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PRODUCTION OF OPTICALLY ACTIVE COMPOUNDS BY MICROBIAL BIOTRANSFORMATION

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Abstract. The production of phenylacetylcarbinol with the yeast Hansenula polymorpha was studied by our research team. Performing the selection of yeast strains adapted to 3.0 g/L benzaldehyde. we succeeded in obtain a final product concentration bigger than 6 g/L and a biotransformation rate of 0.03 g/L/min.

INTRODUCTION

There is substantial interest in the use of microbial biotransformation processes for organic syntheses because of the unique regio- and stereo- selective properties of enzymes and their capacity to operate in non-extreme conditions [1]. From an economic point of view. yeasts have attracted wide attention as a potential catalyst in biotransformation processes because they are cheap and easy to obtain [2].

L-ephedrine is a natural plant alkaloid isolated originally from the dried young branches of *Ephedra*. a plant with interesting pharmacological activities. Extracts of *Ephedra* sp.. particularly *Ephedra sinica. E. equisetina* and *E. distachya* commonly called "Ma Huang" in China. have been used for several thousand years as folk remedies for inducing sweat. soothing breath and easing excretion of urine. The active ingredient of this extracts. L-ephedrine. was first isolated in 1855. and international interest in this drug was stimulated by the classical investigations of Chen and Smith in 1930. who reported on its cardiovascular effects and its similarity to epinephrine [3].

Benzaldehyde is enzimatically converted to L-phenyl acetyl carbinol when added to a fermentation medium previously inoculated with yeast cell mass in the presence of fermentable sugar. Pyruvic acid. a product of glycolysis. is decarboxylated to "active acetaldehide" which reacts with benzaldehyde to produce the final product. L-PAC [4.5]. The key enzyme is pyruvate decarboxylase (EC 4.1.1.1) [6]. In a parallel. undesired reaction. part of benzaldehyde is also reduced by alcohol dehydrogenase (EC 1.1.1.1) and/or other non-specific dehydrogenases to benzyl alcohol –Fig.1-[7]. The major problem associated with this reaction is the concomitant yeast-mediated reduction of benzaldehyde to benzyl alcohol. an unwanted by-product. Small amounts of other byproducts (benzoic acid. acetyl benzoil) has been reported [11].

Most of the authors cited in the literature studied the PAC production mainly in baker's yeast (*Saccharomyces cerevisiae*). There is little data about the production of this compound by other yeast species [12-15].

MATERIAL AND METHOD

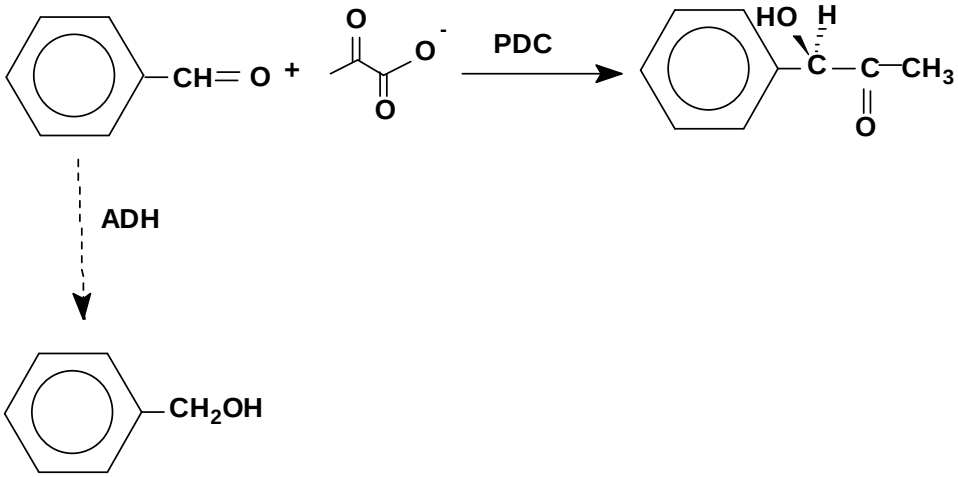

Fig. 1. Bioconversion of benzaldehyde to L-PAC

Strains The strain used in our experiments was *Hansenula polymorpha*. The strain were maintained on a solid medium containing 0.5% yeast extract. 0.5% peptone. 2% glucose and 2% Difco agar. cultivated for 48 h at 35°C. stored at 5°C and transferred every 3 weeks.

Adaptation of microorganism to benzaldehyde Gupta [13] and Long & Ward [17.18] tested the effect of benzaldehyde on living cells. They showed that a benzaldehyde concentration about 0.5 g/l slowed down the growth rate. at a concentration between 1-2 g/l C_6H_5-CHO. cell viability was affected. These informations determined us to select a strain of *Hansenula polymorpha* resisting at higher benzaldehyde concentrations. The cultivations were performed on a GYE agar medium. containing increasing substrate concentrations. We started at 1 g/L C_6H_5-CHO. Three subcultures were done at the same benzaldehyde concentration before transferring to the next stage.

We succeeded to obtain the strain *H.polymorpha*-3.0 which was capable of growth at 3.0 g/L C_6H_5-CHO (Table 1)

Medium For the cultivation and preliminary tests for PAC production. the medium had the following composition: glucose 7.5%. corn steep (50% dry mass) 0.8%. KH_2PO_4 0.1%. $(NH_4)_2SO_4$ 0.4 %. $MgSO_4.7H_2O$ 0.06%. pH was 4.5-5.0. The medium was sterilized 20 min at 110°C.

Cultivation conditions Yeasts were cultivated in 500 ml flasks containing 150 ml medium and closed with cotton stoppers. Flasks of the first generation were inoculated from agar slants and cultivated on a orbital shaker (240 r.p.m). After 24 h. the cultures were transferred (10% v/v) to flasks of the second generation. where the cultivation continued for further 24 h. pH was maintained at 4.5-5 with $NH3_{(aq)}$ 12.5 %.

The batch fermentation phase and the fed-batch bioconversion phase proceeded in a New Brunswick 15 L fermenter. In the first batch fermentation phase. the development of yeast biomass and the formation of pyruvate decarboxylase enzyme complex responsible for the bioconversion occur. The increasing of yeast biomass correlates well with the carbon source consuming rate.

Bioconversion Conditions. Bioconversion of benzaldehyde takes place in fermentation flasks at the optimum age of the yeast cells. determined experimentally. Benzaldehyde and acetaldehyde were added in several doses in proportion benzaldehyde 1 volume : acetaldehyde (50 % in water) 1.5 volumes.

Benzaldehyde concentrations were determined during and of the end of the bioconversion period. and a further dose of the two components was always added when the benzaldehyde concentration decreased below 0.05%.

The optimum time of biotransformation was experimentally determined as 6 hours.

Analytical Methods Benzaldehyde. benzyl alcohol and L-PAC were determined by gas chromatography. Analysis were carried out on a FID Carlo-Erba FRACTOVAP 4200 gas cromatograph. using a glass 2 meters long 4 mm i.d. column with a stationary phase S.P.2250 (50% phenylsilicone) on Gas-Chrom Q (80-100 mesh). Oven temperature was 80^{o}C for 4 minutes. then growing at a rate of 10^{o}C/min until it reached 180^{o}C. Quantitative determinations were achieved by external standard method.

Measured volumes of fermentation broth were centrifugated at 4000 rpm for 20 minutes. upper layer was extracted twice with an equal volume of benzene. The combined extracts were GC analysed.

Evaluation of cell mass development was appreciated by dry cell weight. The glucose consumption during the cultivation was determined spectrophotometrically. by o-toluidine reaction (glucose determination kit –I.C.C.F.). when a blue complex was formed. The determination was made on a Perkin-Elmer λ12 Spectrophotometer. at λ=630nm.

RESULTS AND DISCUSSIONS

The further experiments were performed with strain *H.polymorpha* H1. It was adapted to benzaldehyde up to 3.0 g/L. in order to eliminate the lethal effect of cosubstrate on living cell and to improve the biotransformation rate. The comparative viabilities of parental and adapted strains are presented below.

Table 1

Comparison of cell viabilities of adapted strain versus parental one

Culture Medium Viability	**YPG**	**YPG +3.0 g/L C_6H_5CHO**
H.polymorpha	3.4×10^8	2.8×10^6
H.polymorpha – 2.5	3.2×10^8	3.1×10^7
H.polymorpha – 3.0	2.8×10^8	4.4×10^7

Viability of parental strain is comparable with those of strains adapted to benzaldehyde. In the case of cultivation on GYP medium without benzaldehyde. the growth of *H.polymorpha*-3.0 is weaker than the parental strain. The superiority of adapted strains is obviously in the case of growth on media containing increased concentrations of benzaldehyde.

In a second series of experiments the influence of pH on the PAC levels was examined. The experiments were performed with *H.polymorpha*-3.0. bioconversion time was 30 min. T= 30^{o}C. WCW was about 30 g/L.

Table 2

The influence of pH on PAC production

pH value	**3.5**	**4.0**	**4.5**	**5.0**	**5.5**	**6.0**	**6.5**	**7.0**
L-PAC (g/L)	0.4	0.48	0.6	0.8	0.93	1.05	0.91	0.81

A pH value between 5.5 and 6.5 seems to be optimum for PAC formation by *H. polymorpha*-3.0. in our conditions of bioconversion.

Data presented in the next table indicate that the final concentration of PAC produced. gradually decreased when the age of cells was more than 18 hours from the beginning of bioprocess.

Table 3

Effect of cell age on biotransformation of benzaldehyde to L-PAC

Cell age [h]	**12**	**14**	**16**	**18**	**20**	**24**
L-PAC [g/L]	0.9	0.94	1.2	1.1	0.97	0.92

Maximum L-PAC was obtained with 16-18 h old cells (30-32 g/L WCW). as it is evident from data presented in Table 3. The experimental conditions were bioconversion time 45 minutes. T=30^{o}C. pH = 5.5.

Lineweaver-Burk plot of the experimental data (Fig. 2) revealed that strain accomodation at higher cosubstrate concentration increased r_{max} value (from 1.53 to 1.72 g/L×h). a modification of K_s was also observed.

For diminishing the detrimental effects of cosubstrate we choosed fed-batch cultivation system for the biotransformation stage. We maintained benzaldehyde concentration between 1-2.5 g/L by supplying the cosubstrate mixture in fed batch system. As it is illustrated in Fig. 3. the specific L-PAC and benzylic alcohol production rates are constantly diminished with time.

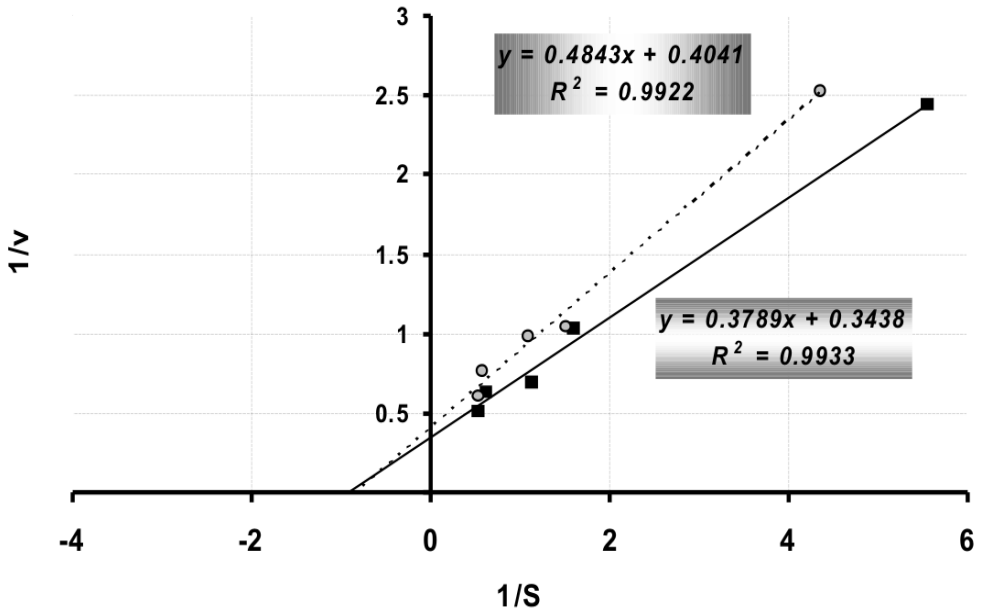

Fig. 2 Effect of initial benzaldehyde concentration [g/L] on the specific rate of L-PAC production [g/L×h] by *H.polymorpha* parental (-!-!-) and adapted (___) strains (Lineweaver-Burk plot)

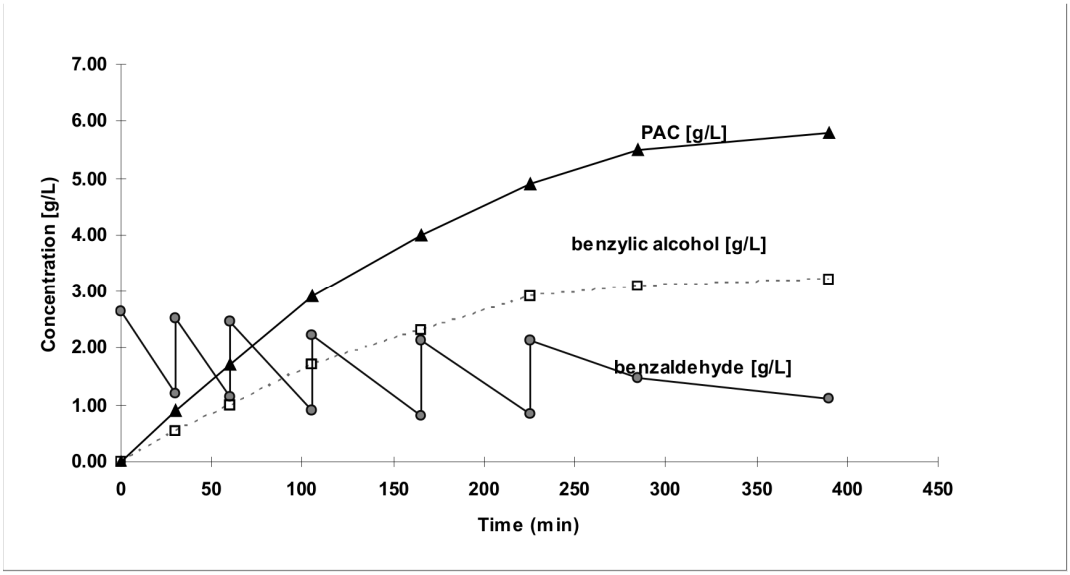

Fig. 3 Products and substrate concentration in fed-batch bioconversion of benzaldehyde with *H.polymorpha* -3.0

CONCLUSION

By performing the adaptation of the yeast strain *H.polymorpha* to a concentation of 3.0

g/L benzaldehyde. our team obtained a final concentration of 6.3 g/L PAC in 400 minutes. the maximum biotransformation rate being of 0.03 g/L/min.

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