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ORIGINAL ARTICLE

Comparison of the synthesis of the alpha-amylase enzyme by the native strain *Bacillus licheniformis* in immobilized and immersed cells

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ABSTRACT

Background and Objectives: The study focused on the amylase enzyme, widely used in the industrial starch liquefaction process. We looked into the best way to immobilize the native strain *Bacillus licheniformis*, which is the only alpha-amylase-producing bacterium, by trapping it in calcium alginate gel. This is a promising way to increase enzyme output.
Materials and Methods: We examined the effects of alginate content, biomass age, initial cell loading (ICL), bead size, and solidification duration in calcium chloride solution on enzyme synthesis. We conducted batch fermentations using both immobilized and free cells.
Results: Alpha-amylase production significantly increased with the alginate concentration ratio, achieving a maximum enzyme yield of 23.5 U/mL at a 30 g/l alginate concentration, utilizing an initial cell loading of 1.5 g in 150-200 beads per flask. These involved cells from a 12-hour culture with a bead size of 5.0 mm, were solidified for 24 hours in a 2.5% (w/v) calcium chloride solution. The yield of the immobilized cells was approximately 111.71% higher than that of the free cells, which produced 11.1 U/ml. The immobilized cells consistently generated alpha-amylase over five repeated cycles, attaining a peak value of 23.5 U/ml during the first cycle, which was 2.2-fold more than the control (free cells).
Conclusion: We used a basic mass balance analysis to understand the growth of both fractions and the dynamics of amylase production in free cells and cells immobilized in Ca-alginate beads. The production of alpha-amylase in immobilized cells results in enhanced volumetric activities during fermentation. Notable advantages of this technique encompass prolonged stability, reuse and recycling, and the potential for adaptable regeneration.

Keywords: Enzyme; Bacterial proteins; Alpha-amylase; *Bacillus licheniformis*; Immobilization; Entrapment; Alginate

INTRODUCTION

Amylase is an enzyme that hydrolyzes starch into sugars. All amylases are classified as glycoside hydrolases and target α-1,4-glycosidic linkages. Alpha-amylases are calcium-dependent metalloenzymes that cannot operate in the absence of calcium. Alpha-amylase degrades long-chain carbohydrates at random sites along the starch chain, producing maltotriose and maltose from amylose, or glucose, maltose, and "restrict dextrin" from amylopectin. Due to its ability to function universally on substrates, alpha-amylase is often more rapid in action. It is a principal digestive enzyme in mammals, with an optimal pH range of 6.7-7.0 (1).

Researchers have thoroughly examined the production of alpha-amylase using immobilized Bacillus cells, affirming the method's dependability. Industry extensively uses this approach, which offers the benefit of increased enzyme synthesis, for starch liquefac-

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tion. The major immobilization technique is trapping inside gel matrices. Numerous individuals have documented the submerged synthesis of alpha-amylase using a synthetic medium (2, 3).

However, because of the relatively low enzyme titers, *Bacillus licheniformis* grown in immersed cultures is not a suitable generator of alpha-amylase. One method to enhance production efficiency is the long-term continuous synthesis of alpha-amylase via cell immobilization. Researchers often examine the cell immobilization technique for its potential to enhance fermentation processes and bioremediation.

The immobilization of entire cells for the production of extracellular enzymes has many benefits, reinforcing trust in the selected method. This involves isolating cell mass from the bulk liquid for potential reutilization, enabling sustained operation over an extended duration, and improving reactor efficiency. Nonetheless, appropriate selection of immobilization methods and auxiliary materials is essential to reduce the limitations of immobilization. The capture of calcium alginate is one of the most appropriate ways for cell immobilization due to its simplicity and cost-effectiveness. Sodium alginate is easily accessible and a non-toxic biopolymer. Consequently, it is appropriate as an immobilization medium for biomolecules and microbes. We synthesized calcium alginate beads under gentle circumstances and widely used them for microencapsulation and cell entrapment. This study's goal was to find the best conditions for immobilizing *Bacillus licheniformis* cells in Ca-alginate gel and to test how stable the biocatalyst was in producing alpha-amylase while the culture was running in a semi-continuous mode (4, 5).

MATERIALS AND METHODS

Bacterial strain and culture parameters. Bacterial strain used in this study, a local strain of *Bacillus licheniformis*, was isolated from wastewater generated by wheat mills in Kashan, a city in Iran. The isolation technique was a crucial component of the investigation, yielding a distinctive strain for further research. We previously discovered the strain and determined its nucleotide sequence (GenBank Accession Number AF438149) (6). We cultivated 50-ml cultures in 250-ml Erlenmeyer flasks, agitating them at 130 rpm and 36.5°C (2). All materials used in this study were obtained from Merck Company. The thoroughness of our study, from the isolation of the strain to the cultivation conditions, ensures the validity of our results.

Immobilization of cells by the capture technique with calcium alginate. There are many different kinds of alginates, each with a different molecular mass, mannuronate-to-glucuronate ratio, and arrangement of units in blocks and alternate sequences. Alginate solutions with concentrations ranging from 0.5% to 10% are suitable for cell immobilization.

Boiling water solubilized the alginate, which then underwent autoclaving at 121°C for 15 minutes. Cells were collected during the phase of logarithmic growth by centrifugation (3000 g, 20 min), washed in 2 ml of saline, and then introduced into 100 ml of sterilized alginate solution. The alginate/cell combination was extruded drop wise into a cool, sterile 2.5% (w/v) $CaCl_2$ solution using a sterile 5 ml pipette while stirring. Gel beads with a diameter of about 5 mm were acquired. The beads were solidified by resuspending in a new $CaCl_2$ solution for 24 hours at 4°C with moderate agitation. Subsequently, these beads were rinsed with distilled water to eliminate surplus calcium ions and unencapsulated cells. The beads were then transferred to a 50-ml production medium and incubated for the specified duration (4, 7, 8).

Optimizing effective factors in cell immobilization. The optimization process was done for each component involved in cell immobilization. These factors included the following: sodium alginate concentration, starting biomass loading, age of biomass, bead dimension, and solidifying time.

The alginate entrapment process in this study was not just a routine procedure. It was conducted with meticulous attention to detail, ensuring the reliability of the results. Various sodium alginate concentrations (10, 20, 30, 40, and 50 g/l), initial biomass loading (0.2 to 3.0 g of biomass in 150-200 beads/flask), biomass age (using cells from 12, 24, 36, 48-hour culture), bead size (2.5, 3.5, 5.0, 7.5 mm), and solidification time (2, 6, 12, 18, 24, 32, 48 h) in a 2.5% (w/v) calcium chloride solution were examined. The entrapment of cells in alginate was conducted following the previously described approach (4, 7, 8).

Enzyme assay. The dinitro salicylic acid (DNS) method was used to carefully test the amylase activity. This method was chosen because it can accurately measure the rise in reducing sugars that happens

when amylase works on starch. The principal issue in this test is the gradual fading of color and the degradation of glucose by components of the DNS reagent. It is essential to address these constraints since a revised approach for assessing and lowering sugars is urgently required. The updated technique has now been widely used to quantify lowering sugars without further adjustments. In order to test alpha-amylase's activity, 500 µL of enzyme supernatant was mixed with 500 µL of 1% (w/v) soluble starch in 0.01M phosphate buffer at pH 6.5. We incubated the mixture for 10 minutes at 90°C. The reaction was stopped, and the amount of free reducing sugars was measured accurately using colorimetry. This was done by adding 500 µL of a reagent called 3-4-dinitro salicylic acid (9). An enzyme unit catalyzed the release of 1 µmol of glucose from the substrate in 1 minute at 96°C (6, 9, 10).

The potential for recycling immobilized cells in the enzyme manufacturing process. The primary benefit of using immobilized biocatalysts is their exceptional efficiency in repeated and continuous applications. This was clearly shown in our analysis of the reusability of *Bacillus licheniformis* cells entrapped in alginate gel. The procedure included decanting the used medium bi-daily and substituting it with new medium after the sterilization of the alginate beads with saline solution. This efficient process was compared with a similar experiment using free cells, further highlighting the impressive superiority of immobilized cells for producing alpha-amylase under these conditions (11).

RESULTS

Influence of alginate concentration. We performed an accurate investigation to determine the optimal alginate concentration for the immobilization of *Bacillus licheniformis* cells, using alginate solutions at several concentrations (10, 20, 30, 40, and 50 g/l). Fig. 1 displays the results of these tests. The synthesis of alpha-amylase enhanced with increased alginate concentration, with a peak yield of 23.5 U/ml at 30 g/l alginate. This was about 111.71% more than the value obtained from free cells (11.1 U/ml).

Furthermore, at low alginate concentrations (10 and 20 g/l), the beads exhibited relative softness and demonstrated quick cell leakage compared to the higher alginate concentration of 30 g/l. Nevertheless, a further elevation in alginate concentration above 30 g/l (to 40 and 50 g/l) resulted in a reduction in enzyme production, either attributable to heightened diffusion constraints or alterations in the microenvironment inside the beads.

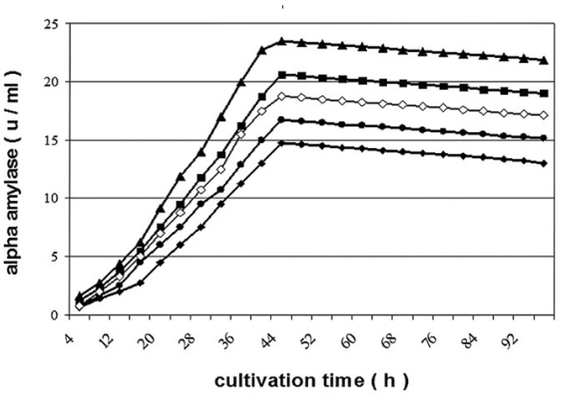

Fig. 1. Influence of alginate concentration on alpha-amylase synthesis by entrapped cells (Concentrations of alginate (g/l): 10 (⬥), 20 (●), 30 (▲) , 40 (■), 50 (◇)

Influence of initial cell loading (ICL). The influence of initial cell loading (ICL) was examined by ranging the initial amount of biomass from 0.2 g to 3.0 g in flasks containing 150 to 200 beads, utilizing a 30 g/l alginate concentration. The experiment entailed establishing cultures with differing initial biomass concentrations and quantifying the resultant alpha-amylase production. It was hypothesized that an increase in ICL, represented by the number of beads, would enhance alpha-amylase production. The findings indicated that elevating the initial biomass amount up to 1.5 g correlated with an increase in alpha-amylase concentration (Fig. 2). In conclusion, the study determined that an initial biomass amount of 1.5 g/flask was optimal, yielding a maximum enzyme output of 23.5 U/ml. This finding clearly indicated that deviations from this concentration, whether higher or lower, diminished enzyme yield.

Influence of alginate bead size. The size of alginate beads significantly influences mass transfer, which is crucial for the growth of immobilized cells. Alginate allows small substrates to diffuse similarly to water, but it may limit the diffusion of larger molecules. The square of the bead diameter may affect the growth and production of cells, as they predominantly proliferate near the bead surface. This study evaluated the

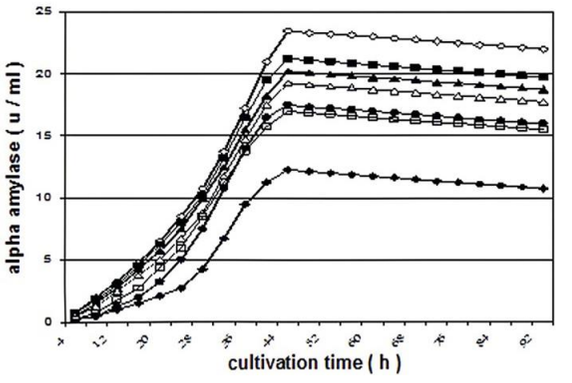

Fig. 2. Influence of initial amount of biomass on alpha-amylase synthesis by entrapped cells (biomass concentration g/flask in 150 to 200 beads: 0.2g (♦), 0.5g (●), 1.0g (▲), 1.5g (◇), 2.0g (■), 2.5g (△), 3.0g (□)

production behavior of *Bacillus licheniformis* cells immobilized in beads measuring 2.5, 3.5, 5, and 7.5 mm. The results, shown in Fig. 3, showed that the 5 mm beads produced more alpha-amylase than the bigger beads. This is likely because they had a larger surface area, which made it easier for mass to move between them. These findings not only advance our understanding of the influence of the alginate bead dimension on cell growth and synthesis behavior but also inspire potential applications in biotechnology and bioengineering, motivating further research and innovation in the field.

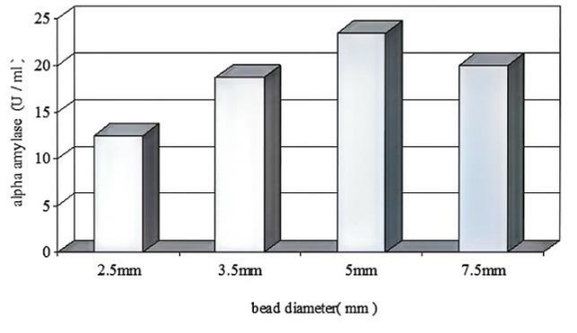

Fig. 3. Influence of alginate bead dimension on alpha-amylase synthesis by entrapped cells.

Influence of solidification time. To get more accurate results, we did an experiment to see what happened to calcium alginate beads that were solidified for 2, 6, 12, 18, 24, 32, and 48 hours at different times. The beads were made with 3% alginate and 2.5% (w/v) $CaCl_2$ solution.

The solidification durations of immobilized beads were used to generate enzymes; the findings are presented in Fig. 4. Beads that underwent a 24-hour solidification period were identified as the most effective enzyme producers. This practical knowledge may be used in the development of stable calcium alginate beads to enhance enzyme synthesis in industrial contexts.

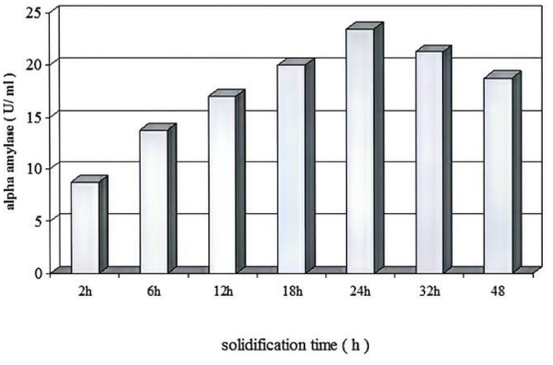

Fig. 4. Influence of solidification duration on the stability of alginate beads and the synthesis of alpha-amylase

Influence of biomass age. We learned a lot about alpha-amylase synthesis by changing the age of the biomass used to start the alginate gel for immobilization. The isolated beads containing cells from 12, 24, 36, and 48-hour cultures were fabricated using a 3% sodium alginate and 2.5% (w/v) calcium chloride solution (Fig. 5). Maximum alpha-amylase level was obtained in the case of cells from a 12-hour culture, a finding that could reshape our approach to enzyme production. Increased biomass age led to a significant decrease in alpha-amylase production, suggesting the need for further research to fully understand this relationship.

Comparison of amylase synthesis between free and entrapped cells. Fig. 6 displays how alpha-amylase is made by cells that are trapped in calcium alginate compared to *Bacillus licheniformis* cells that are free. In the instance of free cells, the synthesis of alpha-amylase escalated after around 6 hours. The logarithmic phase concluded at 24 hours, achieving a maximal activity of 11.1 U/ml, which remained relatively stable until the culture terminated. Simultaneously, encapsulated *Bacillus licheniformis* cells inside calcium alginate beads exhibited a considerable increase in alpha-amylase enzyme production from the start of the culture procedure. Fig. 3 illustrates that the synthesis of alpha-amylase by entrapped cells attained 11 U/mL after about 20 hours, a level

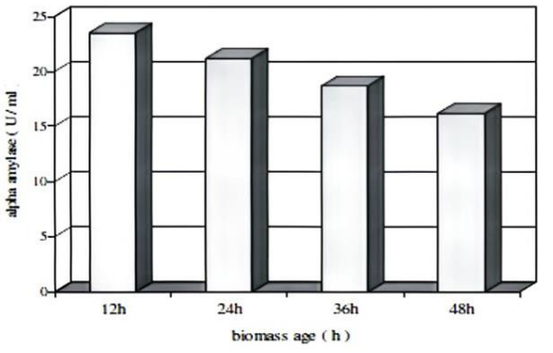

Fig. 5. Effect of biomass age for immobilization in alginate beads and the synthesis of alpha-amylase

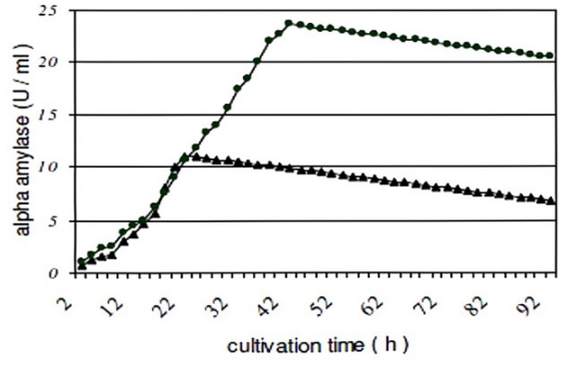

Fig. 6. Alpha-amylase synthesis by free (▲) and entrapped cells (●)

that free cells achieved after 24 hours. This activity persisted in its escalation, attaining a peak value of 23.5 U/ml at 48 hours and thereafter remained quite stable. The concentration of alpha-amylase from entrapped cells was 111.71% more than that of the control-free cells.

Reutilization of entrapped cells for alpha-amylase synthesis in successive batch cultures. The semi-continuous fermentation was finished to look at how stable the biocatalyst was and how well it could make alpha-amylase in repeated batch culture settings. Consequently, we monitored the operational stability of the biocatalysts produced under optimal immobilization conditions (alginate concentration of 30 g/l, initial biomass concentration of 1.5 g in 150-200 beads per flask, biomass derived from a 12-hour culture, bead size of 5.0 mm, and solidification for 24 hours in a 0.2 M calcium chloride solution) over five cycles (Fig. 7).

Alpha amylase was generated in repeated batch

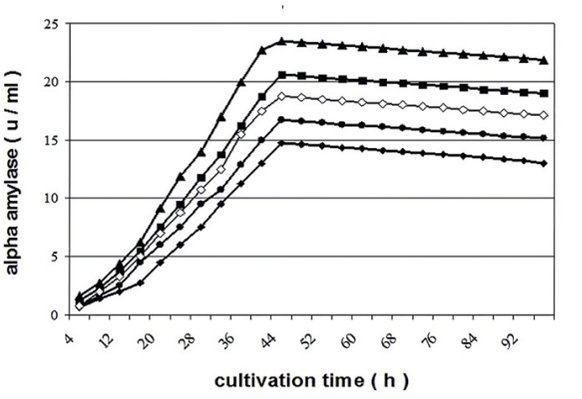

Fig. 7. Synthesis of alpha-amylase by entrapped cells in alginate beads in a repeated batch

shake cultures, with each batch lasting 48 hours. Once the culture reached its projected maximal activity level, we decanted the culture supernatant and introduced 50 ml of new medium into the Erlenmeyer flasks. The findings at the conclusion of the first cycle demonstrated a significant increase (111.71%) in alpha-amylase activity in immobilized cells relative to free cells cultivated concurrently. After transferring to new media in the third culture cycle (192 hours), the biocatalysts continued to generate high alpha-amylase, and we evaluated their alpha-amylase yield against the control (free cells). The peak activity of 23.5 U/ml achieved during the repeated batch shake flask experiment occurred in the third batch, about six days following the initiation of the repeated batch series. Subsequent modifications of the medium did not enhance enzyme production above 23.5 U/ml after the fifth cycle (10 days).

DISCUSSION

Cell encapsulation is a conventional method for enhancing total cell concentration and production. Isolating products from entrapped cells is simpler than from immersed cell systems. Cell encapsulation may facilitate the operation of continuous culture processes at elevated dilution rates. Finally, immobilization serves as a method for safeguarding cells from shear stresses. Researchers have presented numerous strategies for cell immobilization (12).

The use of polymers for entrapment or encapsulation has evolved into a complex technique, with alginate gel beads emerging as the most promising and adaptable approach to date. Alginic acid and

its related compounds are available commercially in many forms, exhibiting varying viscosities and gelling characteristics. Calcium alginate gels quickly develop under moderate circumstances, offering an appropriate medium for the trapping of entire microbial cells. This study used just one kind of sodium alginate (Merck), which exhibited sufficient flexibility for the effective encapsulation of native strains of *Bacillus licheniformis* cells. Thus, de Alteriis et al. produced glucoamylase from the immobilized cells of *Kluyveromyces lactis*, Adinarayana et al. produced the alkaline protease enzyme from the immobilized cells of *Bacillus subtilis*, and Chia and colleagues used calcium alginate after examining different polymers (11-13).

This research involved varying the amounts of alginate solutions used to create biocatalyst beads to assess the effects of various conditions on the physiological processes of the native strain of *Bacillus licheniformis* cells. The gel network varied significantly based on the parameters used during the acquisition of gel beads. This case matched the research of Le-Tien and colleagues, who investigated modified alginate matrices with different concentrations to stabilize bioactive agents (14).

The native strain of *Bacillus licheniformis* cells that were trapped changed shape and biological functions when the macromolecular organization of the matrix was changed. Fig. 1 demonstrates that an increase in alginate concentration above 30 g/l correlates with a reduction in alpha-amylase production. Higher alginate concentrations may enhance bead stability, but the robust gel consistency's diffusion constraints result in diminished enzyme outputs. These findings are in line with those of Dobreva et al., who looked at how immobilization conditions affect the ability of *Bacillus licheniformis* to make α-amylase and with those of Le-Tien et al. (4, 14).

Researchers may attribute the decline in alpha-amylase synthesis with increased alginate concentration to potential effects on substrate flow rate and the reduced density of the beads. The studies of Shinmyo et al. and de Alteriis et al., which looked at the physiology of α-amylase synthesis by immobilized *Bacillus amyloliquefaciens* and the continuous synthesis of a heterologous glucoamylase by *Kluyveromyces lactis* cells encapsulated in Calcium alginate beads, made this point very clear (1, 11).

The relationship between enzyme yield and initial cell loading (ICL) ranging from 0.2 to 3.0 g in 150 to 200 beads per flask, using a 30 g/l alginate solution, was examined (Fig. 2). The beneficial impact of enhanced cell loading, resulting in increased enzyme output, mirrored the findings of Rao & Panda on gluconic acid synthesis by *Aspergillus niger* entrapped in calcium alginate beads (15).

Enzyme production reduced with elevated initial cell loading (up to 1.5 g in 150-200 beads per flask). This may be due to the rise in cell biomass concentration, which results in a decreased nutrient/bead ratio, potentially becoming limiting. Moreover, transfer of mass is a critical factor for the development of entrapped cells. It is crucial to emphasize the substantial influence of bead diameter on the development. The square of the bead dimension may affect the growth and production characteristics of the cells, as cell proliferation primarily occurs at the bead surface. This may be attributed to the increased surface area of the beads, which improves mass transmission. Beads solidified for 24 hours exhibited superior enzyme production compared to those solidified for shorter or longer durations. An extended solidification duration led to complex beads with decreased enzyme output. This may be attributed to the reduced nutrient transfer ratio of the beads, aligning with the findings of Dobreva et al. and Chia et al., as previously referenced (4, 12).

Moreover, altering the age of biomass used to start alginate gel for immobilization could influence alpha-amylase production. Increased biomass age caused a significant decrease in alpha-amylase production because biomass cells were exited in the enzyme production cycle. The results show that it is possible to reuse calcium alginate catalysts to make alpha-amylase in a semi-continuous way. Batch culture is a conventional method for industrial production. Alongside fermentation duration, the production cycle includes turnover time required for sterilization, inoculation, and subsequent processes which decreases the overall productivity and escalates production expenses. Similar results were noted in Dhanasekaran's research on alpha-amylase synthesis in both submerged and entrapped cells of Bacillus species (16).

We conducted continuous cultivation to evaluate the biological catalysts' longevity and their capacity to produce alpha-amylase in successive batch culture settings using modified alginate beads. The reutilization of entrapped cells on alginate beads across three consecutive reaction cycles (each lasting 48 hours)

without any decline in catalytic activity and five runs with 60% remaining activity represents a notable advancement compared to the prior research conducted by Karandikar et al., Ahmed, and Dobreva et al., who implemented five cycles of entrapped cells for the production of enzymes (4, 17, 18).

We detected no pieces of alginate beads in the culture liquid, indicating that the structural integrity of the enzymes was sufficiently good. Conversely, floating cells exhibited reduced enzyme production compared to entrapped cells, and their activity significantly diminished with successive batch runs. We used the immobilized catalysts for about two weeks, during which they continued to exhibit substantial alpha-amylase synthesis. This indicates a favorable outlook for enzyme technology, with the capacity for sustained alpha-amylase synthesis. Researchers have found that cells that are immobilized can consistently produce alpha-amylase. This supports the work of Oliveira et al. on immobilizing *Bacillus subtilis* in alginate microfluidic-based particles to increase lipase output. It also supports Mrudula and Shyam's studies on the Ca-alginate entrapment method for the encapsulation of *Bacillus megaterium* to improve the secretion of alkaline protease (19, 20).

The findings indicate that calcium alginate entrapment is an effective technique for the immobilization of the native strain *Bacillus licheniformis* for alpha-amylase production. When cells are immobilized, they make more alpha-amylase than when cells are free, which means that more volumetric activity happens in the same amount of time. This approach offers distinct benefits, such as longevity, reusability, and the potential for adaptive reproduction and data scalability (21-23).

CONCLUSION

The study findings suggest that adhesion, accumulation, capture, or containment can attain the immobilization of live microbial cells. This technique confines the cells to a specific area and enables the recurrent use of their enzymatic activity. The capability to design and produce immobilized cell fragments in various shapes and sizes with certain densities enables their use as a distinct phase in the bioreactor, therefore decoupling their mechanical activity from other current parts. Consequently, various bioreactor arrangements, such as stirred container, fixed-base, fluidized border, gas excited, and barrier bioreactors, can utilize immobilized cells, allowing the user to benefit from operational flexibilities such as short residence times and high loading rates. Extended stability, improved biomass retention, reaction selectivity, elevated product output, and streamlined subsequent processing are other beneficial attributes of immobilized cell bioreactors. Investigations into the fundamental and practical dimensions of cell immobilization have resulted in several applications, including environmental biological engineering, beverage and food manufacturing, drugs, chemical compounds, and notably, biomedical engineering and medicine.

REFERENCES

1. Willaert RG (2018). Cell immobilization and its applications in biotechnology: current trends and future prospects. 4 Edition. In: *Ferment Microbiol Biotechnol* pp. 324-358. CRC press.
2. Ikram -ul-Haq, Hamad A, Qadeer MA, Iqbal J. Pearl millet, a source of alpha amylase production by *Bacillus licheniformis*. *Bioresour Technol* 2005; 96: 1201-1204.
3. Guo J, Chen C, Chen W, Jiang J, Chen B, Zheng F. Effective immobilization of *Bacillus subtilis* in chitosan-sodium alginate composite carrier for ammonia removal from anaerobically digested swine wastewater. *Chemosphere* 2021; 284: 131266.
4. Sharma A, Satyanarayana T. Production of acid-stable and high-maltose-forming α-amylase of *Bacillus acidicola* by solid-state fermentation and immobilized cells and its applicability in baking. *Appl Biochem Biotechnol* 2012; 168: 1025-1034.
5. Kumaravel V, Gopal SR. Immobilization of *Bacillus amyloliquefaciens* MBL27 cells for enhanced antimicrobial protein production using calcium alginate beads. *Biotechnol Appl Biochem* 2010; 57: 97-103.
6. Shahhoseini M, Ziaee AA, Pourbabai AA, Ghaemi N, Declerck N. A natural variant of *Bacillus licheniformis* α-amylase isolated from flour mill wastewaters sheds light on the origin of high thermostability. *J Appl Microbiol* 2005; 98: 24-32.
7. Sekoai PT, Awosusi AA, Yoro KO, Singo M, Oloye O, Ayeni AO, et al. Microbial cell immobilization in biohydrogen production: a short overview. *Crit Rev Biotechnol* 2018; 38: 157-171.
8. Blandino A, Macias M, Cantero D. Calcium alginate gel as encapsulation matrix for coimmobilized enzyme systems. *Appl Biochem Biotechnol* 2003; 110: 53-60.

9. Bano S, Qader SA, Aman A, Azhar A. Partial purification and some properties of-amylase from *Bacillus subtilis* KIBGE-HAS. *Indian J Biochem Biophys* 2009; 46: 401-404.
10. Tanaka A, Hoshino E. Calcium-binding parameter of *Bacillus amyloliquefaciens* α-amylase determined by inactivation kinetics. *Biochem J* 2002; 364: 635-639.
11. de Alteriis E, Silvestro G, Poletto M, Romano V, Capitanio D, Compagno C, et al. *Kluyveromyces lactis* cells entrapped in Ca-alginate beads for the continuous production of a heterologous glucoamylase. *J Biotechnol* 2004; 109: 83-92.
12. Chia SM, Wan AC, Quek CH, Mao HQ, Xu X, Shen L, et al. Multi-layered microcapsules for cell encapsulation. *Biomaterials* 2002; 23: 849-856.
13. Adinarayana K, Jyothi B, Ellaiah P. Production of alkaline protease with immobilized cells of *Bacillus subtilis* PE-11 in various matrices by entrapment technique. *AAPS PharmSciTech* 2005; 6(3): E391-E397.
14. Le-Tien C, Millette M, Lacroix M, Mateescu MA. Modified alginate matrices for the immobilization of bioactive agents. *Biotechnol Appl Biochem* 2004; 39: 189-198.
15. Pucci EFQ, Buffo MM, Del Bianco Sousa M, Tardioli PW, Badino AC. An innovative multi-enzymatic system for gluconic acid production from starch using *Aspergillus niger* whole-cells. *Enzyme Microb Technol* 2023; 171: 110309.
16. El-Banna TE, Abd-Aziz AA, Abou-Dobara MI, Ibrahim RI. Production and immobilization of alpha-amylase from *Bacillus subtilis. Pak J Biol Sci* 2007; 10: 2039-2047.
17. Martínez D, Menéndez C, Echemendia FM, Pérez ER, Trujillo LE, Sobrino A, et al. Complete sucrose hydrolysis by heat-killed recombinant Pichia pastoris cells entrapped in calcium alginate. *Microb Cell Fact* 2014; 13: 87.
18. Ahmed SA. Invertase production by *Bacillus macerans* immobilized on calcium alginate beads. *J Appl Sci Res* 2008; 4: 1777-1781.
19. Oliveira AF, Bastos RG, de la Torre LG. *Bacillus subtilis* immobilization in alginate microfluidic-based microparticles aiming to improve lipase productivity. *Biochem Eng J* 2019; 143: 110-120.
20. Mrudula S, Shyam N. Immobilization of *Bacillus megaterium* MTCC 2444 by Ca-alginate entrapment method for enhanced alkaline protease production. *Brazil Arch Biol Technol* 2012; 55: 135-144.
21. Potumarthi R, Subhakar Ch, Pavani A, Jetty A. Evaluation of various parameters of calcium-alginate immobilization method for enhanced alkaline protease production by *Bacillus licheniformis* NCIM-2042 using statistical methods. *Bioresour Technol* 2008; 99: 1776-1786.
22. Seifan M, Khajeh Samani A, Hewitt S, Berenjian A. The effect of cell immobilization by calcium alginate on bacterially induced calcium carbonate precipitation. *Fermentation* 2017; 3: 57.
23. Konsoula Z, Liakopoulou-Kyriakides M. Thermostable α-amylase production by *Bacillus subtilis* entrapped in calcium alginate gel capsules. *Enzyme Microb Technol* 2006; 39: 690-696.