Original Research Article

A comparative study on microencapsulation of tryptophan synthase using *Escherichia coli* whole cells entrapped in different natural polymers

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ABSTRACT

Tryptophan (L-Trp) is a fundamental precursor to a number of drugs. In this research, three natural polymers included agar, agarose and alginate were investigated for immobilization of *E. coli* cells for L-Trp production to increase enzymatic stability. Also, the capability of beet molasses as serin (L-Ser) substitution in production reaction was investigated. According to the results, optimum conditions for L-Trp production by immobilized biocatalyst were: agar with 2 % (w/v) as support matrix, 2 g of immobilized bacterial cells as biocatalyst. The immobilized biocatalyst showed acceptable operational stability, maintaining more than 80 % of the initial activity after 5 cycles and 0.604 g/l L-Trp was produced by 2 g of immobilized cells in comparison to 0.140 g/l by 3 g of free biocatalyst. Furthermore, results showed that beet molasses can be used as a cheap source of L-Ser in the L-Trp production reaction. As a consequence, combination of immobilization and cheap substrate was successfully developed.

***Keywords*:** L-Tryptophan, tryptophan synthase, immobilization, agar, alginate, molasses

INTRODUCTION

L-Trp is a fundamental precursor to a number of drugs that used in the treatment of alcoholism, anorexia nervosa, depression, insomnia and schizophrenia [1-4]. Tryptophan synthase is widely used within the biotechnological industry for the production of L-tryptophan [5]. In previous papers, we reported that free *E. coli* cells can be used for production of L-Trp from indole and L-Ser of cane and beet molasses by the catalytic activity of tryptophan synthase [6, 7]. For further improvement of this process in point of economic view, we investigated different carriers and techniques for the immobilization of *E. coli* cells having tryptophan synthase for L-Trp production from indole and L-Ser of molasses. Microencapsulation of whole cell is a widely applicable technique for increasing the productivity due to high cell density in the production medium, protection of cells from harsh environmental conditions, reusability of the cells, and easy separation of the products from the cells [8]. Many different methods have been proposed for cell immobilization. Microencapsulation of the cells in the polymer beads is one of the most suitable techniques [9]. In the earlier researches, the polymers such as k-carrageenan, alginate, polyacrylamide, epoxy, eudragit, chitosan, polyvinyl alcohol were employed for L-tryptophan production by immobilized cells and immobilized L-tryptophan synthase enzyme [10-12]. Whole cell immobilization has many advantages over immobilized free enzyme. It could be eliminated the process of enzyme extraction and purification, and is generally relatively facile [13]. This approach could offer higher stability, and carry out cofactor requiring bioconversion [14].

In the present research, the L-tryptophan production was studied using agar, agarose and alginate as carrier matrices for immobilization of *E. coli (ATCC11303)* cells having L-tryptophan synthase activity. Alginate is widely used carrier due to its eco-friendly nature, mild conditions for immobilization and nontoxic nature. Agarose is a natural polymer that form strong gels even at low concentration due to its chemical structure. It has several characteristics such as high porosity, hydrophilic character, ease of commercial availability and absence of charged groups. Agar is a polysaccharide polymer composing of agarose and has a strong gelling capability, is acid stable and easily available, do not show protein reactivity and moreover, the cost of this matrix is low in comparison with other materials that is important in the point of industrial view [15-17]. As mentioned above agar and agarose are not used as carriers for

microencapsulation of cells for L-Trp production. In this research, the capability of cane and beet molasses as L-Ser substitution in L-Trp production by immobilized cells was investigated.

MATERIALS AND METHODS

Agar, calcium alginate, agarose, L-Ser, L-Trp, indole, and sodium chloride were from Merck (Germany). Molasses were gifts from three sugar production companies (Iran). All other chemicals and materials were of analytical grade or the highest purity commercially available.

Microorganism and culture conditions

E. coli (ATCC 11303) obtained in lyophilized form from ATCC was revitalized in nutrient broth for 24 h at 37 °C. The biomass were harvested by centrifugation at 12000 rpm for 10 min at 4 °C and after twice rinsing with normal saline, it was resuspended in normal saline. The resulting cell suspension was inoculated in a bioreactor containing medium which previously optimized with respect to L-Trp production [6]. The cells of stationary phase were harvested, frozen and then used in L-Trp production medium as a biocatalyst either in the free and immobilized forms.

Microencapsulatio methods

Preparation of alginate beads

Microencapsulation of tryptophan

1 g of biomass was mixed with 20 ml of 1.5 % and 2 % (w/v) sodium alginate solution, separately. This mixture was taken in the syringe and was dropped in the beaker containing 1.5 % (w/v) $CaCl_2$ solution. The system was kept under stirring until complete formation of the spherical beads. The beads were separated and rinsed with 1.5 % (w/v) $CaCl_2$ [18].

Preparation of agar and agarose beads

1 % and 2 % agarose solutions and 1, 2 and 3 % agar solutions were prepared in distilled water by warming them at 50 °C. After cooling down to room temperature, 1 g of biomass was mixed with 20 ml of agarose and agar solutions. The mixed solution was pipetted into vegetable oil. The beads were separated and rinsed twice with 0.1 % (v/v) tween-80 solution [19].

Production of L-Trp

The biocatalyst (either 3 g of free or 2 g of immobilized biocatalyst) was transferred to the production medium that contained components as follow: 0.05 g indole and 0.05 g L-Ser dissolved in the potassium phosphate buffer (0.1 M, pH 8) and incubated in a rotary shaker (180 rpm) at 37 ˚C [6].

Investigation of the biomass load capacity of beads

The biomass that could be immobilized into alginate, agar and agarose supports was investigated offering increasing L-Trp production (1, 2, 3, 4 and 5 mg of biomass per 20 ml of gel solution). The L-Trp production was monitored during the reaction time and at the end of the process.

Determination of time course of L-Trp production by immobilized biocatalyst

In order to determine the time course, a production process of 24 h, was performed as mentioned above, using the obtained optimum conditions: 2 g of biomass immobilized in 2 % agar beads. Samples (200 µl) of the production medium were withdrawn at 6 h intervals up to 24 h and amount of L-Trp production and indole consumption were determined.

Investigation of immobilized cells reusability

The reusability of the immobilized biocatalyst was studied to assay the L-Trp produced by the beads in the several cycles. L-Trp was checked every 12 h. The production in each cycle was calculated by taking the first batch of L-Trp production as 100 %. At the end of each cycle, the immobilized cells were collected, washed twice with sterile normal saline and then re-inoculated into a new fresh production medium.

L-Trp production from indole and molasses by immobilized biocatalyst

In order to investigate L-Trp production by immobilized biocatalyst in the presence of cane and beet molasses as a cheap L-Ser substitution, 0.05 g of L-Ser in the production medium was substituted by molasses that were processed [6].

Analytical methods

The tryptophan content of production medium was analyzed by HPLC using RP-18 column (4×300 mm) (MZ-Analysentechnik, Germany) phosphate buffer (0.05 M, pH 4.2). The measurement was carried out at 220 nm.

Enzymatic activity assay

L-Trp synthase activity was measured by a colorimetric method on the basis of the remaining indole in the supernatant fluid of production medium which was determined spectrophotometrically at 490 nm [20].

Statistical method

All the experiments were done in triplicates and the average values of the obtained data were determined. All the results were analyzed statistically by one-way analysis of variance and Tukey's test with 95 % confidence level using SPSS ,22.0.

RESULTS

Effectiveness of support matrices and their concentrations on L-Trp production

Agar, agarose, and alginate in various concentrations were investigated to achieve the highest immobilization efficiency. These support matrixes are common natural hydrogels that were used for biocatalyst immobilization [21]. Alginate is the most common carrier that used for biocatalyst immobilization. Agar and agarose also used for this purpose especially for enzymes and thermophilic bacteria immobilization. Agar is relatively cheap and preferred matrix rather than agarose and alginate for industrial applications. As shown in the figure 1, maximum L-Trp production was obtained by biocatalyst entrapped in the 2 % agar beads and biotransformation decreased with increasing agar concentration to 3 %.

Optimization of microbial load of beads

Initial microbial load of beads effects on the mass transfer in and out of beads and biotransformation yield thus different microbial loads were investigated. L-Trp production and indole consumption increased with increasing of biocatalyst load but as regard to mass transfer limitations, maximum production yield contributed to 2 g of biocatalyst entrapped in 2 % agar beads (figure 2).

Determination of time course of L-Trp production by immobilized biocatalyst

Time course of production by immobilized biocatalyst was determined. Results showed that L-Trp production and indole consumption gradually increased for up to 12 h and then production decreased due to degradation by TPase (figure 3). Therefore the best time course for L-Trp production was 12 h and L-Trp production was stopped at 12 h in subsequent tests.

Investigation of immobilized cells reusability

The immobilized cells is very important from the point of view of reducing the cost of the biocatalyst. The reusability pattern of immobilized cells is depicted in figure 4. The beads retained activity up to 5 cycles showing a subsequent decrease in production after each cycle. This result is important which would consequently improve the process efficiency, productivity.

L-Trp production from indole and molasses by immobilized biocatalyst

As mentioned above, one of the most important reason of low demand market for L-Trp is L-Ser that could be costly substrated

for L-Trp production. The results showed that Orumiyeh beet molasses was suitable cheap substitution for L-Ser for L-Trp production by immobilized biocatalyst as well as demonstrated for free biocatalyst in the previous studies [6, 7] (figure 5). It was also concluded that higher of L-Trp was produced in the presence of molasses and 0.01 g of L-Ser instead of 0.01 g of L-Ser (figure 5). Also it was observed that L-Trp production increases with increasing of molasses.

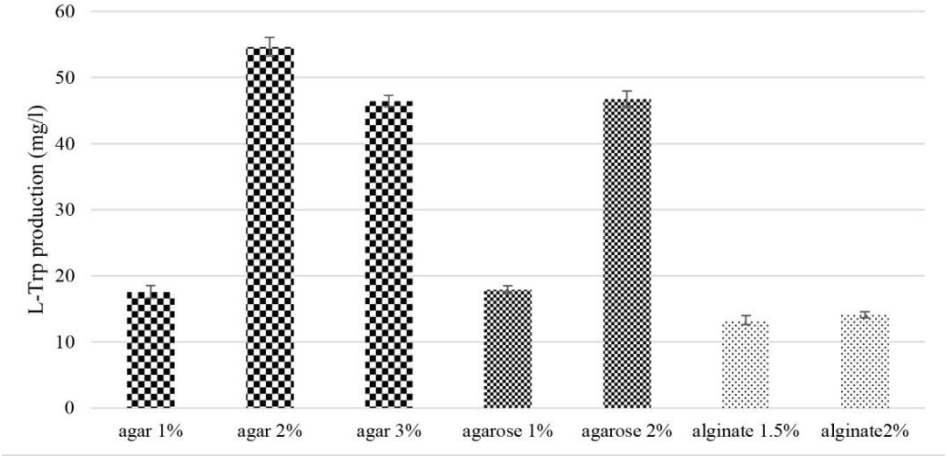

Figure 1. Effectiveness of support matrices and their concentrations on L-Trp production by E. coli cells immobilized in the different matrices in the production medium containing L-Ser and indole (0.5 g/L) incubated at 37 ˚C (180 rpm for 6 h).

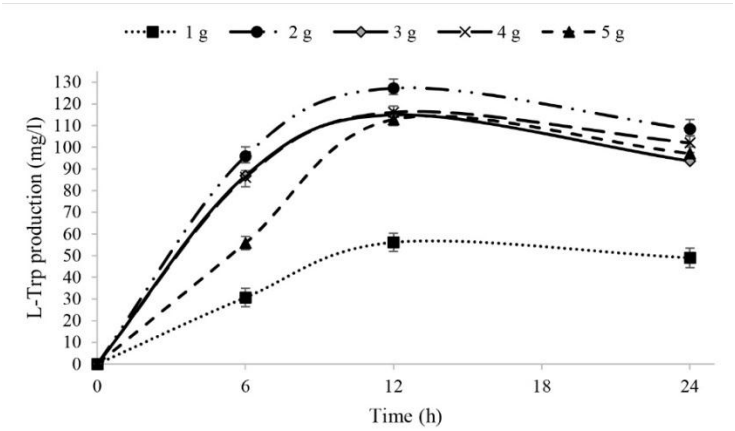

Figure 2. Investigation of the biomass load capacity of beads by 1-5 g *E. coli* biomass immobilized in the 2 % agar in the production medium containing L-Ser and indole (0.5 g/L) incubated at 37 ˚C and 180 rpm for 24 h.

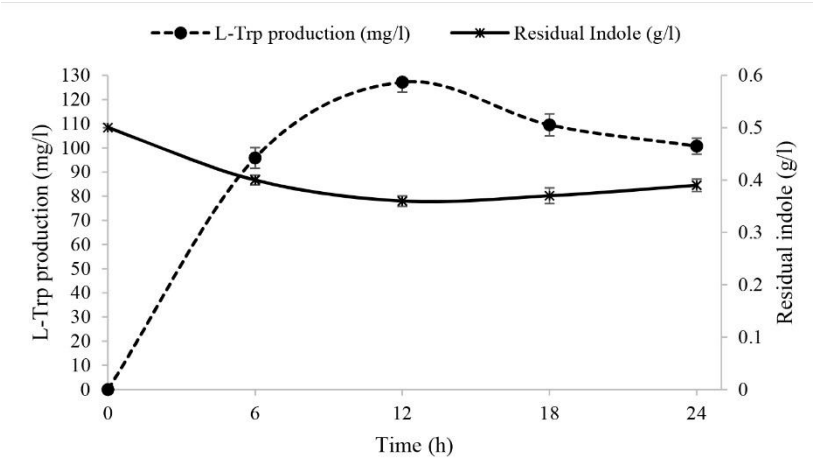

Figure 3. Time course of L-Trp production and indole consumption by immobilized *E. coli* cells in the production medium containing L-Ser and indole (0.5 g/L) incubated at 37 ˚C and 180 rpm for 24 h.

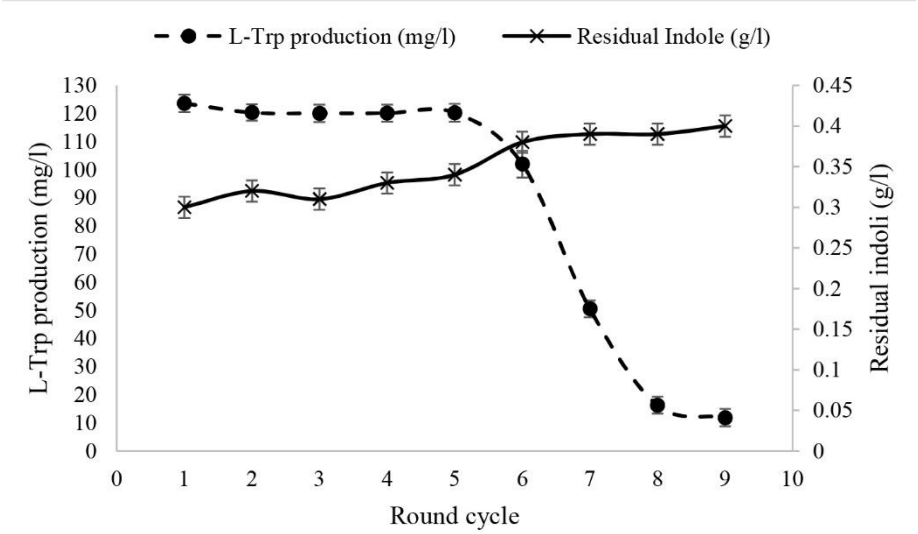

Figure 4. Investigation of reusability of immobilized *E. coli* cells in the production medium containing L-Ser and indole (0.5 g/L) incubated at 37 ˚C and 180 rpm for 12 h. At the end of each cycle, the immobilized cells were collected, washed twice with sterile normal saline and then re-inoculated into a new fresh production medium.

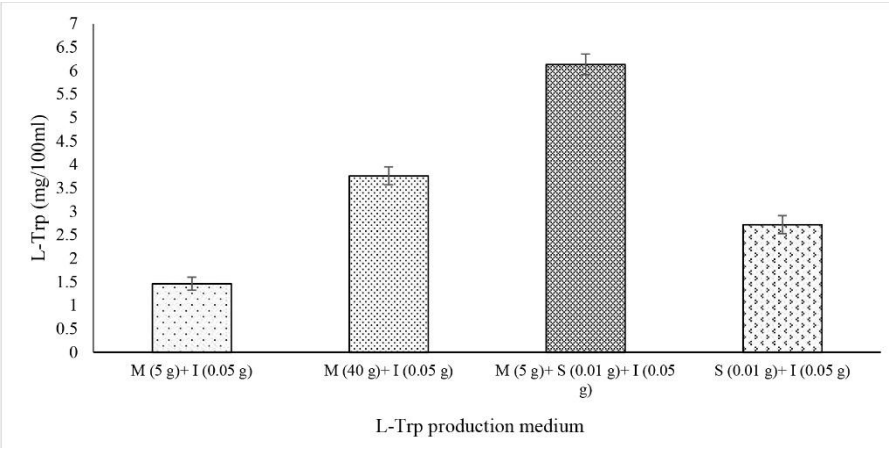

Figure 5. Investigation of L-Trp production from indole (I), molasses (M) and Serine (S) by immobilized biocatalyst.

DISCUSSION

As mentioned above, in the present research we investigated different carriers and techniques for the immobilization of E. coli cells having tryptophan synthase for L-Trp production from indole and L-Ser of molasses. Agar, agarose and alginate were used as cell immobilization matrices. Agarose 0.5 % and alginate 1 % had not been stable and lost the stability and shape gradually in the production medium. L-Trp production by cell immobilized in the 2 % agarose, 3 % agar and 2 % agarose was approximately equal and was 4-times lower than two other carriers. Reason of this observation is that the agar and agarose preserve the viability of immobilized catalyst more than alginate [22]. On the other hand, indole consumption by immobilized catalyst in the three matrices was equal. There are following probabilities for equal indole consumption and lower L-Trp production by trace amounts of poly phenols presented in the alginate have been caused the death of a little fraction of biocatalyst [23] and calcium ions presented in the alginate gelling network had entered into the cells and decreased the tryptophan synthase activity [24]. According to the results, 2 % agar was selected as optimized carrier. In the previous literatures, various polymers such as alginate was introduced as optimized carrier [11, 26]. As agar is a cheap natural polymer [18], it is important as an industrial point of view. Another advantage of the immobilization is decrease of inhibitory effect of substrate and final product on the enzymatic activity due to

formation of the barrier between biocatalyst and product and substrate [25-27]. The obtained results showed that L-Trp produced by 1g immobilized biocatalyst was 1.5 fold more than by 1g of free biocatalyst. Quantity of the immobilized biocatalyst effects on the production yield. Hence, the bacterial loads of beads increases the production yield due to the increase of the biocatalyst and on the other hand, polymer pores are occupied by increase of bacterial load, limited the mass transfer and so production yield is decreased [28]. According to the results, the maximum L-Trp was produced by immobilization of 2 g of biomass into the 2 % agar beads and production was decreased by increase of biomass more than 2 g. One of the most important causes of industrial attention to the immobilization technology is the reusability of the biocatalyst and so decrease of the biocatalyst preparation costs. Our results showed that biocatalyst activity preserved for 5 production cycles and then L-Trp production decreased gradually. Langrene et al reported that L-Trp synthase activity of carrageenan immobilized biocatalyst remained constant for 4 cycles [10]. Lysis and death of immobilized cells due to high indole concentration was to be mentioned as the reason of the biocatalyst activity decrease in the L-Trp production process [29].

CONCLUSION

As a consequence, in this research combination of immobilization and cheap substrate was successfully developed and could be used in the industrial scale as affordable process for L-Trp production.

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