). Immobilized microbial systems have numerous advantages over free or suspended cells; some of which are higher product yields, ease of product recovery and suitability for repeated or continuous use (GUPTA et al, [6]). The immobilized cells are also more resistant to toxic chemicals than the freely suspended cells (ELAKKIYA et al, [5], LIU et al, [7]).

Presently, one major challenge confronting the application of cells immobilized by entrapment in polymer gels is the limitation to transport of nutrients, metabolites and wastes across the gel matrix. Immobilization in alginate beads leads to the formation of nutrient and oxygen-deficient micro environments at the core of the gel beads thereby restricting the growth of the cells to the periphery of the beads with sufficient oxygen and nutrients (OGBONNA et al, [8], PARASCANDOLA and DE-ALTERIIS, [9], OYEAGU et al, [10]). In order to solve the problem of mass transfer limitation in entrapped cells, many early studies focused on reduction in the bead diameters (OGBONNA et al, [1], PEREGO and PERATELLO, [11]), reducing the packed bed volumes or operating at high flow rates (HUSSAIN et al, [12]).

The present study is aimed at demonstrating that increasing the porosity of the beads (microporous beads) can reduce mass transfer limitations in cells immobilized by entrapment in polymer gels. The term ‘microporous’ is used to describe the occurrence of micro-pores in the calcium-alginate gel beads formed by leaching out of fillers (i.e. starch or sugar granules previously mixed with the sodium alginate) into the curing solution during the induction of gel formation. The concept and methods of immobilization have been described in a previous report (OYEAGU et al, [10]). The present paper aims to demonstrate the effectiveness of microporous beads in improving productivity of aerobic processes, using enzyme production as a case study.

Materials and Methods

Preparation of spores for immobilization

Aspergillus niger LC 269109 was cultivated for 3 to 4 days on Potato Dextrose Agar (PDA). The spore suspension was prepared by adding 10 ml of sterilized distilled water to the slant. Inoculation loop was then used to dislodge the spores from the slant into the distilled water. The tube was shaken vigorously and the resulting spore suspension was decanted into a clean sterile receptacle, making sure that hyphae and debris were not transferred together. The number of spores in the suspension was accurately counted, using a Thoma Haemocytometer. The spore suspension was appropriately diluted with distilled water so that an appropriate number of spores was used for each immobilization.

Immobilization in the conventional alginate beads

Sodium alginate (2%) and calcium chloride (2%) solutions were sterilized by autoclaving at 121°C for 15 min. Thereafter, a 5 ml of the standard spore suspension (1.0×10^6 spores/ml) was added to the cooled sodium alginate solution and shaken gently to mix. The mixture was then added drop wise into a beaker containing the calcium chloride solution using a 5 ml automatic pipette. The beaker was gently stirred at 70 rpm (Magnetic Stirrer OP-912/3, Radelkis, Hungary). The alginate droplets solidified upon contact with the solution, forming alginate gel beads. The average spore concentration in each bead was 2.5×10^4 spores/bead. Stirring of the resulting beads was continued for 24 h to promote cross linking and stability of the beads (OGBONNA et al, [8]).

Immobilization in microporous beads

The steps and processes were the same as those described for the conventional alginate beads except that the spores were added to a mixture of sodium alginate and filler (soluble starch or granulated sugar), before adding drop-wise to the 2% $CaCl_2$ solution. During curing in calcium chloride solution, the fillers leached out of the beads and created micro pores (micro-porous beads). Starch and granulated sugar were used as fillers because they are soluble in calcium chloride solution, and are non-toxic to the cells. Furthermore, they can easily be metabolized by *Aspergillus* sp so that even those that did not leach out of the beads during curing are metabolized by the immobilized cells during the cultivation (OYEAGU et al, [10]).

Amylase production by the immobilized cells

The gel beads were recovered and washed in sterile distilled water to remove excess calcium and un-entrapped spores. Two hundred beads each containing 2.5×10^4 spores/bead were inoculated into 100 ml soluble starch medium composed of (in g/l): soluble starch, 15; yeast extract, 4; K_2HPO_4, 1 and $MgSO_4.7H_2O$, 0.5 in 250 ml Erlenmeyer flask. The cultivation was done on a rotary shaker (Bioshaker BR-43FL) operated at 200 rpm for

8 days at 28±2°C. The spores immobilized in the conventional calcium alginate beads served as the control. Broth samples were taken at time intervals and filtered through Whatman No 1 filter paper. The filtrate was used as the crude enzyme source for the amylase activity assays.

Gluco-amylase assay

Gluco-amylase was determined as described by Mohammad (MOHAMMAD et al, [13]). A 0.5 ml of 1% (w/v) soluble starch was added to 0.2 ml of 0.1 M sodium acetate buffer (pH 5.6) in test tubes and 0.3 ml of the crude enzyme was added. The mixture was incubated in a water bath for 30 min at 40°C. A 1 ml of 3, 5 dinitrosalicylic acid was added to each test tube and boiled for 15 min in a water bath. After cooling, 4 ml of distilled water was added. The absorbance was taken at 540 nm. The glucose concentration was extrapolated from a calibration curve. One unit of glucoamylase was defined as the amount of enzyme that liberated 1 microgram of glucose per minute under the assay condition.

Alpha-amylase assay

The same method described for gluco-amylase was used except that after incubation at 40°C, 1.0 ml of 1M acetic acid was added and distilled water was used to make up the volume to 10 ml. A 1.0 ml of iodine solution was added and the absorbance was measured at 600 nm. The concentrations of starch before and after the enzymatic reactions were determined. One unit of alpha-amylase was defined as the amount of enzyme that hydrolyzed one microgram of starch per minute under the assay condition.

Effects of immobilized spore concentration on enzyme production

Various spore concentrations were entrapped in gel beads (2.5×10^1 to 2.5×10^5 spores/bead) and 200 beads were used to inoculate 100 ml of the soluble starch medium in 250 ml flasks. Three types of beads were used: (**a**) normal calcium alginate beads, (**b**) microporous beads prepared by co-immobilization with 0.4% starch (STA) and (**c**) microporous beads prepared by co-immobilization with 1.2% granulated sugar (GSA) (OYEAGU et al, [10]). The average diameter of the beads was 3.0 mm. The cultivation conditions were the same as described earlier.

Effects of various parameters on enzyme production

The three types of beads were used while the method and culture conditions were the same as described above. The effects of temperature and initial pH were investigated by varying the incubation temperature from 25 to 40°C, and the initial pH from 3 to 8 using 1M HCl or NaOH. For these two experiments, the initial spore concentration was 2.5×10^3 spores/bead and the bead diameter was 3 mm. The effects of bead diameters were investigated by varying the diameters of the beads from 2.0 to 5.0 mm. This was achieved by using micropipette tips with different diameters for cell immobilization. The initial spore concentration, temperature and pH were 1.0×10^5 spores/ml, 35°C and pH 6 respectively. The effects of $CaCl_2$ addition to the culture medium were also investigated by varying the concentrations of calcium chloride added to the medium from 1 to 3%.

Measurement of cell growth inside the gel beads

Growth of the cells inside the beads was determined by measuring the number of colony forming units after solubilization of the beads. A total of ten beads were randomly selected from the fermentation broth and transferred into a 50 ml-flask containing 20 ml of 0.2 M sodium citrate buffer (pH 6). The beads were solubilized by stirring with a magnetic stirrer at room temperature (OGBONNA et al, [8]). The cell suspension was appropriately diluted and 0.1 ml was plated on PDA agar plates and incubated at 30°C for 48 h. The number of colony forming units was then counted. To visualize the *Aspergillus niger* mycelia growth inside the beads, transverse sections of the beads were made, using a sharp blade razor. They were examined under a phase-contrast microscope to which a motic camera (Version 2.0, Motic China Group Co, Ltd.) was attached.

Statistical analysis

All the experiments were performed in triplicates. The data were statistically analyzed by One Way Analysis of Variance (ANOVA) while the Least Significant Difference was used to separate the means. Results of the experiments were presented as averages ± standard error of the mean.

Results and Discussion

A comparison of amylase production by *A. niger* LC 269109 immobilized in microporous beads with those immobilized in the conventional gel beads (control) is shown in Table 1. Alpha-amylase activity increased with increase in incubation period up to 96 h after which the activity declined steadily. Generally, the cells immobilized in microporous beads produced significantly higher enzymes than those immobilized in the control beads ($p<0.01$). The microporous beads prepared with starch (STA) had higher enzyme activity compared to those prepared with granulated sugar (GSA). In cultures with cells immobilized in the STA gel beads, alpha amylase

activity rose from 1.80±0.11 U/ml after 24 h to a peak activity of 4.71±0.09 U/ml (96 h) before dropping finally to 1.59±0.09 U/ml after 192 h. The maxima alpha-amylase activities obtained with cells immobilized in the GSA and control gel beads were 3.03±0.11 and 2.24±0.08 U/ml respectively.

The same trend was observed with gluco-amylase production except that the concentrations of gluco-amylase were about 2-folds higher than those of alpha amylase irrespective of the type of gel bead used. Gluco-amylase activity increased with cultivation time up to the peak at 96 h. The maximum gluco-amylase activities in the cultures with STA and GSA gel beads were 9.72±0.17 and 8.31±0.11U/ml respectively while that in the control was 7.18±0.19 U/ml. The activities of both enzymes were significantly higher with STA gel beads than with GSA beads due to better chemical and physical stabilities of the STA gel beads over the GSA beads (OYEAGU et al, [10]). The STA beads displayed significantly ($p<0.05$) longer solubilization time during incubation in 0.2 M sodium citrate buffer compared to the conventional alginate and GSA gel beads (OYEAGU et al, [10]). The porosity of the gel beads is another important factor that might have contributed to higher amylase production in STA microporous beads. The STA gel beads had larger micro-pores (10-600 nm) than those of the gel beads made with granulated sugar (10-500 nm) (OYEAGU et al, [10]).

Table 1. Alpha and gluco amylase production by *A. niger* immobilized in microporous beads.

	Alpha amylase activity(U/ml)			Gluco-amylase activity(U/ml)		
	STA(±SD)	**GSA(±SD)**	**Control(±SD)**	**STA(±SD)**	**GSA(±SD)**	**Control(±SD)**
24 h	1.80±0.11	1.42±0.09	1.27±0.06	3.21±0.04	3.05±0.10	2.89±0.10
48 h	1.91±0.13	1.69±0.32	1.51±0.03	3.80±0.19	3.49±0.11	3.05±0.19
72 h	2.80±0.05	1.97±0.08	1.78±0.10	6.24±0.24	5.80±0.24	5.02±0.16
96 h	4.71±0.09	3.03±0.11	2.24±0.08	9.72±0.17	8.31±0.11	7.18±0.19
120 h	2.95±0.13	2.10±0.06	1.80±0.10	6.59±0.14	5.99±0.13	5.20±0.14
144 h	2.01±0.13	1.89±0.01	1.57±0.08	4.02±0.07	3.80±0.07	3.23±0.09
168 h	1.84±0.11	1.60±0.09	1.50±0.09	3.90±0.13	3.78±0.12	3.11±0.21
192 h	1.59±0.09	1.58±0.09	1.32±0.05	3.56±0.14	3.41±0.14	2.92±0.14

STA = Microporous beads prepared from a mixture of sodium alginate, spore suspension and starch (0.4%)
GSA = Microporous beads prepared from a mixture of sodium alginate, spore suspension and granulated sugar (1.2%)

Another possible reason for higher enzyme activities obtained with microporous beads is that microporous beads supported better mycelia growth. As shown in Plate 1, in comparison with the control bead, mycelia growth in microporous beads were much higher than in the normal gel beads. It is well known that in normal gel beads, both yeasts (OGBONNA et al, [8]), bacteria (SONDERHOLM et al, [14]) and fungi (KWAK and RHEE, [15]) grow preferentially near the surface of the gel beads. This is because oxygen penetrates only into a very narrow periphery of the beads. The extent of oxygen penetration depends on the diameter of the beads, the cell concentration inside the bead and the dissolved oxygen concentration in the culture broth (OGBONNA et al, [1], KWAK and RHEE, [15], OGBONNA et al, [16]). In the present study, the immobilized fungi grew even at the centre of the GSA and STA microporous beads due to high porosity, and thus oxygen penetration to the centre. The growth of the mycelia even at the centre of microporous beads resulted in higher cell concentrations in the microporous beads. The number of colony forming units recorded for GSA and STA were 2.44×10^8 CFU/bead and 2.98×10^8 CFU/bead respectively. These are significantly higher than the 1.96×10^8 CFU/bead recorded for the control bead. Thus, the higher enzyme activities obtained with the STA can be attributed to higher mycelia concentration in the microporous beads. The STA gel beads were thus selected for use in further experiments.

Figure 1 shows the effect of immobilized spore concentration on production of both alpha and gluco-amylases. In the medium inoculated with cells immobilized in the STA gel beads, the alpha-amylase activity increased with increase in the immobilized spore concentration until reaching a peak of 5.0 U/ml at 2.5×10^3 spores/bead (1.0×10^5 spores/ml).

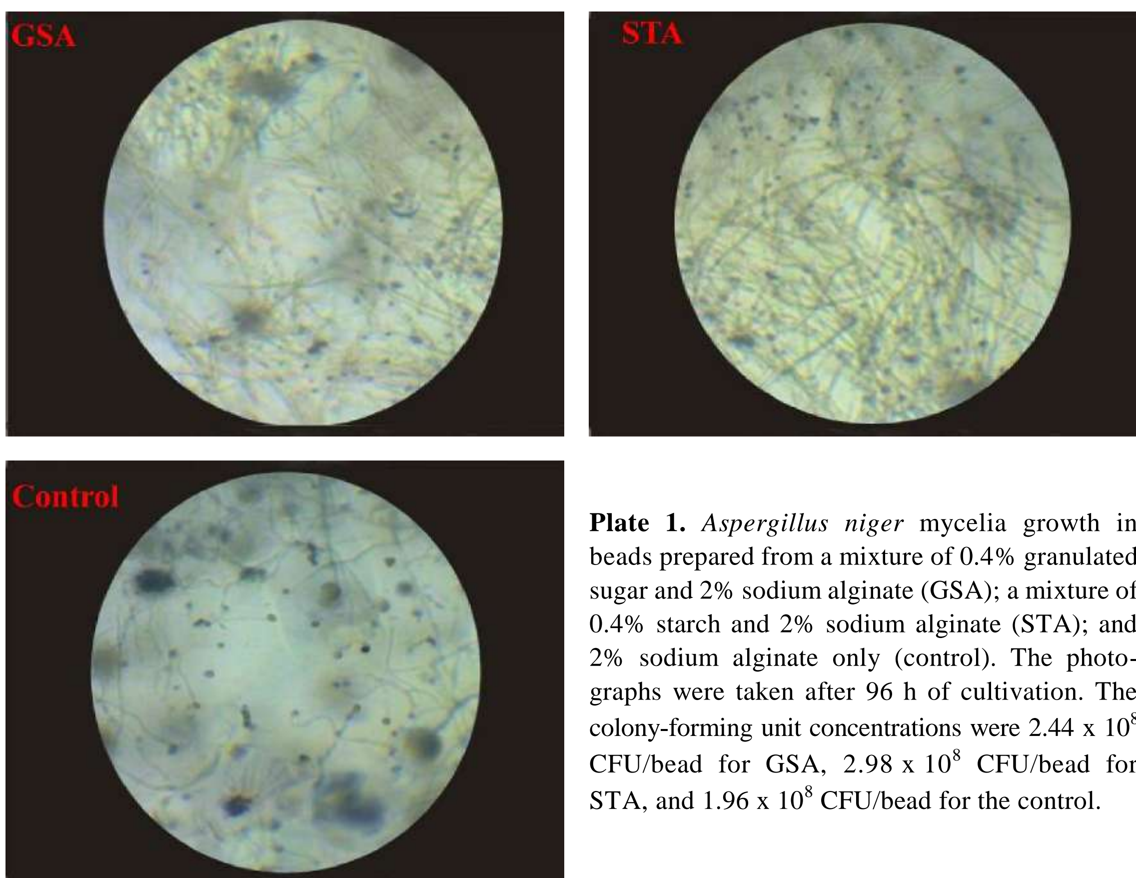

Plate 1. *Aspergillus niger* mycelia growth in beads prepared from a mixture of 0.4% granulated sugar and 2% sodium alginate (GSA); a mixture of 0.4% starch and 2% sodium alginate (STA); and 2% sodium alginate only (control). The photographs were taken after 96 h of cultivation. The colony-forming unit concentrations were 2.44 x 10^8 CFU/bead for GSA, 2.98 x 10^8 CFU/bead for STA, and 1.96 x 10^8 CFU/bead for the control.

However, further increase in the immobilized spore concentration resulted in decrease in enzyme activity. Although gluco-amylase activity in the STA gel beads was higher (8.43-8.93 U/ml) than the activity reported for the control beads, the beneficial effect of the microporous beads over the control beads was more clearly evident with alpha amylase. Of all the concentrations evaluated, 1.0 x 10^5 spores/ml (2.5 x 10^3 spores/bead) gave the highest activity for both alpha and gluco-amylases (Fig. 1). This is in agreement with OGBONNA et al, [8] who reported that 10^5 spores/ml was optimum for immobilization of yeast cells. However, other authors have reported 10^9 cfu/ml (SCHOEBITZ et al, [17], CRUZ et al, [18]) for bacterial concentrations and 10^6cfu/ml for microalgae (CRUZ et al, [18]). Lower immobilized spore concentration resulted in decreased enzyme activity due to decreased growth of the mold. It appears that the optimum immobilization spore/cell concentration depends on the cells. Obviously, aerobic cells and processes would require lower immobilized cell concentration than anaerobic or microaerophilic processes.

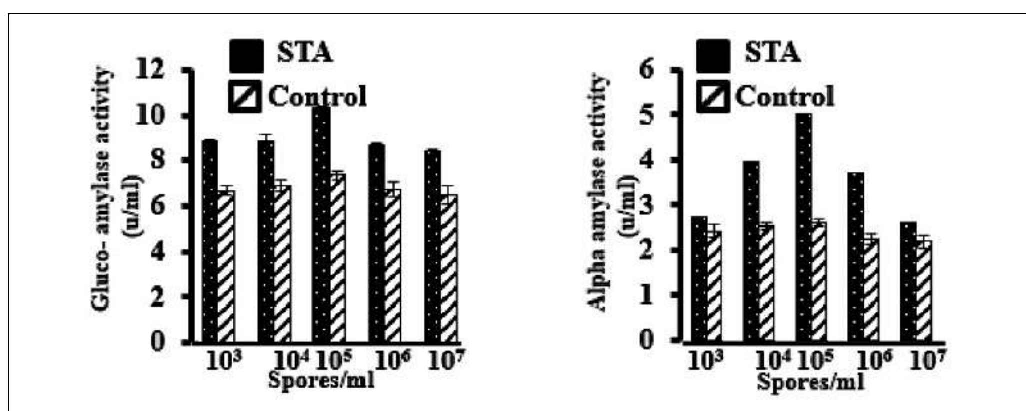

Figure 1. Effect of immobilized spore concentration on alpha and gluco-amylase production by *Aspergillus niger* immobilized in calcium alginate microporous gel beads.

With the microporous beads, the optimum temperature for both alpha and gluco-amylase production was 35°C. In the case of those entrapped in the control beads, the optimum temperature was 30°C (Fig. 2). Further increase in temperature resulted in decreased enzyme production which agrees with the results reported by SHAZIA, [19].

As shown in Figure 3, the activities of the enzymes were the least in the media adjusted to pH 3 but increased with increase in pH up to pH 6 before it declined.

At all the pH values evaluated, immobilization in microporous beads resulted in higher amylase activities than those immobilized in normal gel beads. However, at lower pH values, the differences were less apparent for gluco-amylase. Gluco-amylase activities were statistically ($p<0.01$) higher than alpha amylase under all the pH conditions investigated. This observation agrees with earlier reports by ABRAHAM et al, [20] which stated that gluco-amylase production by immobilized strains of *A. niger* was several folds higher than alpha amylase produced by the same organism. Alpha and gluco-amylases are known to be very sensitive to pH changes. At lower pH conditions, (pH 3 to 5), enzyme production dropped significantly. Similar results have been reported by ABDALWAHAB et al, [21] and KHAN and YADAV, [22]. Although the mechanism of action of pH on the amylase production is not well understood, pH can affect the substrate solubility and charge, as well as enzyme conformation and activity.

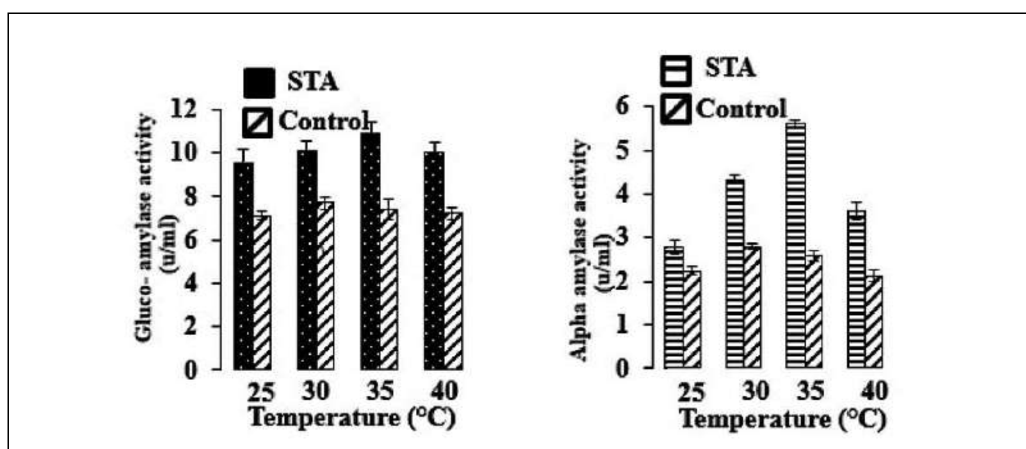

Figure 2. Effect of temperature on alpha and gluco-amylase production by *Aspergillus niger* immobilized in microporous beads.

The effects of bead diameter on enzyme production by the immobilized spores are shown in Fig. 4. The optimum bead diameter for alpha and gluco-amylase production was 3 mm for microporous beads but 2 mm for normal beads. The activities of the two enzymes decreased with increase in bead diameter above 3 mm. The activities of enzymes by the cells immobilized in the microporous beads were significantly ($p<0.01$) higher than those from the control beads.

Oxygen and nutrient diffusion are important considerations on the choice of gel bead diameter. OGBONNA et al, [1] achieved effective oxygenation in entrapped cells by reducing the diameter of the beads. SMRDEL et al, [23] developed a method of optimizing the production of spherical beads suitable for different technological operations.

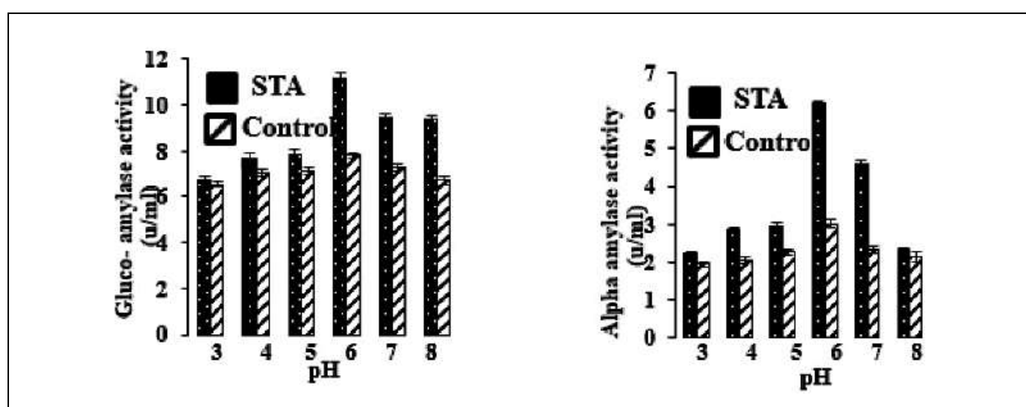

Figure 3. Effect of initial pH on alpha and gluco-amylase production by *A. niger* immobilized in microporous beads.

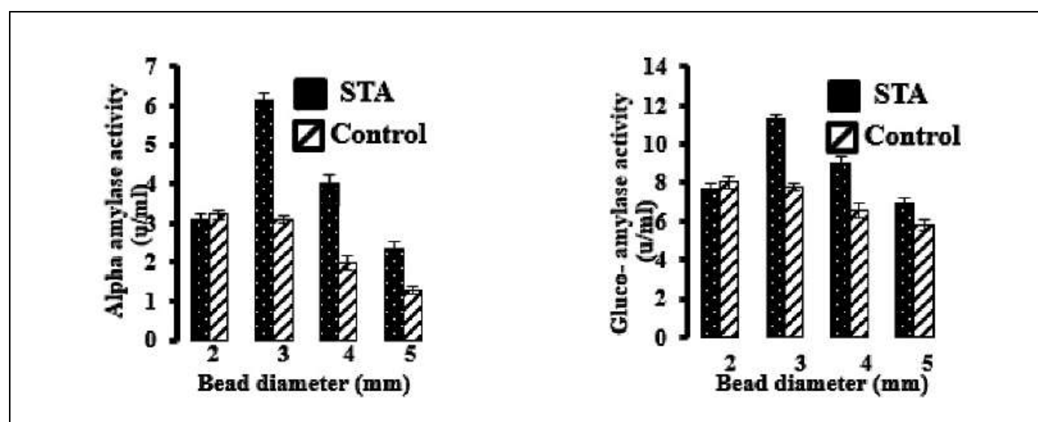

Figure 4. Effect of bead diameter on alpha and gluco-amylase production by spores immobilized in microporous beads.

Cells immobilized in microporous beads had reduced enzyme activity when the 2 mm bead was used perhaps due to inadequate space for cell entrapment. Increasing the bead diameter to 3 mm promoted higher enzyme activities but further increase resulted in decreased activity. Noteworthy is that even with 4 mm microporous beads, the enzyme activities were higher than those immobilized in the 2 and 3 mm normal beads. The 3 mm bead diameter provided sufficient spaces after starch had leached out thus enabling efficient entrapment and mass diffusion. The increase in the optimum bead diameter for the microporous beads is an indication of improved mass transfer.

Cells immobilized in both the microporous and control beads displayed varied responses to different concentrations of calcium chloride (Fig. 5). However, both alpha and gluco-amylase produced by *A. niger* LC 269109 cells immobilized in microporous beads were higher than those entrapped in the control beads. Gluco-amylase activity was approximately double the activity of alpha-amylase. The results showed that 1.5% $CaCl_2$ gave the highest concentrations of alpha and gluco-amylases. Previous studies showed that as fermentation progressed, normal beads got weaker and addition of $CaCl_2$ was necessary to stabilize the beads.

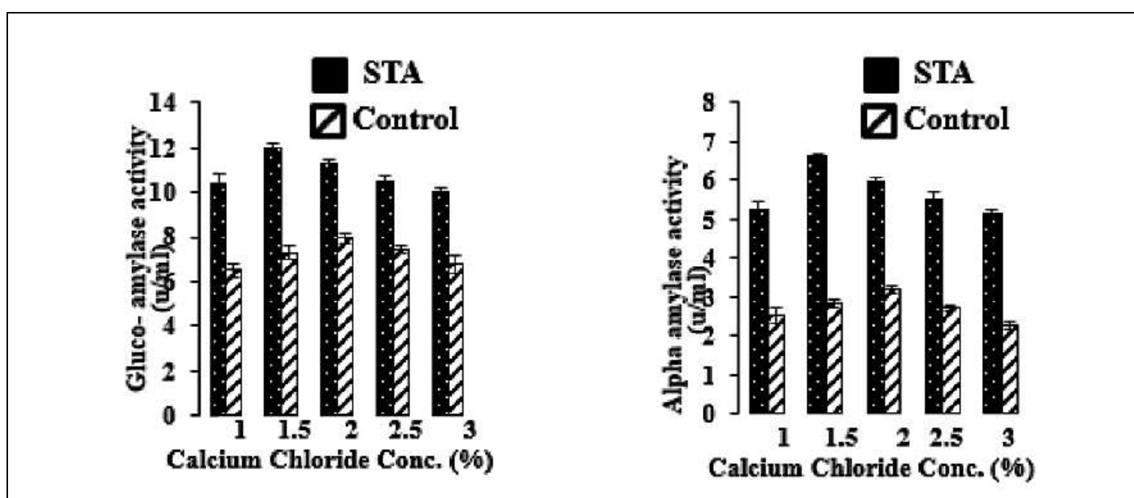

Figure 5. Effect of calcium chloride concentration on alpha and gluco-amylase production by spores immobilized in microporous beads.

In the present study, the higher the concentration of $CaCl_2$ added to the medium, the more stable the beads were. At 3% $CaCl_2$ concentration, the activities of both enzymes were still significantly high, indicating potential for long term application. Apart from stabilizing the beads, the presence of calcium in the medium is believed to confer special benefits to the activity of the enzymes. Metal ions, particularly calcium, potentiate the production (MATHEW et al, [24]), activity (JEONG-BIN et al, [25], ADEJUWON, [26], HERTADI and WIDHYASTUTI,

[27]), and stability of hydrolytic enzymes (particularly amylases) to heat and non-polar organic solvents (HERTADI and WIDHYASTUTI, [27]).

Different authors have reported different values for amylase concentrations produced in suspended cultures of *A. niger*. MONGA et al, [28] reported amylase activity within the range of 1.9 to 7.4 U/ml. MUHAMMAD et al, [29] reported gluco-amylase activity of 3.2 U/ml while ADEJUWON et al, [30] reported values between 0.47 and 3.53 U/ml. The activity reported by OMINYI et al, [31] were between 0.785 and 28.8 U/ml. The reports by MALIK et al, [32] and ASRAT and GIRMA, [33] showed maximum activities between 2.08 and 9.36 U/ml, and between 0.483 and 1.24 U/ml respectively. GUPTA et al, [6] optimized enzyme production by both free and immobilized *Aspergillus niger* and reported that under optimal conditions of substrates, temperature, pH and surfactants, the activity of α-amylase produced by the immobilized cells of *A. niger* was as high as 68 U/ml, which was significantly higher than the value obtained with free cells. However, it is difficult to compare these values because of variations in the types and concentrations of carbon and nitrogen sources used (GUPTA et al, [6]), variation in the strains of organisms used (NWUCHE et al, [34]), and variations in the culture conditions (GUPTA et al, [6]). Furthermore, there were variations in the conditions (temperature and pH) under which the enzyme activities were measured, and these have significant effects on the values obtained (MONGA et al, [28], ASRAT and GIRMA, [33]). More importantly, the definitions of one unit of enzyme activities were very different (GUPTA et al, [6], MONGA et al, [28]), making simple comparison of reported values difficult. Some of them did not even define their unit of enzyme activity (OMINYI et al, [31], MALIK et al, [32]). Nevertheless, the values of alpha and gluco-amylase activities obtained in the present study (6.7 and 11.98 U/ml respectively) are very high and significantly higher than the values obtained with the conventional calcium alginate gel beads using the same strain under the same culture conditions.

Conclusion

The approach used in the present study was to make calcium alginate beads more porous by first co-gelation of sodium alginate and fillers (starch or granulated sugar), and subsequently leaching out the filler from the calcium alginate beads. In our previous report, the micro-pores formed on the gels acted as appendages to the entrapped cells. This resulted in increased porosity of the beads, even distribution of immobilized cells, increased cell holding capacity and better mass transfer. Apart from facilitating oxygen and nutrient diffusion, the pores also aided cross linking of the gel matrices which made the microporous gels chemically and physically more stable than the conventional calcium alginate beads (OYEAGU et al, [10]). The present paper shows that *A. niger* immobilized in microporous beads were more effective than those entrapped in conventional gel beads in amylase production. To the best of our knowledge, this is the first report on amylase production by cells entrapped in microporous beads. The enzyme production by cells entrapped in microporous beads were significantly higher ($p<0.01$) than those entrapped in the conventional (control) gel beads under all the conditions investigated. It is concluded that the microporous gel beads can be used for improved production of extracellular metabolites since they are more stable, have higher mass transfer and lead to increase in immobilized cell concentrations.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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