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SOLID-PHASE TRYPTOPHAN SYNTHASE BIOCATALYSTS AND THEIR APPLICATION IN INTEGRATED BIOCONVERSION SYSTEM

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A trpAB gene cloned *E. coli* strain of high tryptophan-synthase activity was cultivated then the separated biomass was permeabilized [1]. Agglomeration of the cells, entrapment into Ca-alginate gel and adsorption onto Celite-560 beads were investigated to get solid-phase biocatalysts. Biotransformation experiments for production of tryptophan and its 5-hydroxy derivative *via* coupling L-serine with indole/5-hydroxy indole were carried out in aqueous /organic system. Productivity has been found to the highest in case of using agglomerated biomass and Celite-560 adsorbed biomass but the reusability of the former catalyst was not acceptable.

Setting-up possibilities of an integrated system consisting of the tryptophan biosynthesis step and of a product separation step have been studied. The aim was to increase the productivity of the bioprocess through reducing the effect of product inhibition. Adsorption of the product onto XAD-4 neutral resin has proved to be selective enough [2] for its separation from the substrates. The system with alternate operation of the catalytic bioconversion step and the product adsorption step seems to be applicable for production of tryptophan but enhancement of the working stability needs further investigations.

Introduction

Solid-phase biocatalysts play an important role in enzyme catalytic production of numerous amino acids and their derivatives. If it follows from the character of the process that non-conventional reaction medium should be chosen, the significance of application of immobilized enzymes increases because they are considerably more stable than native enzymes [3] in such conditions. On the other hand, if the efficiency of bioconversion can be enhanced by simultaneous product removal the reusability of the biocatalyst can be improved by its immobilization [2,4].

According to the literature [2,4,5], and to our previous results [6,7], the use of non-aqueous medium and the application of integrated system both have importance in the biocatalytic synthesis of tryptophan and its derivatives: on one hand, indole substrate can be hardly dissolved in aqueous medium, on the other hand, the *in situ* removal of the formed amino acid is reasonable to decrease the product inhibition.

The aim of this article is to show our results in the matter of the biosynthesis carried out by solid-phase biocatalysts produced in different ways as well as concerning the studies on the integrated system including the biocatalysis step and the product removal step.

Experimental part

Microorganism:

Escherichia coli strain with cloned trpAB gene was constructed at the Agricultural Biotechnology Center, Gödöllő, Hungary.

Maintenance:

The *E. coli* culture was maintained on solid substrate containing 1 % tryptone, 1 % NaCl, 5% yeast extract, 2-3 % agar, 20 µg/10 cm^3 Ampicillin.

Fermentation and harvesting:

Tubes were washed into 50 cm^3 medium in 300 cm^3 E. flask, shaken at 37 °C, 190 rpm for 24 h. (Medium: 0.1 mole phosphate buffer pH=7.8 supplemented with 5 g NaCl, 1 g yeast extract, 10 g tryptone, 5 mg Ampicillin in 1 dm^3). Then 5 cm^3 broth was transferred to 200 cm^3 medium and was shaken again for 24 h. A mechanically stirred fermenter (*BR-97/INEL)* of 5 dm^3 was inoculated with 500 cm^3 broth, using the aforesaid medium. Cells were harvested by centrifugation then permeabilized by freezing at -20 °C [1].

Preparation of solid-phase biocatalysts:

Biocatalyst A: Agglomeration of the cells: The wet, permeabilized biomass was dried on air [6] at room temperature and was stored in refrigerator at 5 °C.
Biocatalyst B: Entrapment into Ca-alginate gel: 0.5 g cell mass was suspended in 5.5 cm^3 dist. water and the suspension was mixed with 10 cm^3 Na-alginate solution of 4 w/w% then it was dropped into 0.5 M $CaCl_2$.
Biocatalyst C and Biocatalyst D: Adsorption onto Celite-560 beads: The autoclaved Celite-560 (Fluka) particles (5 g) were added to 200 cm^3 fermentation broth at the 15th (C) and the 48th (D) hour of the fermentation, respectively. The separated particles were dried on air [6] at room temperature and were stored in refrigerator at 5 °C. Efficiency of immobilization was evaluated *via* productivity measurements. The productivity was given in mg Trp / mg fixed protein /h and mg Trp / mg catalyst /h, respectively.

Biotransformation conditions:

10 µmol/dm^3 pyridoxal-5-phosphate, 0.38 mol/dm^3 L-serine, 5 mg/dm^3 Ampicillin were solved in dist. water and mixed with cyclohexane containing 0.25 mol/dm^3 indol or with 2-etil-hexanol containing 0.25 mol/dm^3 5-hidroxy-indole. The volume ratio of aqueous and organic phases was changed between 1:4 and 1:9. Solid-phase biocatalysts (A),(B),(C) or (D) were added with concentration of 0.15-10 mg biomass / cm^3 reaction mixture and were shaken in 50-100 cm^3 E. flasks at 37 °C for 72-96 h. Concentration of indole or 5-hidroxy-indole in the aqueous phase was 0.05-0.1 M.

Adsorption studies:

AMBERLITE XAD-4 neutral resin (Rohm and Haas) [2] was used as adsorbent. L-tryptophan or 5-hidroxy-L-tryptophan were dissolved in 0.1 M phosphate buffer and shaken in flasks with the adsorbent at 37 °C and 175 rpm for 1 hour. Studies on parallel adsorption of the substrates and products were carried out in a mixture consisted of 90 % phosphate buffer and 10 % cyclohexane or 2-etil-hexanol.

Analytics:

Tryptophan and indole content of samples were analyzed by HPLC (HP-1050) UV detector at 254 nm. (Column HYPERSIL 10 µm (250 x 4 mm), eluent: 20:20:60 mixture of acetonitrile, methanol and phosphate buffer (0.0175 M KH_2PO_4-H_3PO_4), eluent flow rate 0.7 cm^3/min.)

Integrated biotransformation – product adsorption system:

The equipment for testing the operation of the integrated system (*Fig. 1*) consisted of 2 parts applied

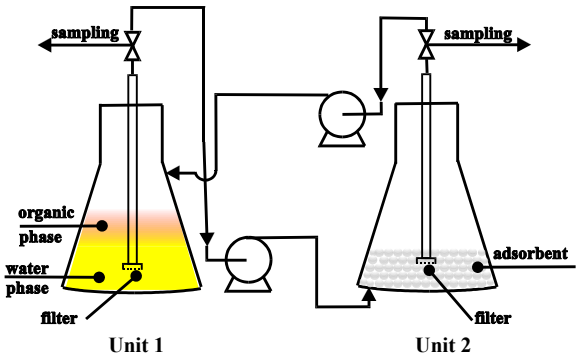

Figure 1: Equipment for testing the operation of the integrated biotransformation - product adsorption system

alternately to perform the catalytic bioconversion step and the product adsorption step. Biotransformation was carried out in a 500 cm^3 E. flask (Unit 1) with 200 cm^3 reaction mixture consisted of 20 % organic phase with 0.1 M indole and 80 % aqueous phase with 0.15-0.3 M L-serine. The mixture containing 10 and 5 g biocatalyst B and D, respectively was shaken at 37 ^oC and 175 rpm. After achieving a sufficiently high amino acid level the reaction mixture was settled then the aqueous phase was transferred into Unit 2 where the product adsorption was performed. The adsorbent, AMBERLITE XAD-4 neutral resin was mixed with the liquid phase at 37 ^oC, 175 rpm for 1 hour. Then the cyclic functioning of the system carried on and the two-step process was repeated several times in succession.

Results

Comparison of different types of immobilized biocatalyst:

Table 1: Specific tryptophan production of different solid phase biocatalysts of different type during 72 hours reaction

Type of immobilized biocatalyst	***Tryptophan production in72 hours*** *(mg Ttrp /mg protein)*
Agglomerated permeabilized biomass (Biocatalyst A)	0.157
Ca-alginate entrapped biomass (Biocatalyst B)	0.004
Adsorbed cells onto Celite-560 beads in the 15th hour of fermentation (Biocatalyst C)	0.223
Adsorbed cells onto Celite-560 beads in the 48th hour of fermentation (Biocatalyst D)	0.417

Among the solid-phase biocatalysts, the agglomerated permeabilized biomass showed the best productivity expressed in mg Trp / mg catalyst /h but its reuse was very difficult because – due to the small particle size – the loss was high. Using Ca-alginate entrapped biocatalyst the productivity was low because of the internal diffusional resistance. Cell adsorption onto Celite-560 beads has resulted in sufficient productivity and recycling properties.

On the basis of the above presented results, the third type immobilized catalysts were chosen to examine its stability properties, accordingly cells were adsorbed onto Celite-560 beads by method 'C' and 'D'.

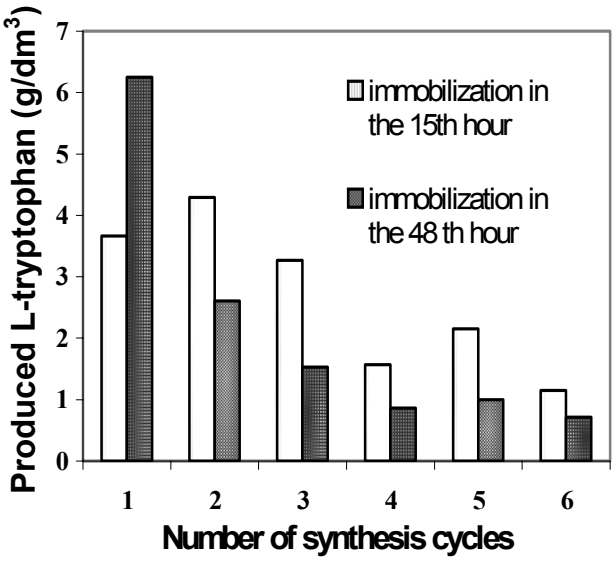

Figure 2: Catalytic stability of Celite-560 supported *E. coli* cells during tryptophan production cycles

The results of stability studies show that the time of addition of the support particles to the fermentation broth influences the catalytic stability of the immobilized cells. If Celite-560 was added in the 15th hour (Biocatalyst C), the initial activity was significantly lower than in the case of the support addition in the 48th hour (Biocatalyst D). On the other hand, the residual activity of Biocatalyst C, left after the 6th cycle was almost two times higher than that of Biocatalyst D.

Comparison of formation of tryptophan and 5-hydroxy-tryptophan:

As it can be seen in Fig. 3 the kinetics and the productivity of the amino acid formation catalyzed by biocatalyst A are very similar both in case of production of tryptophan and its 5-hydroxy derivative.

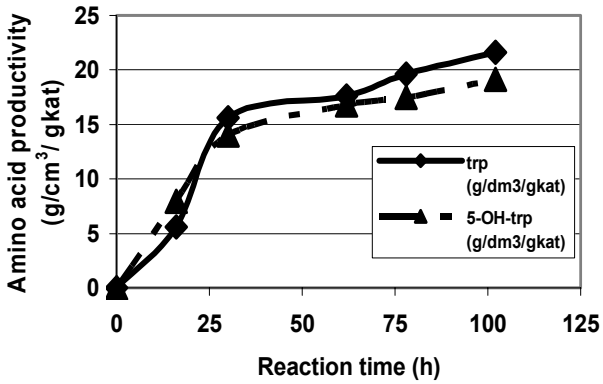

Figure 3: Formation of tryptophan and 5-hydroxy-tryptophan as functions of reaction time, using agglomerated biomass as catalyst

Adsorption experiments

To develop the adsorption effectiveness we considered the previous results [2] and our selectivity and capacity measurements [7] in aqueous / organic two-phase system as a model of the reaction mixture, which contains the substrates and the product, too. It was found that the capacity of XAD-4 resin adsorbent was considerably lower for tryptophan in the presence of indole than without it. The capacity reducing effect was more pronounced in case of adsorption of 5-hydroxy derivatives (Fig. 4). The presence of L-serine moderated the indole effect in case of the tryptophan [7].

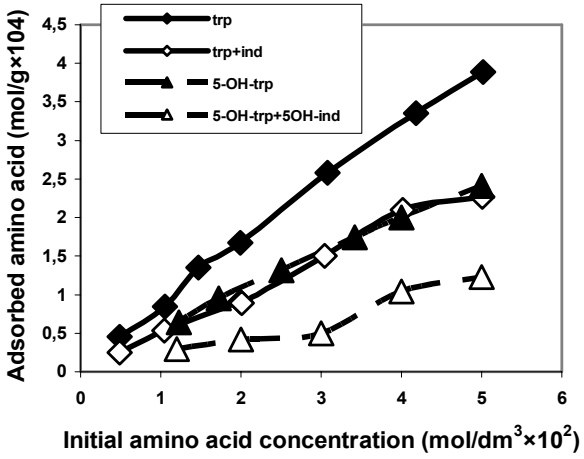

Figure 4: Adsorbed L-tryptophan and 5-hydroxy tryptophan quantities on XAD-4 neutral resin as functions of composition of the aqueous liquid phase

It was concluded from the adsorption results that the effective tryptophan separation is not possible from the two-phase reaction mixture system. Therefore - after settling of the phases - adsorption experiments were carried out in the aqueous phase saturated with indole. In point of all the facts, the reduced capacity seemed to be high enough to use the adsorbent for on-line tryptophan removal.

L-tryptophan production experiments in integrated system

The integrated biotransformation - product adsorption system was carried out with alginate entrapped (Biocatalyst A) and Celite-560 adsorbed (Biocatalyst D) cells. The latter catalyst proved to be more applicable because of its higher productivity and better stability. The productivities of the simple biotransformation system and the integrated biotransformation / product adsorption system were compared. The data are summarized in Table 2. It can be seen that efficiency enhancement of 30 % can be achieved using the cyclic integrated system.

Table 2: Comparison of the tryptophan productivity of the simple biotransformation system and the integrated biotransformation - product adsorption system

Type of solid phase biocatalyst	*72 hours tryptophan production in simple biotransformation process (g Ttrp / cm³)*	*72 hours tryptophan production in integrated biotransformation / adsorption process (g Ttrp / cm³)*	*Ratio of efficiencies of simple biotransformation and integrated systems*
Ca-alginate entrapped biomass (Biocatalyst B)	0.89	1.12	1.26
Adsorbed cells onto Celite-560 beads in the 15th hour of fermentation (Biocatalyst B)	7.2	9.5	1.32

Conclusion

Comparing different methods to produce solid–phase biocatalysts from genetically manipulated *E. coli* K-12 cells of tryptophan synthase activity it was found that the catalyst immobilized by adsorption on Celite-560 showed the best catalytic character considering both its productivity and stability.

Carrying out the bioconversion of indole or 5-hydroxy indole and serine in aqueous/organic reaction mixture it was stated that the kinetics and productivity were very similar both in case of tryptophan and in case of 5-hydroxy-tryptophan formation.

An integrated system constructed as a combination of the alternately working bioconversion and product adsorption steps has been proved to be applicable for tryptophan production and showed about 30 % higher productivity than the simple biotransformation system. XAD-4 neutral resin operated quite efficiently to remove tryptophan from indole and serine containing aqueous phase. Further developments are necessary to increase the working stability and productivity of the system.

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REFERENCES

1. TERASAWA M., FUKUSHIMA M., KURUSU Y. and YUKAWA H.: Process Biochem. Int., 1990, 25, 172-175
2. RIBEIRO M. H. L., PRAZERES D. M. F. CABRAL J. M. S. and DA FONSECA M. M. R.: Bioprocess Eng., 1995, 12, 95-102
3. LANGRENE S., SICSIC S. and LE GOFFIC F.: Enzyme Microb. Technol., 1984, 6, 81-84
4. DA FONSECA M. M. R., MATEUS D. M. R. and ALVES S. S.: Performance of a Liquid-Impelled Loop Reactor with Immobilized Cells. Progress in Biotechnol. Vol. 11., Int. Conf. Immobilized Cells: Basics and Applications, Noordwijkerhout, The Netherlands, Elsevier Sci. Publ. Amsterdam, pp. 511-517, 1996
5. FERNANDES P., CABRAL J. M. S. and PINHEIRO H. M.: J. Molec. Catal. B: Enzymatic, 1998, 5, 307–310
6. SISAK CS., BOROSS L., OROSZ L. and SZAJÁNI B.: Configuration Studies on Immobilized-Cell Fluidized-Bed Reactor System for Bioconversion of Tryptophan and Its Hydroxy Derivative, Int. Congress CHISA'2000 CD-ROM Full texts. Magicware Ltd. CHISA'2000/1003, 2000
7. KOVÁCS Á., LŐVITUSZ É., SZAJÁNI, B., BOROSS L. and SISAK CS.: Application Possibilities of Selective Product Adsorption in Case of L-tryptophan Production by Biotransformation, Conf. "Chemical Engineering Days 2001". pp. 216-221, 2001 (in Hungarian)
8. WOOD W. A., GUNSALUS I. C. and UMBLREIT W. W.: J. Biol. Chem. 1947, 170, 313-321
9. ZAFFARONI P., VITOBELLO V., CECERE F., GIACOMOZZI E. and MORISI F.: Agric. Biol. Chem., 38 1335-1342 1974