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Biosynthesis Of Indole-3-Acetic Acid By *Bacillus cereus* Immobilized Cells

Murat ÖZDAL[1,2], Özlem GÜR ÖZDAL[1], Alev SEZEN[1], Ömer Faruk ALGUR[1]

[1]*Ataturk University, Faculty of Science, Department of Biology, 25240 Erzurum, Turkey*

[2]*Ataturk University, Ispir Hamza Polat Vocational School, 25900-Ispir, Erzurum, Turkey*

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Abstract. Four different strains of Bacillus cereus producing indole-3-acetic acid (IAA) were isolated from various rhizospheric soils and characterized biochemically. The isolates were screened for (IAA) production and BC-On strain was found to be the best IAA producer with 46.25 mg/l. Maximum IAA production was obtained in the stationary growth phase at 72h. Significant correlation was also observed between IAA production and growth of the B. cereus strains. Among the isolates, BC-On strain was further used for immobilization studies. Maximum IAA production was obtained at initial pH of 8.0 and temperature of 35 °C after 18 h of fermentation. The immobilized cells could be effectively reused thirteen times and the IAA concentration of 300 mg/l was determined during this period. Results showed that immobilized cells can be used in the continuous process for the production of IAA. The productivity obtained by immobilization was higher than the one obtained by submerged cultivation and immobilization reduced the fermentation time.

Keywords: Indole-3-acetic acid, Rhizobacteria, Bacillus cereus, Immobilization

Immobilize *Bacillus cereus* Hücreleri Tarafından İndol-3-Asetik Asitin Biyosentezi

Özet. İndol-3-asetik asit (İAA) üreten dört farklı Bacillus cereus straini çeşitli rizosfer topraklarından izole edildi ve biyokimyasal olarak karakterize edilmiştir. İzolatlar İAA üretimi için taranmış ve BC-On straini 46.25 mg/l ile en iyi İAA üreticisi olarak bulunmuştur. En fazla IAA üretimi 72. saatte durağan büyüme fazında elde edilmiştir. B. cereus strainlerinin IAA üretimi ve büyüme arasında önemli bir ilişki gözlenmiştir. İzolatlar arasında, BC-On straini immobilizasyon çalışmalarında kullanılmıştır. En fazla IAA üretimi 18 saatlik fermentasyon sonrasında başlangıç pH'sı 8.0 ve 35 °C elde edilmiştir. İmmobilize edilmiş hücreler etkili olarak on üç kez kullanılabilmiş ve bu süre boyunca 300 mg/l İAA konsantrasyonu tespit edilmiştir. Sonuçlar immobilize hücrelerin IAA üretimi için sürekli bir işlemde kullanılabilir olduğunu göstermiştir. İmmobilizasyon ile elde edilen verimlilik, derin fermentasyon ile elde edilenden daha yüksek olduğu bulunmuş ve immobilizasyon fermentasyon süresini kısaltmıştır.

Anahtar Kelimeler: İndol-3-asetik asit, Rizobakteriler, Bacillus cereus, İmmobilizasyon

1. INTRODUCTION

Indole 3-acetic acid (IAA) is a commercially important and one of the most widely studied plant growth promoting hormone. L-tryptophan is the precursor of IAA production and it is converted into IAA by different types of microorganisms such as bacteria, fungi, cyanobacteria (plant growth promoting rhizobacteria and plant pathogens) and plants. Bacteria belonging to the genera *Azospirillum*, *Bacillus*, *Pseudomonas*, *Enterobacter*, *Xanthomonas*, *Streptomyces*, *Pantoea* and *Rhizobium* have been shown to produce IAA [1-3]. *A variety of* Bacillus *species* have been isolated from soil and rhizosphere environments, and reported to have the ability to produce of IAA in the presence of tryptophan [4, 5].

* Corresponding author. *Email address:* murat.ozdal@yahoo.com

Immobilized cells and enzymes are often used for industrial production of high value products. Immobilizing biocatalysts provides many benefits such as higher stability, lower operational costs, repeated use of biocatalysts, higher resistance to contamination, easy separation from the solution and increased reaction yield [6-8]. In addition, immobilization of cells ensures high retention of cell viability and allows the continuous use of the biocatalysts. A number of support materials (carrageenan, polyurethane, polyethylene glycol) have been suggested for cell immobilisation. The use of calcium alginate beads seems to be useful because it is a rapid, nontoxic, cheap, and versatile method [8]. Although many high value products produced by the immobilized cells and enzymes are available, there are very few reports on the production of IAA from tryptophan by immobilized systems. Considering the economic importance of IAA, this study aimed at repeated production of IAA by a new strain of *Bacillus cereus* BC-On and evaluation of this biosynthesis by immobilized cells of *Bacillus cereus*.

2. MATERIALS AND METHODS

2.1 Isolation and identification of microorganisms

Bacillus cereus strains (BC-XA, BC-On, BC-DS and BC-SO) were isolated from rhizospheric soils of different plants (Table 1), and were identified according to their cytological and metabolic features [9]. Analysis of Fatty Acid Methyl Esters (FAMEs) from whole cell fatty acids of bacterial isolates were carried out using GC according to the method described by the Kurbanoglu et al. [10]. *B. cereus* strains were grown and maintained on Nutrient Agar (NA; Merck) slants at 28 °C and 4 °C, respectively.

2.2 IAA production in submerged culture

For IAA production, the bacteria were grown in Nutrient Broth (NB; Merck) at 30 °C for 24 h at 150 rpm. One millilitre (2%, v/v) of culture was added to 50 mL of Luria-Bertani (LB) broth containing L-tryptophan (0.1%, v/w) in a 250 ml flask and incubated in a shaker at 150 rpm and 30 °C. The pH was adjusted to 7.2 before autoclaving at 121 °C for 15 min. The amount of IAA and the growth of microorganisms were determined at different times.

2.3 Immobilization of *B. cereus* BC-On cells

B. cereus BC-On cells were immobilized in calcium alginate gel beads as described previously by Kurbanoglu et al. [6] and Okay et al. [8]. *B. cereus* BC-On was grown in NB at 30 °C for 24 h. The culture was centrifuged at 5.000 rpm for 15 min and the biomass was washed two times with sterile 0.85% (w/v) saline solution. Sodium alginate solution (4%, w/v) was prepared by dissolving sodium alginate in sterile distilled water at 70 °C. Wet cells (4 g) were thoroughly resuspended in 40 ml of sterile distilled water. The cell suspension was mixed with an equal volume (1:1, v/v) of sodium alginate solution and stirred until a homogenous solution was formed. The mixture was dropped into a well-stirred sterile $CaCl_2$ solution (3.5%, w/v) using a syringe. Each alginate drop solidified upon contact with $CaCl_2$ and formed beads that encapsulated the *B. cereus* BC-On cells. The beads were left to harden for 30 min at room temperature, than they were washed with SSS to remove excess calcium ions and cells. The average bead diameter was approximately 2–3 mm.

2.4 IAA production in immobilized culture

The immobilized cells (3 g) were incubated in 25 ml of Tris buffer (100 mM, pH 7.2) containing L-tryptophan (0.1%, v/w) at 150 rpm and 30 °C. Optimization of process parameters for IAA production was carried out using immobilized cells. To evaluate the effect of glucose on the production of IAA, 10 g/l glucose was added into Tris buffer. Influence of temperature on the production of IAA was studied by incubating the inoculated flasks at different temperatures (25, 30, 35 and 40 °C). The effects of pH (pH 6-9) and incubation time (12-36 h) were also determined.

2.5 Determination of IAA

The production of IAA was measured by colorimetric assay at 530 nm using Salkowski's reagent as described by Patten and Glick [1]. After incubation, the fermentation broths were centrifuged (3.000 rpm for 10 min at 4 °C) and 1 ml of supernatant was combined with 2 ml of Salkowski's reagent and incubated for 30 min at room temperature. The measurement of IAA was carried out using a standard curve with known concentrations of pure IAA (Sigma−Aldrich, Co.).

2.6 Statistical analysis

Experiments were repeated three times in a randomized block design. The statistical analysis of the data was performed by one-way analysis of variance (ANOVA) using SPSS 15.0 software. The level of significance was $P<0.05$.

3. RESULTS AND DISCUSSIONS

3.1 Morphological and Biochemical Characterization of *Bacillus cereus* isolates

Bacillus cereus strains were isolated from various rhizospheric soils. Morphological and biochemical tests showed that they were gram positive, aerobic, endospore forming, catalase positive and oxidase negative, rod shaped, unpigmented and mobile organisms. Although all isolates grew at pH 5.7 and 45 ºC, none of them could grow at 5 °C and 65 ºC. Other biochemical characteristics of the bacterial isolates were given in Table 1.

Cellular fatty acids of strains were given in Table 2. As shown in Table 2, totally 15 different fatty acids were detected in strains. Twelve of them were saturated fatty acids. Isopentadecanoic acid (15:0 iso) had higher relative mass comparing to remaining FAMEs, whereas 12:0 iso and 18:1 w7c were found at quite low relative mass ratios. Fatty acid profiles of *Bacillus* are noticeably uniform in many respects. Terminally methylbranched iso and anteiso fatty acids containing 12 to 17 carbons constitute 55-95% of the total acids in the *Bacillus* strains. Although the most common fatty acids such as 14:0 and 16:0 are present in the majority of organisms, they are minor constituents in the genus *Bacillus*. Members of the *Bacillus cereus* group (*B. cereus, B. thuringiensis, B. mycoides, B. pseudomycoides, B. weihenstephanensis and B. anthracis*) have 15:0 iso as the major fatty acid [11-14]. The fatty acid

composition of *B. cereus* group is very similar and it may not be used to differentiate them [15,16]. *B. mycoides* and *B. pseudomycoides* show mycoidal growth, whereas *B. cereus* and *B. thuringiensis* do not show mycoidal growth. *B. thuringiensis* produces parasporal crystals in cultures unlike other species in the *B. cereus* group. *B. anthracis* is non-motile and *B. weihenstephanensis* can grow at low temperatures [16]. According to cytological, metabolic and fatty acid properties, the isolated strains (BC-XA, BC-On, BC-DS and BC-SO) were recognized as *Bacillus cereus*.

Table 1. Distinctive biochemical and morphological tests for the identification and characterization of the *Bacillus* isolates

	Isolates			
	BC-XA	**BC-On**	**BC-DS**	**BC-SO**
Host plant	*Xeranthemum annum*	*Onosma* sp.	*Dipsacus sylvestris*	*Senecio orientalis*
Motility	+	+	+	+
Endospore	+	+	+	+
Phosphate solubilisation	+	+	+	+
Crystal formation	-	-	-	-
Growth at 5 °C	-	-	-	-
Growth at 45 ºC	+	+	+	+
Growth at 65 ºC	-	-	-	-
Growth at pH 5.7	+	+	+	+
Growth on 7% NaCl	-	-	-	-
Catalase	+	+	+	+
Oxidase	-	-	-	-
Amylase	+	+	±	+
Protease	+	+	±	+
Lipase	+	+	+	+
Glucose	+	+	+	+
Maltose	+	+	+	+
Fructose	+	+	+	+
Sucrose	+	+	+	+

+= positive, - = negative

Table 2. The percentage of fatty acids in the isolates and two other strains of *Bacillus cereus*

Fame Acids	**BC-XA**	**BC-On**	**BC-DS**	**BC-SO**	***[24]**	****[25]**
12:0		4.20	4.10	5.04		
12:0 iso		0.57	0.51	0.49	0.68	
13: 0 ISO	9.43	9.76	9.71	11.35	5.54	
13:0 ANTESİO		1.02	1.00	1.14	0.13	
14: 0	5.18	5.71	5.69	3.49	3.40	
14: 0 ISO	4.19			2.46	5.12	5.5
15: 0 ISO	**33.76**	**32.18**	**31.10**	**33.52**	**33.27**	**31.0**
15:0 2OH		0.82	0.78	0.33		0.5
15: 0 ANTESİO	**3.54**	**3.33**	**3.30**	**4.50**	**2.74**	**3.7**
16: 0	3.61	2.99		4.24	7.37	4.71
16: 0 ISO	4.52	4.09	4.07	2.94	12.62	
17: 0 ISO	5.46	3.73	3.68	6.00	11.99	7.0
ISO 17: 1 w10c	3.94	2.43	2.41	3.65	0.87	
ISO 17: 1 w5c	6.53	5.01		2.20	3.04	3.61
18:1 w7c		0.44	0.37			

3.2 Screening of *B. cereus* isolates for IAA production

As shown in Figure 1, the production of IAA depends on the *B. cereus* isolates and fermentation time. The bacterial growth (Fig. 1a) and IAA production (Fig. 1b) increased with time. The IAA production by the *B. cereus* isolates started after the inoculation and maximum IAA production was obtained in the stationary growth phase at 72h followed by a decrease. As stated previously, the decrease in IAA production after 72 h might be due to the synthesis of IAA degrading enzymes such as IAA oxidase and peroxidase [17, 18]. Unyayar et al. [19] reported that the amount of these enzymes were high in the stationary phase. Among the isolates, strain BC-On was the best IAA producer with 46.25 mg/l and strain BC-SO produced only small amount of IAA (21.25 mg/l) (Fig 1b). It was also observed that there was a relationship between IAA production and the growth of the *B. cereus* strains. Results obtained in the present study were also confirmed by Prashanth and Mathivanan [20] and Blinkov et al. [21].

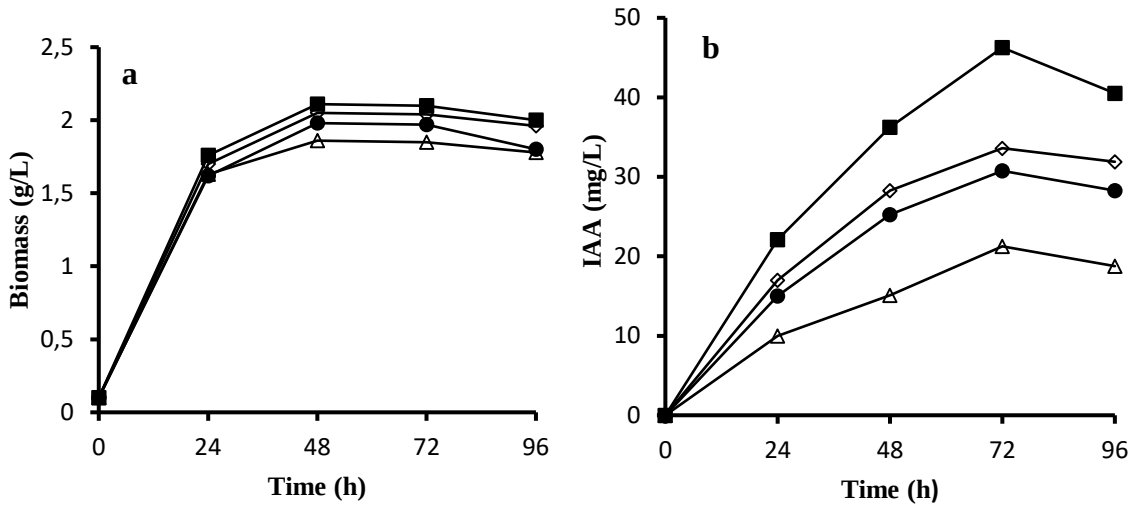

Figure 1. Effects of incubation time (24, 48, 72, 96 h) on the growth (a) and IAA production (b) of the isolates. • BC-XA, ◊ BC-DS, Δ BC-SO, ■ BC-On

Studies in the literature have reported different results associating IAA production with several species of *Bacillus*. *B. licheniformis* MML2501 was able to produce 23 mg/mL IAA, in the presence of tryphtophan at a concentration of 16 mM [20]. Production of IAA was reported for *B. cereus* HZB, *B. pumulis* DH-11 and *B. firmus* 40 at a concentration of 6.31, 109.46 and 138.93 mg/l, respectively, in LB medium containing 0.5 g/l tryptophan, incubated at 25 °C, 160 rpm for 5d [5]. In another study, Kumar et al. [4] found that *B. subtilis*, *B. megaterium*, *B. cereus/thuringiensis*, *B. pumilus* and *B. weihenstephanensis* produced 10-20, 20-22, 12-20, 10-24.5 and 24 mg/l IAA, respectively, in NB with L-tryphtophan (0.5 %, w/v), incubated at 28 °C for 5 days. In this study, we found that *B. cereus* isolates produced 16.25-46.25 mg/L IAA in LB containing 0.1% (w/v) L-tryptophan, incubated at 30 °C, 150 rpm for 3d. From these results, one of the best IAA producers, *B. cereus* BC-On, was selected for immobilization studies.

3.3 Production of IAA by immobilized cells of *B. cereus* BC-On

It is well known that reaction conditions are quite important for the successful production of IAA and optimization of parameters such as media composition, pH, temperature, and incubation time are essential in developing the process.

3.4 Effect of glucose on IAA production

The effect of *glucose on the production of IAA by immobilized cells* is *shown* in *Figure* 2. IAA production *remarkably increased* (*P<0.05*) in the *presence of glucose.* However, in the absence of glucose, low yield of IAA production (40 mg/l) was observed. Addition of glucose to the Tris buffer stimulated IAA production (175 mg/l). This may be due to providing energy for cells and improved

cofactor recycling in the cells. Similar results have been reported in other studies [8, 22]. Many researchers reported that glucose addition to the culture medium increased IAA synthesis [17, 23].

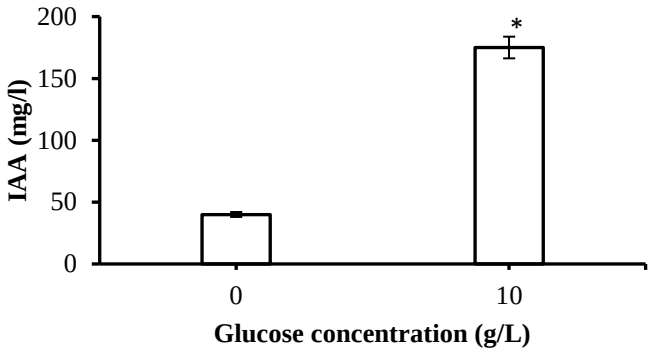

Figure 2. Effect of glucose on IAA production by Ca-alginate immobilized *B. cereus* BC-On.
Reaction conditions: T = 30 °C, pH 7.2, 150 rpm, 24 h.
An asterisk (*) denotes a value significantly greater than the other value (P<0.05).

3.5 Effect of temperature on IAA production

The effect of fermentation temperature (25, 30, 35 and 40 °C) on IAA production was examined (Fig. 3). The highest IAA production was obtained at 35 °C (*P<0.05*). Temperatures above or below 35 °C did not influenced the IAA production. The highest IAA (220 mg/l) production of the immobilized cells was at 35 °C for subsequent experiments.

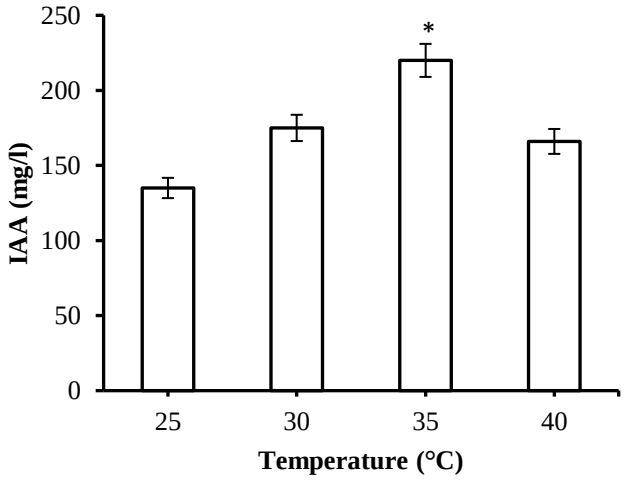

Figure 3. Effect of temperature on IAA production by Ca-alginate immobilized *B. cereus* BC-On.
Reaction conditions: pH 7.2, 150 rpm, 24 h, 1% glucose.
An asterisk (*) denotes a value significantly greater than the other values (P<0.05).

3.6 Effect of pH on IAA production

The effect of pH on IAA production was evaluated by incubating immobilized cells in Tris buffers at pH values ranging from 6.0 to 9.0. As shown in Fig. 4, the highest yield of IAA (260 mg/l) was obtained when biotransformation was performed at pH 8.0 (P<0.05).

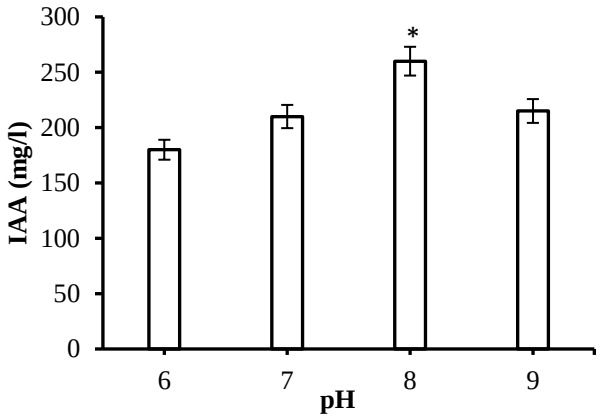

Figure 4. Effect of initial pH on IAA production by Ca-alginate immobilized *B. cereus* BC-On.
Reaction conditions: T = 35 °C, 150 rpm, 24 h, 1% glucose.
An asterisk (*) denotes a value significantly greater than the other values (P<0.05).

3.7 Effect of fermentation time on IAA production

The results of IAA production by immobilized *B. cereus* cells at different incubation times are shown in Fig. 5. The maximum IAA (300 mg/l) was noted after18 h (P<0.05) and further increase in fermentation time did not improve IAA production. It might be due to depletion of nutrients and the release of any IAA degrading enzyme in fermentation medium [26].

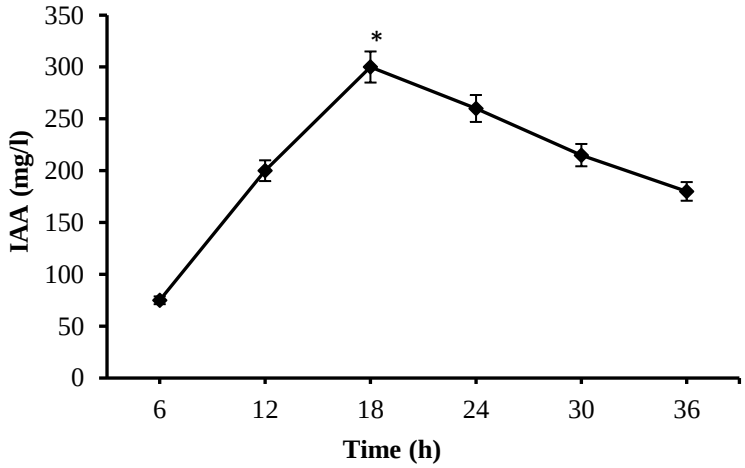

Figure 5. Effect of incubation time on the production of IAA by Ca-alginate immobilized *B. cereus* BC-On.
Reaction conditions: T = 35 °C, pH 8, 150 rpm, 1% glucose.
An asterisk (*) denotes a value significantly greater than the other values (P<0.05).

3.8 Reusability of immobilized cells for IAA production

Figure 6 shows the reusability of immobilized cells to produce IAA in semi-continuous process. The reaction was carried out at 35 °C for 18 h (per cycle). The initial concentration of L-tryptophan was always 0.1% (w/v). The immobilized cells could be repeatedly used for thirteen cycles. The amount of IAA was found to be 300 mg/l during this period (Figure 6). Scheme of the IAA production is summarized in Fig. 7.

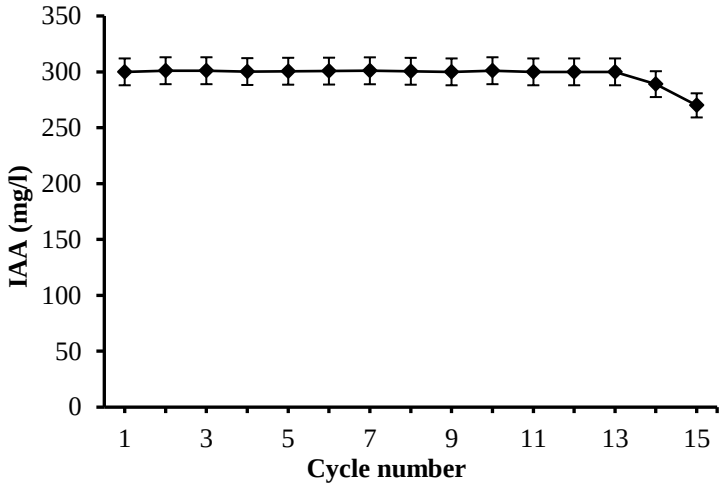

Figure 6. Reusability of the immobilized cells for the production of IAA.

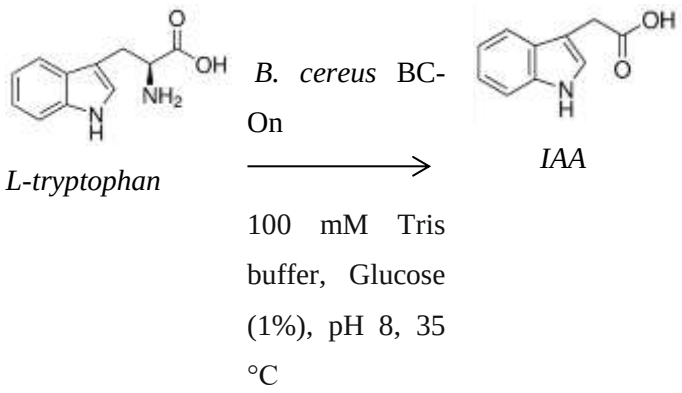

Figure 7. Production of IAA by the immobilized cells of *B. cereus* BC-On.

4. CONCLUSIONS

In this study, successful immobilization of *B. cereus* BC-On in Ca-alginate for repeated production of IAA was demonstrated. Immobilized cells in alginate beads were repeatedly used in thirteen successive reaction cycles (each cycle 18 h). The immobilized *B. cereus* has the advantages of high IAA production even after long term and repetitive fermentation. High IAA producing species should be investigated for IAA overproduction with immobilized cells. In addition, other immobilization materials (chitosan, carrageenan, polyurethane, polyethylene glycol) should be researched for the continuous production of IAA.

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