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Improved L-Methionine Fermentation With Immobilized *Corynebacterium Glutamicum* X300

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ABSTRACT

An experimental study was conducted to examine the advantage of whole cell immobilization of a mutant Corynebacterium glutamicum X300 entrapped into calcium alginate beads for L-methionine production. For this purpose, several parameters were examined to maximize the product yield. Production was increased significantly ($p<0.01$) with immobilized Corynebacterium glutamicum X300 into calcium alginate compared to the production by free cells. Production was comparatively lower with the immobilized cells into chitosan-coated calcium alginate beads. Maximum production of L-methionine was obtained with 2.5% sodium alginate, 0.3 (M) CaCl2, 6.0 mm bead diameter and 24h storage period,using 0.4 (M) CaCl2 in the synthetic medium for bead stability. A continuous production of L-methionine was obtained up to 66th cycle reuse of the beads.

KEYWORDS : Immobilization, Calcium alginate, Chitosan ,Corynebacterium glutamicum

Introduction

L-methionine is an essential amino acid, required in human nutrition. Deficiency of L-methionine may lead to development of various diseases like muscular paralysis, toxemia, childhood rheumatic fever, schizophrenia, hair loss, depression etc [1].Such deficiency can be overcome by L-methionine supplementation in the diet, and thus L-methionine is of significant interest in modern scientific research [2].Recently, L-methionine is produced either by chemical synthesis or by protein hydrolysis [3].Both of these processes are very expensive. In addition to that , chemical synthesis produces a racemic mixture of D and L-methionine from which separation of L-methionine is difficult and protein hydrolysis produces a complex mixture from which L-methionine has to be separated [4,5].

Fermentative accumulation of various amino acids was initiated when Kinishita et al. (1957) discovered an L-glutamic acid producing bacteria Micrococcus glutamicus (subsequently renamed Corynebacterium glutamicum) [6].everal attempts have been made to improve L-methionine fermentation [7-11].

Recent advancement of biotechnology gained the attention of many biotechnologists for the successful application of immobilization technology in this field. Among them, entrapment of whole cells in different matrices including calcium alginate beads has been better choice over the other methods for microbial fermentation [12-16].

The main purpose of this present study was to investigate the potency of the mutant *Corynebacterium glutamicum* X300, immobilized into calcium alginate beads for L-methionine accumulation using selected suitable synthetic medium.

Materials and methods

Microorganism: A multiple analogue resistant mutant Corynebacterium glutamicum X300 developed in our laboratory from Corynebacterium glutamicum X1 was used throughout the study [16].

Composition of Basal salt medium: Microbial growth for inoculum preparation was carried out using the following basal salt medium (Per liter): glucose , 60 g; $(NH_4)_2SO_4$, 1.5g; K_2HPO_4, 1.4g; $MgSO_4.7H_2O$, 0.9 g; $FeSO_4.7H_2O$,0.01g; biotin , 60µg [17, 18].

Optimum Cultural Conditions: Fermentation was carried out using the following cultural conditions : Volume of medium, 25ml ; initial pH , 7.0 ; shaker's speed , 150 rpm ; age of inoculum , 48h; optimum cell density , $4.0x10^8$ cells/ml; temperature , 28^0C and period of inoculum , 72h [19].

Composition of synthetic medium: The experiment was carried out using a synthetic medium composed of (per liter): glucose, 100mg ; $(NH_4)_2SO_4$,8.0g; K_2HPO_4,2.2g; $MgSO_4.7H_2O$,1.5g; $FeSO_4.7H_2O$, 0.03g; KH_2PO_4, 2.0g; $ZnSO_4.7H_20$, 1.6mg; $CaCO_3$, 1.5g; $Na_2MoO_4.2H_2O$, 5mg, $MnSO_4.4H_2O$, 2.5mg; biotin , 80µg and thiamine-HCl, 70 µg [20].

Preparation of inoculum: A full grown slant of 48h old Corynebacterium glutamicum X300 was scrapped off and suspended in 100 ml sterile water. The cell suspension 4.0% (v/v) of the seed culture of microorganism was used as an inoculum [21].

Preparation of Calcium alginate beads: The cell suspension was slowly added to the sterile solution of sodium alginate (2.5%) and mixed thoroughly with sterile glass rod. The mixture was continuously extruded into 50 ml Erlenmeyer conical flask containing 20 ml 0.2 (M) $CaCl_2$ for 30 minutes. Then the beads were filtered aseptically and washed successively with sterile buffer solution (pH 7.0) and with sterile distilled water [15].

Formation of Chitosan-coated Calcium alginate beads: The calcium alginate beads formed by the method as mentioned above were added to 0.25% chitosan (v/v) solution prepared with 5.0% acetic acid in a 250 ml Erlenmeyer conical flask. The beads were then washed with sterile water to be used in the subsequent fermentation trials [22].

Analysis of L-methionine: Descending paper chromatography was employed for detection of L-methionine in the culture broth and was run for 18h on Whatman No.1 chromatographic paper. Solvent system used include: n-butanol: acetic acid: water (2:1:1). The spots were visualized by spraying with a solution of 0.2% ninhydrin in acetone and quantitative analysis of L-methionine in the suspension was done using colorimetric method [18].

Estimation of Dry Cell Weight: The cell paste was obtained from the fermentation broth by centrifugation and dried at 100^0C until constant cell weight was obtained [23].

Statistical analysis: All the data were expressed as mean ± SEM, where n=6. Data were analyzed using One Way ANOVA followed by Dunett's post hoc. Multiple comparison test using a software prism 4.0.

All the chemicals used in this were analytical Reagent (AR) grade and obtained from E-mark. Borosil glass goods and triple distilled water were used throughout the study.

Results

Different parameters for Calcium alginate beads formation and its stability were studied one after another to maximize L-methionine production by the mutant *Corynebacterium glutamicum* X300 as shown in table 1-7.

Table 1: Effect of CaCl2 in the synthetic medium for the bead stability and L-methionine production by the mutant Corynebacterium glutamicum X300

Concentration of $CaCl_2$ (M)	L-methionine (mg. ml^{-1})	Condition of the beads
0.0(control)	28.3±0.981	Disintegrated gradually
0.1	**56.8±1.312	Disintegration rate was decreased significantly

Values were expressed as mean±SEM , where n=6, **p<0.01

Table2: Optimization of CaCl2 in the synthetic medium for the bead stability and L-methionine production by the mutant Corynebacterium glutamicum X300

Concentration of $CaCl_2$ (M)	L-methionine (mg. ml^{-1})	Condition of the beads
0.05	**48.6±0.983	Disintegrated
1.0(control)	56.8±0.991	Disintegrated
0.2	*58.2±0.982	Disintegrated
0.3	**60.1±1.313	Disintegrated
0.4	**62.2±0.886	Unchanged
0.5	**62.2±0.971	Unchanged

Values were expressed as mean±SEM , where n=6,* p<0.05,p<0.01**

Table3:Optimization of Sodium alginate for the bead formation and L-methionine production by the mutant Corynebacterium glutamicum X300

Concentration of Sodium alginate (%)	L-methionine (mg. ml^{-1})	Condition of the beads
1.0	**40.6±0.991	Disintegrated
1.5	**48.1±1.683	Disintegrated
2.0	**54.2±0.926	Disintegrated
2.5(Control)	62.6±0.892	Unchanged
3.0	*61.4±0.668	Unchanged

Values were expressed as mean±SEM , where n=6,* p<0.05,p<0.01**

Table 4:Optimization of CaCl2 for the bead formation and L-methionine production by the mutant Corynebacterium glutamicum X300

Concentration of $CaCl_2$ (M)	L-methionine (mg. ml^{-1})	Condition of the beads
0.1	**54.2±0.983	Disintegrated
0.2(Control)	62.6±0.986	Disintegrated
0.3	**66.2±0.668	Unchanged
0.4	**66.2±0.913	Unchanged

Values were expressed as mean±SEM, where n=6,p<0.01**

Table 5: Optimization of bead diameter for L-methionine production by the mutant Corynebacterium glutamicum X300

Bead diameter (mm)	L-methionine (mg. ml^{-1})	Condition of the beads
2.0	**48.2±0.961	Disintegrated
4.0(Control)	66.2±0.681	Unchanged
6.0	**69.1±0.761	Unchanged
8.0	**69.1±0.683	Unchanged

Values were expressed as mean±SEM, where n=6,p<0.01**

Table 6:Optimization of the storage period of the bead for L-methionine production by the mutant Corynebacterium glutamicum X300

Storage Period(h)	L-methionine (mg. ml^{-1})	Condition of the beads
0.0	**36.2±0.913	Disintegrated
24(Control)	69.1±0.661	Unchanged
36	69.1±0.732	Unchanged
48	69.1±0.913	Unchanged

Values were expressed as mean±SEM, where n=6,p<0.01**

Table7: Comparison of L-methionine production between free and immobilized cells of the mutant Corynebacterium glutamicum X300

Condition of cells	L-methionine (mg. ml^{-1})	Dry cell weight (mg. ml^{-1})
Cells immobilized into Calcium alginate beads	**69.1±0.862	Equivalent to 2.1±0.661
Cells immobilized into Chitosan- coated Calcium alginate beads	61.2±0.913	Equivalent to 2.1±0.661
Free cells(Control)	52.1±0.993	28.5±0.913

Values were expressed as mean±SEM, where n=6,* p<0.05,p<0.01**

The production of L-methionine by the mutant Corynebacterium glutamicum X300 can be increased significantly ($p<0.01$) by the immobilized cells into calcium alginate beads. But the production was decreased significantly ($p<0.01$) by the cells entrapped into chitosan-coated calcium alginate beads. This low yield has been noticed in fermentations with immobilized cells into chitosan-coated calcium alginate beads which might be due to diffusion problems of essential nutrients in the synthetic medium or production may be inhibited by the products, accumulated within the beads [24]. The activity of the entrapped cells was assayed up to 72 cycles as to check the reusability of the entrapped cells into calcium alginate beads. The investigation revealed that 100% productivity was retained after 66th cycle, 81% after 69th cycle and 58% after 72 cycle.

Discussion

In many reviews, it has been noted that calcium chloride solution used to gel the beads has been of varying concentration, ranging from 0.1-4.0 wt% [25-30].The storage period has also varied. The two important parameters cause the alginate beads to have a tighter alginate matrix. Electrostatic interactions of calcium clusters surrounding the beads could play an important role in the diffusion of certain charged species [27]. Ganguly and Banik (2011) conducted an experimental study to examine the efficiency of whole cell immobilization in calcium alginate beads and agar block for L-glutamic acid production using an auxotrophic mutant *Micrococcus glutamicus* AB100. Production was increased with cells entrapped into calcium alginate beads with the presence of 0.1 (M) $CaCl_2$ in the synthetic medium, 0.2 (M) $CaCl_2$ for bead formation, 3.0% sodium alginate, 24h storage period of beads [28]. Very recently Ganguly and Satapathy (2014) reported an experimental study to evaluate some advantages of immobilization of *Gluconobactor oxydans* GPM60 entrapped into calcium alginate beads for gluconic acid production. Maximum production was obtained with immobilized cells of *Gluconobactor oxydans* GPM60, obtained with 0.2(M) $CaCl_2$, 2.5% Sodium alginate, 6.0mm bead diameter and 24h storage period [29].

Conclusion

From this present study, it can tentatively be concluded that, the production of L-methionine by the mutant *Corynebacterium glutami-*

cum X300 can be increased with whole cell immobilized into calcium alginate beads formed with 2.5%, sodium alginate, 0.3(M) $CaCl_2$,6.0 mm diameter of beads and 24h storage period using 0.4(M) $CaCl_2$ in the synthetic medium.

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