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Enhanced production of extracellular L-methioninase by entire cell immobilization of Streptomyces MDMMH4 and examination of its utilization as an antioxidant agent

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Abstract: *Streptomyces MDMMH4* cells were immobilized in various matrices with two different techniques for the enhanced and semi-continuous production of extracellular L-methioninase. Of these, agarose was proven to be the most suitable matrix for the immobilization of cells. The optimal agarose concentration was approximately 3% and the initial cell concentration was 150mg/ml (wet cell weight). Agarose-entrapped cells increased the enzyme yield by 21% compared to the highest yield obtained with free cells. Even after twelve successive and efficient fermentation operations, the agarose blocks had good stability. They maintained 69.3% of the enzyme yield obtained in the first cycle. Applying this process on an industrial scale using agarose-entrapped cells, an inexpensive and renewable matrix will allow the stable production of L-methioninase. The purified L-methioninase could be successfully obtained after applying the purification protocol as mentioned in the previous studies. Subsequently, the purified enzyme showed that L- methioninase possessed moderate scavenging activity with high IC50 values of 390.4µg/mL (corresponding to 11.62U/mL). To our knowledge, this is the first report on L-methioninase production by whole-cell immobilization.

Keywords: L-methioninase, immobilization, streptomyces and antioxidant.

INTRODUCTION

L-methioninase, also called L- methionine γ-lyase, L-methionine γ-demethiolase and L- methionine methanethiol- lyase, is part of the γ-class of Pyridoxal 5'-phosphate (PLP) dependent enzymes which primarily uses PLP as covalent co-enzyme. It catalyzes α; γ elimination of L- methionine as well as derivatives. The biological unit with the enzyme is a homotetramer. Each monomer includes 398 amino acids and has one PLP molecule as a cofactor, which can be covalently linked to the amino group of Lysine residue (Nakayama *et al.,* 1984a, selim *et al*., 2015 and Abdelraof *et al*., 2019). A primary potential therapeutic application of enzymes is the treatment of cancer. Therefore, much attention has been paid to L- methioninase, which has demonstrated antitumor efficacy *in vitro* and *in vivo* (Tan *et al.,* 2010, Kahraman *et al*., 2011 and Selim *et al.,* 2016). L-methioninase is one of the few microbial enzymes with high therapeutic value. It was reported as a potent anticancer agent against various types of tumor cell lines associated with Breast, Lung, Colon, Kidney and Glioblastoma (Kokkinakis *et al.,* 2001, El-Sayed *et al.,* 2012)and (Kavya, Varalakshmi Kilingar Nadumane, 2023). Many human cancer cell lines and primary tumors have an absolute requirement for L-methionine to survive and proliferate as an essential amino acid (Anderson, 1998). In the past several years, marked trials have been applied to L-methioninase production and efforts to accomplish high productivity under submerged and solid-state cultivation have been carried out (Abu-Tahon and Isaa , 2016; El-Sayed, 2009 and Muharram, 2016).

One of several strategies to enhance enzyme productivity is by long-term conditions production of L-methioninase under cell immobilization conditions. Cell immobilization technology is often studied because of its possibility of improving fermentation processes (Beshay, 2003; Abdelraof, 2020 and Hassabo *et al.,* 2022). The immobilization of whole microbial cells and their applications to bioprocessing have been of great interest (Kar *et al*., 2008) and (Azza A abozeid. 2022) Immobilization of whole cells for extracellular enzyme production offers several benefits. Benefits like the ease of separating cell mass from the bulk liquid for possible reuse, high cell concentrations, facilitating continuous operation for more than a prolonged period, enhancing reactor productivity, ensuring higher efficiency of catalysis and stabilizing varied cell functions (Kar *et al.,* 2008). Immobilized whole-cell systems have already been successfully utilized in producing several extracellular enzymes by microorganisms for example, α-amylase (Duran-Paramo *et al*., 2000); Lipase (Ferrer and Sola, 1992), Protease (El-Aassar *et al.,* 1990), Cellulase (Xin and Kumakura, 1993) and (Mamo and Gessesse, 2000). The production of extracellular enzymes by immobilized cells possesses the following advantage over freely suspended cells. Immobilization of whole cells is likely to

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enhance enzymes' stability by retaining them of their natural surroundings during immobilization and subsequent continuous operation (Tallur *et al*., 2009). The immobilized cell systems tend to tolerate local perturbations such as fluctuations in pH, temperature, the existence of inhibitor compounds, etc. (Tallur *et al*., 2009 and Zheng *et al*., 2009). Although much interest has been focused on the use of immobilized *Streptomyces sp*. for the production of extracellular hydrolytic enzymes such as xylanases (Beg *et al.,* 2000) and pectinases (Kuhad *et al.,* 2004), no reports have appeared in the literature documenting the utilization of cell immobilization technique for the production of L-methioninase from *Streptomyces Sp*. Given the importance of L-methioninase, along with the need to develop economic strategies to improve enzyme production with the overall aim of reducing the expense of industrial processes, whole-cell immobilization can serve as an excellent alternative for improving L-methioninase productivity. Hence, the present work aimed to study the practicability of L-methioninase production from the whole-cell immobilization of *Streptomyces MDMMH4* using entrapped and microencapsulated procedures to improve enzyme productivity. The rates of the production of L-methioninase by the cells immobilized in various matrices were evaluated and compared with that of freely suspended cells of *Streptomyces MDMMH4*. The reusability of immobilized cells for continuous enzyme production under repeated batch conditions with the best matrix will be carried out. On the other hand, the antioxidant activity of the purified enzyme will be evaluated.

MATERIALS AND METHODS

Chemicals and reagents

The source of chemicals and reagents used in this study were as follows: L-methionine was obtained from (Merk, Germany). Pyridoxal-5-phosphate (PLP); five, 5-Dithiobis-2- nitrobenzoicacid (DTNB); Commassi Brilliant Blue G-250; Polyvinyl alcohol; Chitosan; Gelatin and Glutaraldhyde were obtained from Sigma-Aldrich (Sigma, St. Louis, USA). Sodium alginate and Agarose were purchased from (Bio-basic, Canada). All other chemicals were of the highest analytical grade.

L-methioninase assay

Streptomyces MDMMH4 was deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases with accession number: LC021308. This strain was previously isolated from Egyptian soil and chosen as the most potent L-methioninase producer (Selim *et al*., 2015 and Selim *et al.,* 2016). The enzyme production medium used is a modified starch medium consisting of It's contained (g/L): Starch, 20; L- methionine, 3; MgCl2 anhydrous, 1.75; CaCO3, 3 and yeast extract, 0.5. In addition, the growth medium was adjusted to pH 7 using potassium phosphate buffer 0.075 M. After four days of growth with shaking at 150 rpm (New Brunswick, USA), cells were harvested by centrifugation (5500 rpm, Herml, Germany). The clear supernatant was used as a crude enzyme. L-Methioninase activity was determined by the method (Arfi *et al.,* 2003 a, Abdelraouf *et al.,* 2019) using L-methionine as a substrate. Methanethiol (MTL) produced from substrate reacted with 5,5 dithio-bis-2-nitrobenzoicacid added (DTNB) to form thionitrobenzoic acid was detected spectrophotometrically at 412 nm (Agilent UV/Vis. Cary-100). The assay mixture contained 20mM of L-methionine in 0.1M potassium phosphate buffer pH 7.0, 0.02mM PLP, 0.25mM DTNB and the supernatant in a final volume of 1ml. One unit (U) of L-methioninase was expressed as the amount of enzyme that releases 1µmole of methanethiol per minute under optimal assay conditions.

Preparation of cell suspension

The spore suspension was prepared from a 6-day-old culture grown on Starch-nitrate agar slants by adding 10ml of sterile distilled water containing 0.01% (v/v) Tween 80 and suspending the spores with a sterile loop (Lingappa *et al.,* 2009). This spore suspension containing 4x107 CFU/ml (equivalent to 100mg/ml cell wet weight) was used as an inoculum for immobilization and free cell fermentation studies. The cells were immobilized in different matrices utilizing (entrapment and microencapsulation procedures). The entrapment procedure includes sodium alginate (SA); agar, agarose; polyvinyl alcohol (PVA); Sodium alginate-polyvinyl alcohol (SA-PVA); polyacrylamide and gelatin while, microencapsulate procedure, include starch and chitosan. Details are shown below.

Entrapment procedures

Entrapment of cells in Alginate

Entrapment of cells in alginate is one of the simplest and the most frequently used non-toxic and cheapest methods of immobilization for whole cells (Palmieri *et al*., 1994). The entrapment of cells was carried out by the principle of ionotropic gelation (Johnsen and Flink, 1986). Sodium alginate and calcium chloride were used to prepare the whole cells' alginate beads. Sodium alginate solution (3% w/v) was prepared by dissolving sodium alginate in hot water. The contents were stirred vigorously for 10 min to obtain a thick uniform slurry and sterilized by autoclave. A 10% level of cell suspension was transferred aseptically into sodium alginate solution and mixed well. The alginate mixture and cell mass were taken into a sterile tube and extruded gently drop-wise into a sterile 0.2 M calcium chloride solution from 1cm height. The prepared beads were kept for 1h in the refrigerator for curing. After curing, calcium chloride was decanted and the beads were washed with sterile distilled water to 3-4 times and used for production studies (Veelken and Pape, 1982).

Entrapment of cells in Agar or Agarose

A definite quantity of agar-agar or agarose was dissolved in distilled water to get a final concentration of 2% w/v and sterilized by autoclaving. To the molten agar-agar tube maintained at 40°C, a 10% v/v level of cell suspension was added and shaken well for a few seconds, without forming foam or bubbles and then poured into a sterile flat-bottom Petri plate and allowed to solidify. After solidification, the agar-agar or agarose was cut into equal size cubes (4 mm3) and the cubes were added to a sterile 0.1M potassium phosphate buffer (pH 7.0). The cubes were kept for 1h in the refrigerator for curing. After curing, phosphate buffer was decanted and the cubes were washed with sterile distilled water 3-4 (Veelken and Pape, 1982) and used for production studies.

Entrapment of cells in polyvinyl alcohol (PVA)

Immobilizing cells in PVA-H3BO3 gel was done as per the method described by (Wu and Wisecaver, 1991; Abdelraof *et al*., 2020) grams of PVA was dissolved in 90ml of distilled water and heated to boil for complete dissolution. This solution was sterilized and brought to room temperature. 10% v/v level of cell suspension was poured into the cooled PVA and agitated thoroughly with a pre-sterilized glass rod for the uniform distribution of cells. This mixture was taken in a sterile syringe and extruded into a cold saturated solution of boric acid from a height of 20cm and allowed for 24hr for hardening. These beads were washed thrice in sterile distilled water used to produce L-methioninase.

Entrapment of cells in sodium alginate-polyvinyl alcohol (SA-PVA)

The SA-PVA entrapment of the cells was performed according to (Whang and Hu, 2007). PVA (6%, w/v) and SA (4%, w/v) was dissolved in boiling water and autoclaved at 121°C for 15 min. A 10% v/v level of cell suspension was added to sterilize SA-PVA solution (4% and 6%, w/v) and mixed by stirring on a magnetic stirrer. This SA-PVA cell mixture was extruded drop by drop into a cold, sterile CaCl2 (0.2M) solution. Gel beads of approximately 3mm diameter were obtained. The beads were hardened by re-suspending in a fresh CaCl2 solution for 2h with gentle agitation, then frozen for 2 days. Finally, these beads were washed several times with sterile double distilled water and stored at 4˚C for further investigations.

Entrapment of cells in Polyacrylamide

According to (Veelken and Pape, 1982), the polyacrylamide gel entrapment procedure was performed. About 10% V/V level of cell suspension was suspended in 10ml distilled water and chilled in ice. 10ml of 0.2M potassium phosphate buffer, pH 7.0 was also chilled in ice. This buffer adds 2.85g acrylamide, 0.15g bisacrylamide and 10mg ammonium persulphate and mixed dissolve the solids. Immediately, the chilled buffer solution was mixed with the chilled cell suspension, followed by the addition of 10µl of TEMED and poured into 2 or 3 glass Petri dishes. Thereafter allowed polymerization to proceed for 1h; then, the sieved gels were suspended in 100ml of 0.2M potassium phosphate buffer pH 7.0 and allowed to settle. Then were decanted and the gel was decanted and used for L-methioninase production.

Entrapment of cells in Gelatin

Gelatin was accurately weighed to prepare 20% w/v saline solution by gentle heating and sterilization by autoclaving (Adinarayana *et al.,* 2005). To the gelatin solution, 10% v/v level of cell suspension and 0.5ml of 10% glutaraldhyde solution were added, mixed well and poured into a sterile flat-bottom Petri plate. After solidification, the Gelatin entrapped cells were cut into equal size cubes (4 mm3) and cubes were kept in the refrigerator (4°C) for 6 h for curing. These cubes were washed thoroughly with sterile distilled water to remove excess glutaraldhyde and used for production studies ultimately.

Microencapsulation procedure

Coating with starch

The encapsulation way for making microcapsules was a modified version of methods reported by (Donthidi *et al.,* 2010 and Sultana *et al.,* 2000). Briefly, 2g of maize starch was added to 100mL distilled water and boiled until it formed a gel, then sodium alginate and inulin (1%) were added and stirred until they were dissolved or dispersed. Then the cell suspension of *Streptomyces MDMMH4* (10% V/V level of cell suspension) was transferred to the carrier solutions with stirring under sterile conditions to ensure uniform distribution of the cells. The final mixture was suspended in 500mL vegetable oil containing 0.2% tween 80 and mixed (350 rpm for 20 min, Daihan Stirrer, Korea) until it appeared creamy. Capsules were prepared by adding 200mL 0.1M calcium chloride into the mixture and the phase separation of oil/water emulsion occurred. The mixture was allowed to stand for 30 min to settle calcium alginate capsules in the bottom of the beaker at the calcium chloride layer (Water phase). The oil layer was drained and capsules in calcium chloride solution were harvested by low-speed centrifuge at 350g for 10 min and kept in 0.1% peptone solution at 4°C.

Coating with chitosan

The Chitosan aqueous solution was prepared according to (Krasaekoopt *et al*., 20040) In brief, Chitosan (Low molecular weight, Sigma-Aldrich) was dissolved in 90ml distilled water acidified with glacial acetic acid to achieve a final Chitosan concentration of 0.4% (w/v). The pH was then adjusted to between 5.7 and 6 by adding 1m NaOH. The mixture was filtered through filter paper (Whatman No. 41) and autoclaved at 121°C for 15 minutes.

Then 20g of washed microcapsules (alginate-gelatinized starch) were immersed in 100ml of Chitosan solution and

shaken at 200rpm for 40 min on an orbital shaker for coating. The Chitosan-coated microcapsules were washed with peptone solution and kept in 0.1% peptone solution at 4°C. All the above operations were carried out aseptically under sterile conditions in the laminar airflow unit.

Release of entrapped and encapsulated cells

The polymer containing *Streptomyces* was released by citrate-phosphate buffer (pH= 6.0, 1 %) reported by (Mokarram *et al*., 2009). One gram of the beads or blocks was transferred to a 9mL buffer. The solution was stirred vigorously on a shaker for 15 min (Edison, New Brunswick, USA) until bacteria were released from the matrix ultimately. The counts (CFU/g) were determined by plating on Starch nitrate agar plates and incubating for 72h at 30^{o}C. The free bacteria were treated similarly. All samples were counted in triplicates.

Comparison of L-methioninase production of free and immobilized cells

The immobilized cells obtained as above were used to inoculate a 250ml Erlenmeyer flask containing 30ml of the production medium. The composition of the production medium was the same as that optimized for L-methioninase production with free cells, except that 0.5g/L of $CaCl_2$ anhydrous was added to keep the beads intact during prolonged operation case of alginate matrices. Simultaneous experiments with free cells equivalent to those used in immobilized cultures were also conducted. Batch fermentations with free and immobilized cells were carried out in a rotary shaker for 96h at 30^{o}C and 150rpm. Aliquots (1ml) were withdrawn at regular time intervals of 12h. The culture supernatant obtained after centrifugation at 8,000 rpm for 20 min was used as the enzyme preparation (Anisha and Prema, 2008).

The effectiveness factor= Cimm/Cfree

Where Cimm is the L-methioninase yield produced by immobilized cells and Cfree is the L-methioninase yield produced by free cells.

Repeated batch fermentation with immobilized cells:

Optimized immobilization conditions were selected for effective cell immobilization in repeated batch fermentation. Repeated batch fermentation was conducted by running each fermentation cycle. At the end of each fermentation cycle, the matrix collected from the spent medium was washed with sterile distilled water and transferred aseptically to a fresh production medium to continue the fermentation (Anisha and Prema, 2008). The following equation determined the operational stability of the immobilized system.

Operational efficiency (%) = 100 x (Cx/C1)

Where C1 is the L-methioninase yield produced in the first (1st) fermentation cycle and Cx is the L-methioninase yield produced in the (Xth) fermentation cycle.

Storage stability

The stability of freely suspended cells and immobilized cells was tested after storage for up to three months at 4^{o}C was tested.

Cell growth and Cell leakage estimated

Growth in the medium surrounding the immobilized beads or blocks was the result of cell leakage and the subsequent growth of these cells. Both cell growth in freely suspended cultures and cells leaked from the gel matrix was determined as cell wet weight by measuring the colony forming unit (CFU/ml). The CFU was then converted into wet weight mg/ml.

Purification of L-methioninase

L-methioninase purification scheme was applied according to our previous report of Selim *et al*. (2016) and Neven Ahmad Salah *et al*. (2023).

Antioxidant activity of purified L-methioninase

The antioxidant activity of L-methionine γ-lyase was determined by the DPPH free radical scavenging assay according to Yen and Duh (1994).

DPPH Radical scavenging activity

Freshly prepared (0.004% w/v) methanol solution of 2,2-diphenyl-l-picrylhydrazyl (DPPH) radical was prepared and stored at 10°C in the dark. A methanol solution of the test compound was prepared. A 40µl aliquot of the methanol solution was added to 3ml of DPPH solution. Absorbance measurements were recorded immediately with a UV-visible spectrophotometer (Milton Roy, Spectronic 1201). The decrease in absorbance at 515 nm was continuously determined, with data recorded at 1 min. Intervals until the absorbance stabilized (16 min). The absorbance of the DPPH radical without antioxidant (control) and the reference compound ascorbic acid were also measured. All the determinations were performed in three replicates and averaged. The percentage inhibition (PI) of the DPPH radical was calculated according to the formula: PI = [{(*A*C-*A*T)/*A*C} x 100]

Where *A*C= Absorbance of the control at t = 0 min and *A*T= absorbance of the sample + DPPH at t = 16 min.

The IC_{50} value of each antioxidant assay was calculated and expressed as µg/mL.

STATISTICAL ANALYSIS

All experiments were carried out in triplicate and their results are presented as mean± standard deviations (SD) at P ≤0.05 by software Prism 9 using ANOVA test.

RESULTS

Cell immobilization is one of the standard strategies for increasing overall cell concentration and continuous productivity. The division of items from immobilized cells is easier contrasted with the suspended cell system (Muharram, 2016). L-methioninase generation by immobilized *Streptomyces MDMMH4* in a different matrix, including entrapment (for example, Ca-alginate; gelatin; polyacrylamide; polyvinyl alcohol; agar and agarose) and microencapsulation (for example, starch and chitosan) were contrasted and that of freely suspended cells.

Table 1 shows the comparative L-methioninase production pattern by free and immobilized cells of *Streptomyces MDMMH4* by different matrices along with cell leakage. The estimation of enzyme production was carried out at 12-h intervals. The free cell system started the enzyme production after 48h and reached a maximum activity of (214 U/ml) at 96h of incubation, On further incubation, the enzyme activity gradually decreased, this may be due to the depletion of essential nutrients required for the growth of the organism (Lingappa *et al.,* 2009) Moreover, the accumulation of metabolites in the culture medium led to an increase in medium pH to an alkaline condition which also decreased the stability of enzyme (Anisha and Prema, 2008).

Considering the data obtained for L-methioninase generation by *S. MDMMH4* cells in various matrices and by free cells noticed in table 2, agarose is displayed as the best support for immobilization and possesses some desirable features over other gels. For example, it does not require polymerization like polyacrylamide as well as does not need any cations like alginate. Subsequently, the agarose gel continued the work, because of security; high efficiency; the minimal effort of reagents and the effortlessness of the immobilization procedure.

Optimization of immobilization process in agarose

Optimizing the immobilization parameters offers the advantage of improving the matrix characteristics, such as permeability and rigidity. Among various supporting matrices studied for whole-cell immobilization of *S. MDMMH4*, agarose was found to be a better entrapment matrix for L-methioninase production. The effect of various concentrations of agarose, the effect of inoculum size (block weight) the effect of cell concentration (cell loading in the matrix) and the effect of curing time on L-methioninase production was carried out.

Effect of agarose concentration on cells stability and L-methioninase productivity

To decide the best concentration of agarose, various concentrations of agarose (1-5%) (W/V) were utilized to set up the gel. From the data obtained in fig. 1, it was observed that the most noteworthy L-methioninase formation (271 U/ml) was acquired with blocks arranged by utilizing 3% (W/V) agarose, $P \leq 0.05$.

Effect of cell concentration (cell loading in a carrier) on cells stability and L-methioninase productivity

Another factor to be considered in the efficiency of the immobilized system is the initial cell concentration that can be loaded in the gel; six different cell concentrations (50-300mg/ml wet weight in gel) were used for L-methioninase production. L-methioninase productivity by immobilized cells depended on the initial cell concentration in the agarose matrix (fig. 2), $P \leq 0.05$. An increase in initial cell concentration resulted in maximum production of L-methioninase (290U/ml) was achieved at 150mg/ml wet cell weight in blocks. As the cell loading in the gel blocks was increased (200-300mg/ml), the cell leakage into the fermentation medium also increased and L-methioninase production was decreased correspondingly. The low rate of enzyme activity with increased cell loading in the gel blocks might be due to competition between cells. The nutrient concentration available in the medium might not have been sufficient for optimal growth. Thus, the competition between cells would have led to rapid substrate use, resulting in a smaller duration of enzyme activity.

Effect of inoculum size (block weight) on cells stability and L-methioninase productivity

To investigate block size on L-methioninase creation, five unique sizes of the agarose carrier (2-10 g) were prepared to utilize various amounts of agarose before filling plates and utilized for L-methioninase generation. The data appeared in fig. 3, $P \leq 0.05$, and demonstrated that 2 g of immobilized cells (blocks) was the most reasonable inoculum level showing the greatest L-methioninase generation (290 U/ml). In the meantime, the increase in the weight of blocks in the cultivation medium resulted in weak L-methioninase productivity. In addition, smaller agarose sizes led to diminished cell leakage into the cultivation medium compared with bigger sizes.

Effect of curing time for agarose (blocks stability) on cells stability and L-methioninase productivity

To adjust the curing time of agarose for better L-methioninase generation, blocks were arranged with 3% agarose and loaded with 150mg/ml wet cell weight as starting cell concentrations were cured for various times ranging from 2 to 24h (fig. 4) , $P \leq 0.05$ demonstrates that the blocks cured for six h were stable and gave the best yield of L-methioninase (312U/ml). It was shown that the prolongation of hardening time from 2h to 6h resulted in enhanced dependability of blocks, which brought about decreased cell leakage. After six h, the stability of blocks increased with an increase in curing time, bringing about more challenging blocks but with less L-methioninase generation. Therefore, the curing time of 6 is thought to be ideal for the arrangement of stable blocks and better-methioninase formation.

Table 1: Comparative analysis of free and immobilized cells of *Streptomyces MDMMH4* entrapped or encapsulated in the different matrices for L-methioninase production.

Carrier	Time course of L-methioninase production (U/ml) by immobilized and free cells of *S.* DMMHM4 and cell leakage (mg/ml)/ hour											
	36		48		60		72		84		96	
	Activity (U/mL)	Leakage mg/mL	Activity (U/mL)	Leakage mg/mL	Activity (U/mL)	Leakage mg/mL	Activity (U/mL)	Leakage mg/mL	Activity (U/mL)	Leakage mg/mL	Activity (U/mL)	Leakage mg/mL
Ca-alginate	29	0.15	81	1.3	137	2.2	290	3.6	261	4.7	253	5.5
Agarose	0	0	19	0.14	91	1.1	260	1.9	252	2.1	241	2.1
Agar	0	0	6	0.38	47	0.8	224	1.4	220	1.5	220	1.7
Ca-alginate-PVA	11	0	33	0.68	50	1.3	168	1.8	159	2.9	152	4.6
Gelatin	0	0	55	0.94	140	1.6	149	3	122	3.2	94	3.2
Polyvinyl alcohol (PVA)	0	0	0	0	12	0.8	22	1.4	43	1.5	37	1.8
Polyacrylamide	0	0	0	0	0	0	0	0	0	0	0	0
Ca-alginate-gelatinized starch	0	0	0	0	81	0.28	137	0.3	197	0.37	195	0.4
Chitosan	0	0	0	0	39	0.12	121	0.2	179	0.55	188	0.7
Free cells (control)	0	0	41	0	99	0	168	0	190	0	214	0

C.L: Cell leakage (mg/ml wet cell weight)

Table 2: Effectiveness factor for each matrix.

immobilized and free cells	Maximum activity (U/ml)	Hours	Effectiveness factor (Cimm/Cfree)
Free cells	214±22	96	1±0.1
Agarose	260±23	72	1.21±0.3
Agar	224±19	72	1.04±0.2
Ca-alginate-PVA	168±15	72	0.78±0.1
Gelatin	149±16	72	0.69±0.1
Polyvinyl alcohol	43±6	84	0.20±0.02
Poly acrylamide	0	0	0
Ca-alginate- starch	197±15	84	1.03±0.2
Chitosan	188±17	96	0.83±0.1
Ca-alginate	290±26	72	1.35±0.2

Table 3: L-methioninase production by agrose-entrapped cells in repeated batch fermentation. Operational Efficiency %= 100 x (Cx/C1)

Cycles	Agarose		
	Activity (U/ml)	Operational Efficiency %	Cell leakage (mg/ml wet cell weight)
1	312±33	100	1.5±0.4
2	312±32	100	2.3±0.6
3	312±31	100	4±0.3
4	312±30	100	5.6±0.8
5	312±31	100	7.4±0.9
6	312±32	100	9.1±0.9
7	307±30	98.2	12.2±0.7
8	297±29	95.3	16.6±1
9	285±24	91.3	19.9±2
10	265±26	84.9	25.8±3
11	245±27	78.6	28.7±2
12	216±25	69.3	32.4±4
13	157±16	50.2	38.5±3
14	101±11	32.3	47.6±5

Where C1 is the enzyme yield produced in the first cycle and Cx is the enzyme yield produced in the X^{th}cycle.

Table 4: Continuous production of enzyme by immobilized cells and comparison with free cells.

Conditions	Free cells	Immobilized cells on agarose matrix
Fermentation period for each batch/ (hour)	96	72
Number of batches/ (cycle)	1	14
Total fermentation time/(day)	4	42
Total L-methioninase production/(U/ml)	214	3745
Normal volumetric productivity (U/ml/h)	2.2	52

Table 5: Evaluation of the Antioxidant activity of purified L-methioninase using DPPH scavenging.

Sample conc. (µg)	L-methioninase	Reference standard (Ascorbic acid)
	DPPH scavenging %	DPPH scavenging %
800	69.6	100.00
600	61.7	100.00
400	51.1	100.00
200	43.2	100.00
100	32.72	100.00
50	20.37	89.15
25	12.08	55.08
12.5	9.33	21.13
6.25	5.18	11.78
3.125	0	6.44
0	0	0
IC50	390.4µg/ml	14.2µg/ml

To date, the present study is the first study investigating the potential use of L- methioninase as an antioxidant. (Mousumi and Dayan, 2013) found that, The IC50 value of L-glutaminase was 601.13µg/ml in *Streptomyces enissocaesilis* while in the present study IC50 value of L-methioninase enzyme showed significant activity than the earlier reports 390.4µg/ml (11.62 U/mL).

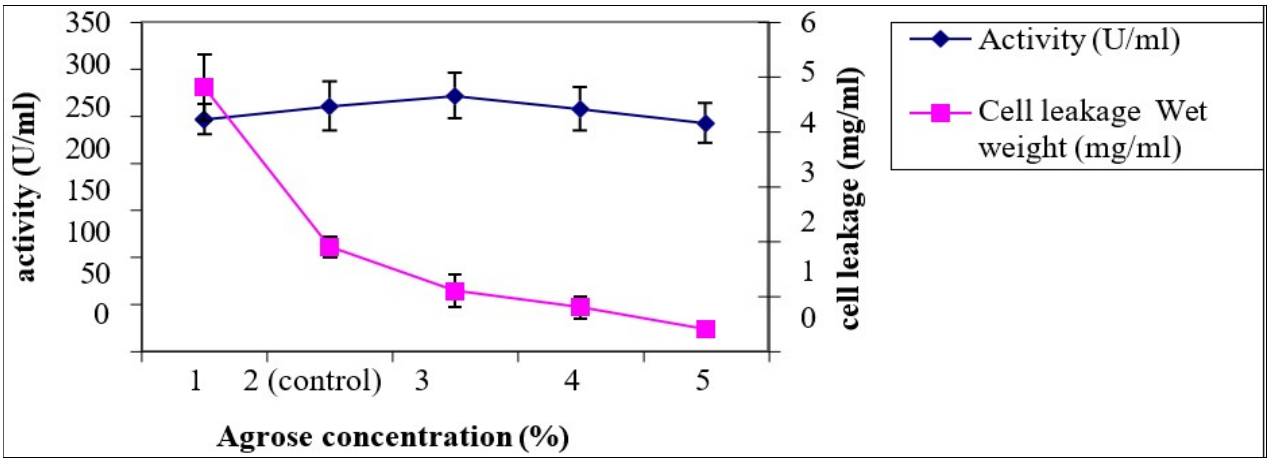

Fig. 1: Effect of agarose concentration (W/V) on cell stability and L-methioninase productivity. P ≤0.05

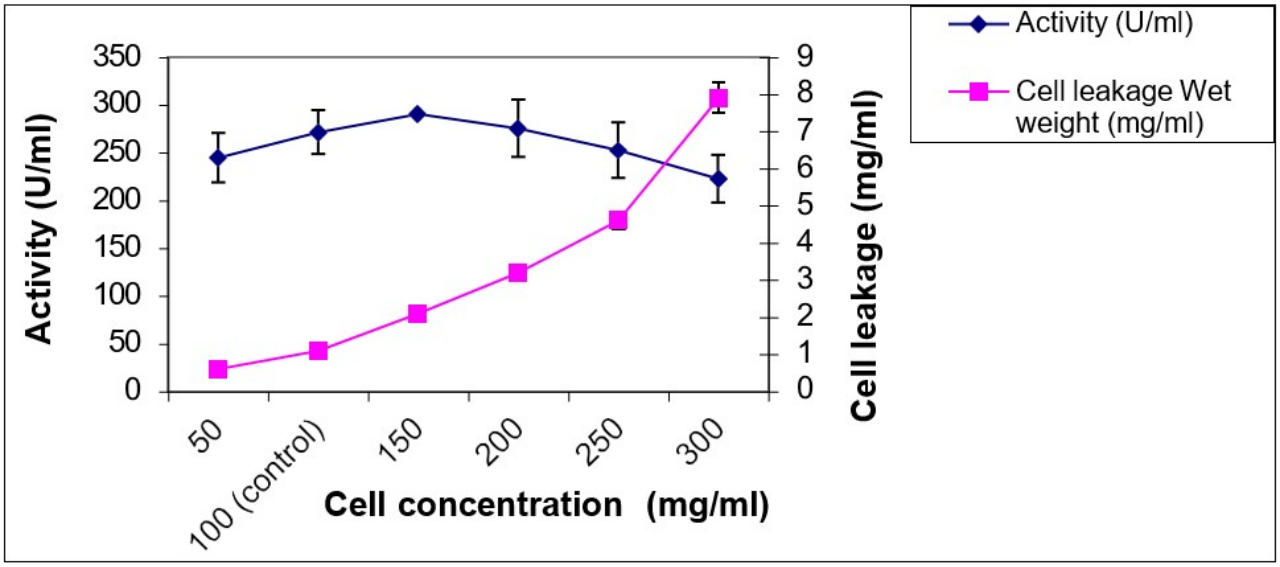

Fig. 2: Effect of cell concentration (mg/ml) on cell stability and L-methioninase productivity. P ≤0.05

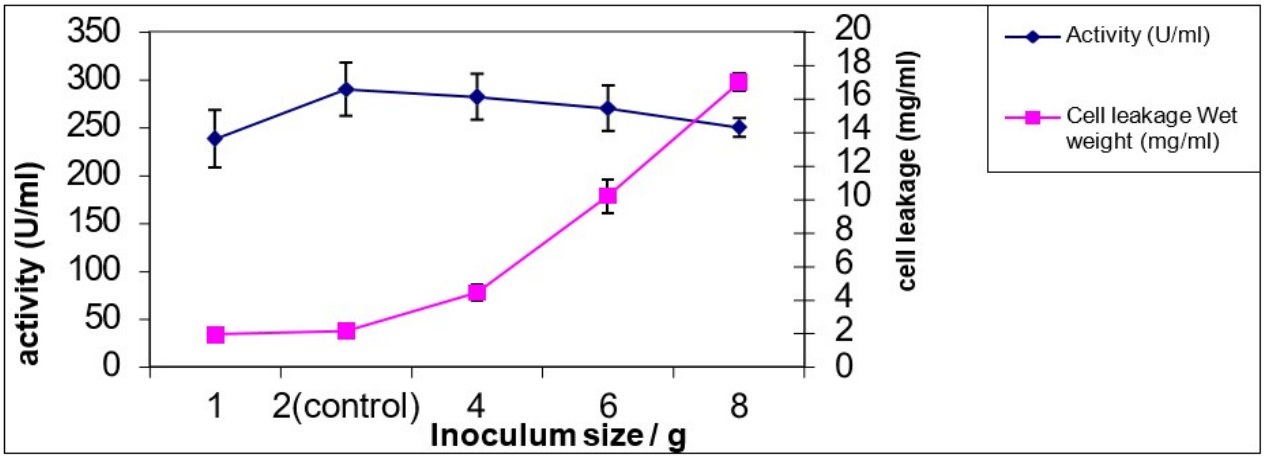

Fig. 3: Effect of inoculum size on cell stability and L-methioninase productivity. P ≤0.05

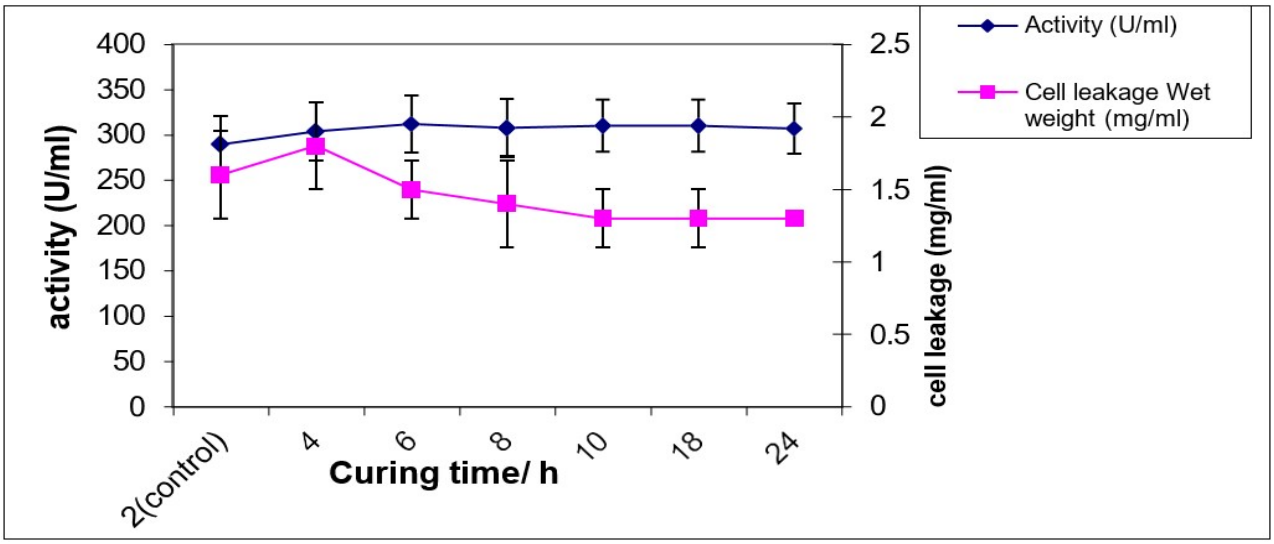

Fig. 4: Effect of curing time for agarose on cell stability and L-methioninase productivity/ h. P ≤0.05

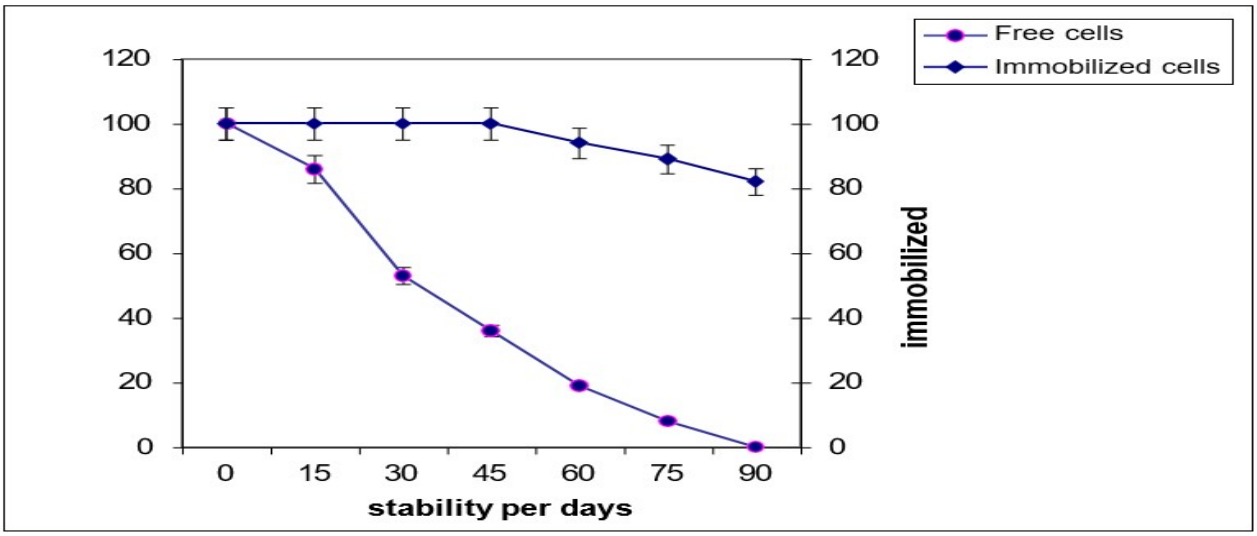

Fig. 5: Effect of storage stability on L-methioninase production by free and immobilized cells. P ≤0.05

L-methioninase production by repeated batch cultures: reusability test of immobilized blocks

The possibility of re-using immobilized cells of *Streptomyces MDMMH4* for L-methioninase production was studied over 42 days, corresponding to 14 cycles of fermentation, as shown in table 3. The duration of a fermentation cycle chosen for repeated batch fermentation was 72h. The results of semi-continuous production showed that the immobilized blocks could be re-used effectively for L-methioninase production in six cycles without any apparent loss of enzyme yield (312U/ml). After the six cycles, there was about an 8.7% loss in activity with each consecutive use till the nine cycles. L-methioninase production by immobilized cells remained in the range of (285U/ml) throughout the nine cycles, which was still higher than that obtained by free cells (214U/ml). Even after twelve successive and efficient fermentation operations, the agarose blocks had good stability and maintained 69.3% of the enzyme yield obtained in the first cycle. At the same time, gradual cell leakage from the agarose blocks was observed from the first cycle to the twelve cycles.

Results of the total L-methioninase production with free and immobilized cells were exhibited in table 4. The data clearly showed that the average volumetric productivity with the immobilized cells was (52U/ml/h), while it was (2.2U/ml/h) in the case of free cells. Improved L-methioninase profitability of 50U/ml/h was accomplished with immobilized cells over the free cells.

Effect of storage stability on L-methioninase production by free and immobilized cells

One of the benefits of microbial cell immobilization is the capacity to utilize them for a long time. The storage stability of immobilized was studied under indistinguishable test conditions from above in agarose after optimization, in which cells were given the highest levels of L- methioninase. Keeping in mind the end goal to recognize the storage stability of immobilized cells, free and immobilized cells that were put away for 0, 15, 30, 45, 60, 75 and 90 days at 4°C were applied for L-methioninase generation. As appears in fig. 5, $P \leq 0.05$ immobilized cells hold a steady L- methioninase creation rate with the augmentation of capacity time. They could form 94% L- methioninase after being put away for 60 days at 4°C. In contrast, the generation rate of free cells decreased after putting them away for 30 days at 4°C and almost lost the more significant part of their action after putting them away for 60 days at 4°C. These results demonstrated that immobilized cells have more robust storage stability than free cells.

Purification of L-methioninase

The purification of L-methioninase was carried out according to our previous scheme as reported by (Selim *et al*., 2016).

Free radical scavenging activity (antioxidant activity of L-methioninase)

The antioxidant capacity of L-methioninase was investigated using DPPH assay (table 5). DPPH is a stable non-physiological radical, which could provide a relative figure of the radical scavenging capacity of a tested probe. The DPPH assay showed that *Streptomyces MDMMH4* had moderate scavenging activity with high IC_{50} values of 390.4µg/mL (11.62U/mL) compared to the scavenging activity of the standard antioxidant (ascorbic acid, IC50 14.2µg/mL).

DISCUSSION

The calcium alginate-entrapment technique increased the production of L-methioninase (290 U/ml) after 72h of the incubation period, which was 35% more than the free cells with cell leakage (3.6mg/ml). L-methioninase production by cells immobilized in calcium alginate (290 U/ml) was more significant at p-value at $P \leq 0.05$ than for cells immobilized in calcium alginate combined with other matrices i.e. starch (197U/ml) at 84 h; chitosan (188 U/ml) at 96 h and polyvinyl alcohol (43 U/ml) at 84h with cell leakage (0.37; 0.7 and 1.5mg/ml respectively). The lowest enzyme production might be due to the attempt to form starch, chitosan, or polyvinyl alcohol around the alginate matrix. Entrapped or encapsulating a more significant number of cells may have decreased the transfer of cells to the fermentation medium and a subsequent decrease in enzyme production (Delani *et al.,* 2012).

In addition, the immobilized beads prepared with sodium alginate such as calcium alginate, calcium alginate-starch, calcium alginate-PVA and chitosan have an inherent problem of dissolving in a phosphate solution. Fast disintegrated in the fermentation medium, instability of Ca-alginate beads are attributed to the presence of potassium phosphate with a high concentration in the production medium that tends to dissolve the beads (Bajpai and Sharma, 2004).

When potassium phosphate concentration was decreased or depleted from the medium, Ca- alginate beads were stable, but the enzyme yield declined remarkably. The results demonstrated that all alginate matrices fast disintegrated in the production medium and proved unstable. Our findings are in agreement with (Bisht *et al.,* 2013) who recorded the Instability of Calcium alginate beads in a production medium containing potassium phosphate. In this respect Anisha and Prema (2008) reported a two-fold increase in α-galactosidase production by *Streptomyces griseoloalbus* using calcium alginate as a support matrix.

Relative good productions were obtained with agar and agarose-entrapped cells, which started the enzyme

production after 48 h and gradually approached maximum yield (224 and 260 U/ml) at 72 h which was 4% and 21% more compared to freely suspended cells. The cell leakage from these matrices was (1.9 and 1.5mg/ml). The maximum enzyme production in immobilized cells required only 72 h, whereas the free cells required 96 h. (Lingappa *et al.,* 2009) reported a considerable reduction in fermentation time and an increase in the L-asparaginase production with immobilized cells of *Streptomyces gulbargensis*.

Gelatin and polyvinyl alcohol entrapped cells exhibited (149 U/ml and 43 U/ml)of enzyme production after 72h and 84 h of incubation which was less than free cells with (31% and 80%). A low level of enzyme production was observed with gelatin and polyvinyl alcohol, which contained glutaraldhyde and boric acid as binding agents. The result suggested that the negative influence of glutaraldhyde and boric acid on enzyme activity may be attributed to the biocidal activity of these compounds led to poor viability of cells (Bisht *et al.,* 2013). This subject [Miner *et al*., 1977 and Rutala, 1991] reported that glutaraldhyde readily alkylates sulfhydryl, hydroxyl, carboxyl and amino groups of nucleic acid and proteins. The amine groups present on bacteria's outer cell wall and cell membrane are cross-linked with glutaraldhyde or boric acid. This is thought to be responsible for their efficacy against bacteria (Chambon *et al.,* 1992 and Simons *et al*., 2000). The utilization of agarose as a support matrix for lipase production from *pseudomonas aeruginosa* was studied by Bisht *et al.* (2013), who showed the negative effect of glutaraldhyde on enzyme activity when used as a binding agent. Abdel-Fattah *et al.* (2008) reported that glutaraldhyde as a binding agent exerted an adverse effect on levansucrase production by *Bacillus circulans* cells entrapped in agar.

Furthermore, no development was seen if there should be an occurrence of polyacrylamide entrapped cells and this might be because of the powerlessness of cells for development with polyacrylamide monomers, (for example, TEMED) which was the excessively toxic impact for cells (Veelken and Pape, 1982).

Blocks arranged with 4% and 5% focuses were harder than different concentrations and rigidity was enhanced. However, L-methioninase yield decreased because of diffusional limitations that restricted the movement of supplements into the gel and enzymes out of the gel into the medium (Ellaiah *et al.,* 2004) Furthermore, blocks arranged with 1% was delicate and the cell leakage into the medium was observed to be more when compared with different concentrations and the blocks broke down quicker. Bisht *et al.* (2013) reported that the high lipase generation was gotten with blocks arranged by utilizing 2% (w/v). Similarly, Joseph *et al.* (2006) likewise reported that the gel arranged with 3% was more reasonable for lipase generation from *Staphylococcus epidermidis.*

Effect of cell concentration (cell loading in a carrier) on cells stability and L-methioninase productivity

Our findings are supported by Jouenne *et al.* (1993) who reported that, the low concentrations of immobilized cells led to rapid enzyme production, while high concentrations caused diffusion limitation of nutrients. In addition, a very high level of cell loading in the beads was less active because the porous structure of the beads was lost (Cheetham *et al*., 1985).

Effect of inoculum size (block weight) on cells stability and L-methioninase productivity

Our results agree with Bisht *et al.* (2013), who found that 2g of immobilized cells (blocks) in 50ml of medium was the most appropriate inoculum level displaying the most excellent lipase yield. They recommended that the rise in the weight of blocks led to a decrease in alkaline lipase efficiency. This could be ascribed to the way that when the weight of blocks increased, the supplement/block proportion decreased, thus declining the lipase generation (Massalha *et al.,* 2007).

The impact of curing time on the stability of the alginate matrices was researched by Anisha and Prema (2008). They found that the prolongation of hardening time from 2 to 10h prompted to enhanced dependability of beads, which led to a reduction in cell leakage and resulted in enhanced enzyme yield.

The stability of the biocatalysts and their ability to produce L-asparaginase from *Streptomyces gulbargensis* under repeated batch cultivation was investigated by Johnsen *et al.* (1986). They reached seven repeated batch cycles with polyurethane foam-entrapped cells, with the time for each batch being 72h. (Srinivasalu and Ellaiah, 2005) Studied neomycin production by *Streptomyces. marinensis* NUV-5 immobilized on polyurethane foam and obtained significant productivity of neomycin under repeated batch fermentation (up to 7 cycles; with each batch lasting 96h). Bisht *et al.* (2013) reported 11 cycles of repeated batches with agarose-entrapped cells by *Pseudomonas aeruginosa* for lipase production.

CONCLUSION

Immobilization studies of *Streptomyces* cells to improve L-methioninase production clearly showed the possibility of re-using agrose-entrapped cells of *Streptomyces MDMMH4* for L-methioninase production was studied over 42 days. The immobilized blocks could be re-used effectively for L-methioninase production in six cycles without any apparent loss of enzyme yield. The significantly high yield of L-methioninase by agarose-entrapped cells (entrapment) warrants their candidature

for L-methioninase production at a pilot scale for clinical and industrial applications. L-methioninase has different practical applications, especially in cancer therapy; our results observed significant continuous production of L-methioninase via the green immobilization approach of the *Streptomyces* MDMMH4 cells. Furthermore, the antioxidant activity of this enzyme showed that *Streptomyces* MDMMH4 L-methioninase possessed somewhat scavenging activity.

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