*Recent Research in Science and Technology 2009, 1(2): 058–062*
*ISSN: 2076-5061*
*www.recent-science.com*

NANOSCIENCE & NANOTECHNOLOGY

# PRODUCTION OF PHENYLACETYLCARBINOL IN A STAND STILL CONDITION USING TWO PHASE SYSTEM BY APPLYING YEAST CELLS CAPABLE OF FERMENTING DRIED EPHEDRA EXTRACT AND MOLASS AS BIOCATALYSTS

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## Abstract

Microbial Biotransformation of benzaldehyde to L Phenylacetylcarbinol (L-PAC) as a key intermediate for L-ephedrine synthesis has been evaluated using immobilized *Hansenula polymorpha.* The cultivation of 10 ml *Hansenula anomala* inoculum for 48 h in the cultivation medium with glucose as a sole carbon source resulted in the highest ethanol concentration of 10.6 ± 0.53 g/l. This was followed by *Hansenula polymorpha* (4.06 ± 0.20 g/l) and *Brettanomyces lambicus* (2.94 ± 0.15 g/l), respectively. The cultivation using 100 ml dried Ephedra extract and molass in 1:1 ratio as a sole carbon source showed that microbes with ability to consume glucose, fructose and sucrose released high level of ethanol (g/l) included *Hansenula polymorpha* (43.4 ± 4.0), (41.8 ± 1.2) and *Hansenula anomala* (38.1 ± 2.5). A biotransformation experiment was conducted using wet biomass of 3.06 g/l dry biomass equivalent from the medium with dried Ephedra extract and molass in 1:1 ratio as carbon source. After the two phase system was stood aside for 24 h, *Hansenula polymorpha* generated an overall *R*-phenylacetylcarbinol (PAC) concentration of 3.97 and 3.72 mM at 4°C and 35°C which was followed by *Hansenula polymorpha* (3.04 and 3.73 mM), *Brettanomyces lambicus* (1.24 and 2.98 mM) as well as *Hansenula anomala* (0.86 and 0.07 mM).

**Key Words**: Microbial Biotransformation; Ephedra extract; Molass; Phenylacetylcarbinol.

## Introduction

The problems that could arise from burning these Ephedra leaves wastes included, space non-availability, difficulties of continuous burning, toxic fumes released to the environment, and wastage of valuable carbon sources. Therefore, the initiatives of employing dried Ephedra leaves as carbon sources for ethanol producing microbes might be more beneficial. In addition, the cells precipitate obtained after fermentation process also had the potential application for extraction of pyruvate decarboxylase (PDC) enzyme which was able to catalyze the ligation reaction between benzaldehyde and pyruvate to produce *R*-phenylacetylcarbinol (PAC) (Rosche *et al*. 2002). PAC is a valuable precursor for pharmaceutical industry in the production of ephedrine and pseudoephedrine that could be used to alleviate the symptoms of allergy and nasal congestion (Hildebrandt and Klavehn 1932, 1934). Besides, Leksawasdi *et al*. (2005) illustrated that the application of two phase biotransformation system between an aqueous phase of MOPS buffer and an organic phase such as octanol could result in the production of PAC up to 92 g/l (613 mM) within 47 h.

This study was the final report in a series of three investigations leading to the application of dried Ephedra leaves in the productions of ethanol and active biomass for biotransformation. The objectives of current study were to (1) investigate the ability of 15 yeast strains for ethanol production in a stand still condition with the

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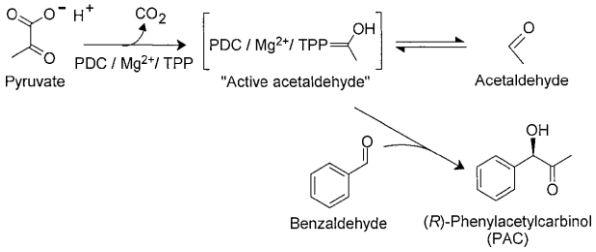
presence of dried Ephedra leaves extract and molasses in 1:1 ratio with nitrogen sources as well as (2) evaluate the possibility of performing *R*-phenylacetylcarbinol, PAC – a precursor for production of the medicinal compounds ephedrine and pseudoephedrine, biotransformation in a stand still two phase system using the biomass obtained previously.




Schematic diagram of PAC

## Materials and Methods

The yeasts strains isolation from Sugar cane juice was added into 100ml conical flasks containing sterile water and incubated at room temperature for two to three days. After incubation one loop full from each flask was added aseptically to separate Petri plates containing YEMA medium. Antibiotics like streptomycin and griseofulvin were added YEMA medium to prevent the growth of bacteria respectively. After inoculation, the plates were incubated for two to three days at room temperature and yeast growth was observed.

The obtained yeast colonies were further purified by streaking on pertriplates containing the same medium. These strains were identified at the basing on sequencing of D/D2 domain of 26S rRNA gene and assigned the species as following; *Brettanomyces lambicus* (B 1,2,3,4,5,6), *Candida utilis* (C 1,2,), *Saccharomyces* sp., *Hansenula polymorpha* (1,2,3) and *Hansenula anomala*(1,2,3). The freeze dried stock of each culture was proliferated in the corresponding medium and stored as glycerol stock at -20°C in 1 ml aliquot.

In order to investigate the effect of 48 h incubation period on the growth of each microbe, the glycerol stock aliquot was transferred to the sterilized inoculum medium of 10 ml. Each culture was stood aside at 35°C with two replicates for the period of 48 h and the collected samples at 0 and 48 h were analysed for glucose and ethanol concentrations, dried biomass, and pH. The obtained results were compared to the study by Kanchanwong *et al*. (2008) and Phrathong *et al*. (2008) to choose the optimum cultivation time of the inoculum.

Further investigation was carried out in a larger scale of 100 ml with dried Ephedra extract and molasses in 1:1 ratio as well as corresponding 1.5 folds of nitrogen source concentration used previously in the inoculum medium. The optimal inoculum age of 48 h was used and the culture was maintained in the stand still condition for the next 48 h at 35°C after addition of 10 ml inoculum. The comparison of the fermentation parameters at 0 and 48 h were made for glucose, ethanol, organic acids, and dried biomass concentrations, optical density at 600 nm (OD600), pH, as well as total soluble solid (TSS) of the separated supernatant by centrifugation. The collected cells were also treated with 0.5 mm glass beads in 3 cycles of 1 min vortexing and ice-water cooling so that the obtained crude extract after centrifugation could be analyzed for total soluble protein (Bradford 1976) and pyruvate decarboxylase (PDC) activity (Bergmeyer & Grabl, 1983). The wet biomass was harvested at 48 h for subsequent biotransformation experiment. Four microbial strains (*Hansenula polymorpha*, *Hansenula anomala*, and *Brettanomyces lambicus*) were subsequently selected for whole cells biotransformation experiment based on their abilities to produce ethanol. The biotransformation was performed at 4 and 35°C in the stand still condition for 24 h with 3.06 g dry biomass equivalent/l. The biotransformation system contained two liquid phases of 5 ml volume in each phase, viz. an organic octanol phase with 500 mM benzaldehyde as well as an aqueous 900 mM $KH_2PO_4$ (pH 6.5/KOH) phase with 600 mM sodium pyruvate. The additional cofactors of 1 mM thiamine pyrophosphate (TPP) and 1 mM $MgSO_4.7H_2O$ were also included in the aqueous phase. The reaction was carried out with two replicates.

Concentrations of benzaldehyde, L-PAC and benzyl alcohol were determined by gas chromatography. Samples were prepared by extraction into dichloromethane (sample: solvent"1:5). The biotransformation sample (0.2 ml) was mixed with 1 ml dichloromethane in a microcentrifuge tube and vortexed for 2 min. A sample from the bottom organic layer was injected into a gas chromatograph with the column and its operating conditions as follows: column material, 6.4-mm glass 1 m long; packing material, Chromosorb in the mesh range of 80-100 lm; carrier gas, nitrogen (30 cm3/min); oven temperature, 115°C (isothermal); injector temperature, 180°C; detector temperature, 180°C with flame ionization detector; injection sample, 3. The concentrations of benzaldehyde and benzyl alcohol were determined by comparison with standard samples (from Biiogeneic) and L-PAC (from Sigma Aldrich). (Rosche *et al*. 2001).

## Results and Discussion

In term of glucose consumption efficiency within the period of 48 h, the inoculum of *Hansenula anomala* was able to consume most of the glucose at 96.2% which was followed by *Hansenula polymorpha* (88.4%), (83.7%), and *Brettanomyces lambicus* (81.1%). As indicated in Fig. 1, *Hansenula anomala* provided the highest level of ethanol concentration (10.6 ± 0.5 g/l) with corresponding ethanol yield of 0.55 g ethanol/g glucose (a slightly higher value than that of the theoretical yield of 0.51 for glucose and 0.54 for sucrose) which preceded *Hansenula polymorpha* (4.06 ± 0.20 g/l, 0.52) and *Brettanomyces lambicus* (2.94 ± 0.14 g/l, 0.37) for inoculum media with the presence of glucose.

The inoculum of *Saccharomyces* sp also produced an appreciable amount of ethanol at 3.67 ± 0.18 g/l. According to Dien *et al*. (2003), *Saccharomyces* sp generated ethanol by converting pyruvate using pyruvate formate lyase (PFL) through an unbalanced fermentative pathway with the consumption of one NADH + $H^+$ molecule in which acetic and succinic acids must be produced to replenish the disappearing NADH + $H^+$. The accumulation of dried biomass after 48 h is shown in Fig. 2 with the highest concentration level and biomass yield belonged to *Hansenula polymorpha* (8.61 ± 0.43 g/l, 0.55 g biomass/g consumed glucose) while the other eight successive strains starting from *Saccharomyces* sp (5.43 ± 0.27 g/l, biomass yield on glucose in not available) to *Hansenula polymorpha 2* (6.21 ± 0.31 g/l, 0.27 g/g) were able to produce only 63.1 - 72.1% of that dried biomass level. Some results of further experiment in a stand still condition of 100 ml with dried ephdra extract and molasses mixed in 1:1 ratio as carbon

Source are given in Fig. 3 to 5. Interestingly, *Hansenula anomala* was among five other microbial strains, viz. *Hansenula polymorpha, Brettanomyces lambicus*, *Candida utilis*, *Saccharomyces* sp which were incapable of metabolizing sucrose as evident from the initial sucrose and remaining sucrose concentrations at 0 and 48 h in Fig. 3(a) and 3(b). In fact, *Hansenula anomala* was only able to utilize 26.1% of overall sugar concentrations when the dried ehedra extract and molasses mixed in 1:1 ratio was used as its carbon source. This was in contrast to findings of Kanchanwong *et al*. (2008) who reported complete sugar consumption (64 g/l) of this bacterial strain after 48 h in a similar condition, however, only glucose was used as a carbon source in their experiment. From Fig. 3(a) and 3(b), *Hansenula anomala* was the best overall sugar consumer (99.1%) and was followed immediately by *Hansenula polymorpha* (98.4%) and (96.5%). In addition, *Brettanomyces lambicus B1,2,3,4,5* were also able to consume almost all sucrose after 48 h but the rate of utilizing glucose and fructose were much slower than the three forefront overall sugar consumers.

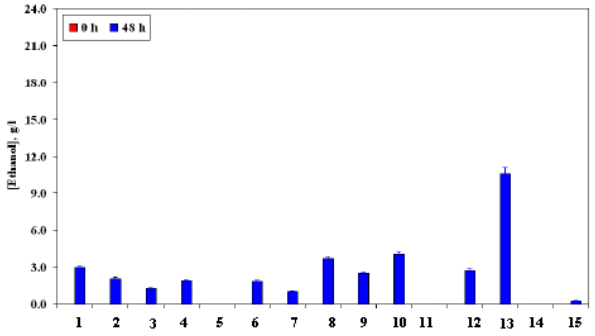



Fig. 1: Ethanol concentration produced by 10 ml inoculum at 48 h.

Verduyn *et al*. (1991) discussed the difficulties of *Brettanomyces lambicus* in uptaking glucose through the cells membrane which required ATP while *Hansenula polymorpha* was able to absorb glucose through facilitative diffusion process in absence of ATP. The corresponding ethanol production of each microbial strain is given in Fig. 4. The highest level of produced ethanol and its corresponding yield on the consumed sugars were 43.4 ± 4.0 g/l and 0.50 g ethanol produced/g sugars consumed for *Hansenula polymorpha* The other high ethanol producers included *Hansenula polymorpha* (41.8 ± 1.2 g/l and 0.53 g/g) and *Hansenula anomala* (38.1 ± 2.5 g/l and 0.43 g/g). Conversely, *Hansenula anomala* which produced much less ethanol concentration (11.1 ± 0.7 g/l) but the respective ethanol yield was still relatively high at 0.45 g/g.

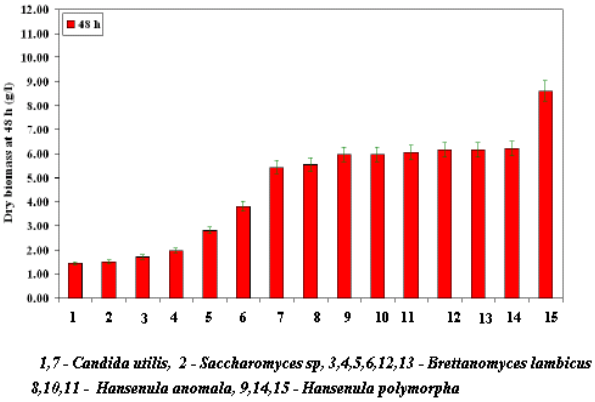



Fig. 2: Dried biomass concentration of 10 ml inoculum at 48 h which had been ordered in an ascending fashion.

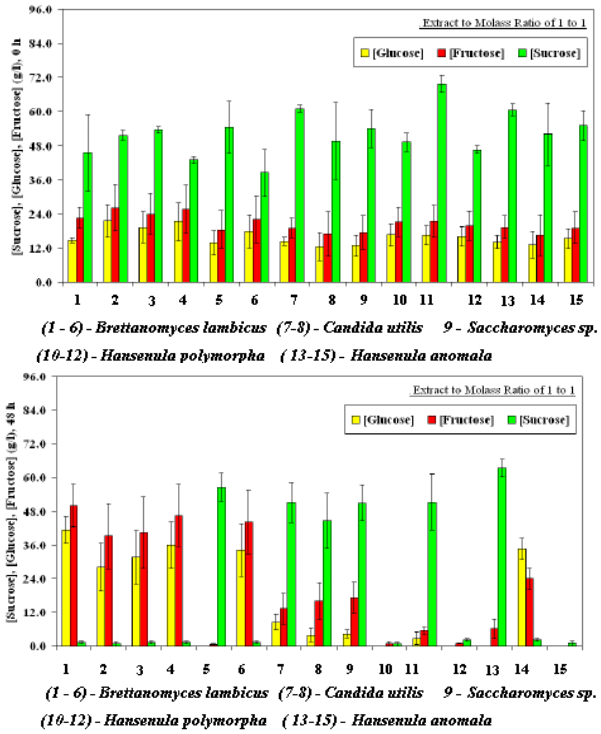



Fig. 3: The sugar concentrations in 100 ml scale experiment at (a) 0 h and (b) 48 h when the carbon source was dried ephedra extract and molasses in 1:1 ratio.

The lower ethanol yield of *Hansenula anomala* in comparison to *Hansenula polymorpha* had been discussed by Bai *et al*. (2008) who stated that even though the bacteria was able to produce an equivalent or even higher amount of ethanol in relation to yeast, its ethanol yield might be lower due to the production of by-products such as levan (from fructose) and sorbitol.

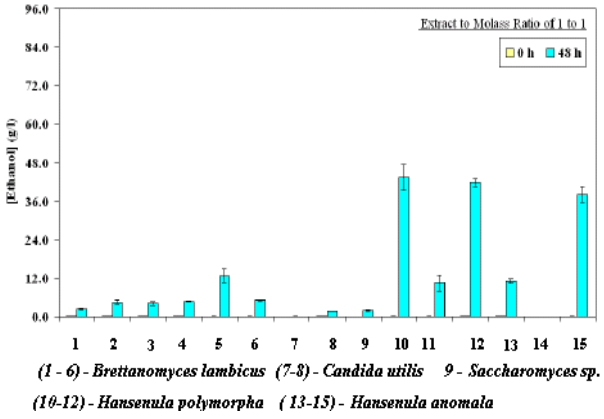



Fig. 4: The production of ethanol after 48 h cultivation period with 100 ml volume when the carbon source was dried ephedra extract and molasses in 1:1 ratio.

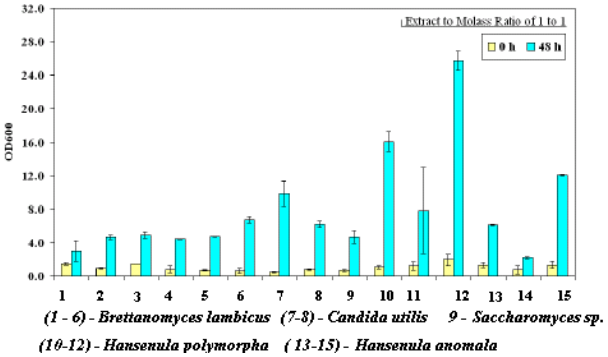



Fig. 5: The level of OD600 after 48 h cultivation period with 100 ml volume when the carbon source was dried ephedra extract and molasses in 1:1 ratio.

Furthermore, the same trend of increased OD600 to ethanol concentration was also observed in Fig. 5 with *Hansenula polymorpha* (23.8 ± 1.3) and (15.0 ± 1.2) as well as *Hansenula anomala* (10.7 ± 0.4). As evident from Fig. 6, *Brettanomyces lambicus* and *Hansenula anomala* were able to produce lesser level of PAC than *Hansenula polymorpha*.

The former group was able to generate PAC at the level of 0.87 – 1.24 mM and 0.07 – 2.98 mM at 4 and 35°C while the latter group produced higher PAC at 3.04 – 3.97 mM and 3.72 – 4.72 mM, respectively. In fact, the current production mode resulted in the higher level of by-product, viz. acetaldehyde, concentration between 5 – 14 mM and 15 – 36 mM at 4 and 35°C for *Brettanomyces lambicus* and *Hansenula anomala* as well as 2 – 3 mM and 13 – 15 mM at 4 and 35°C for *Hansenula polymorpha* in the stand still condition.

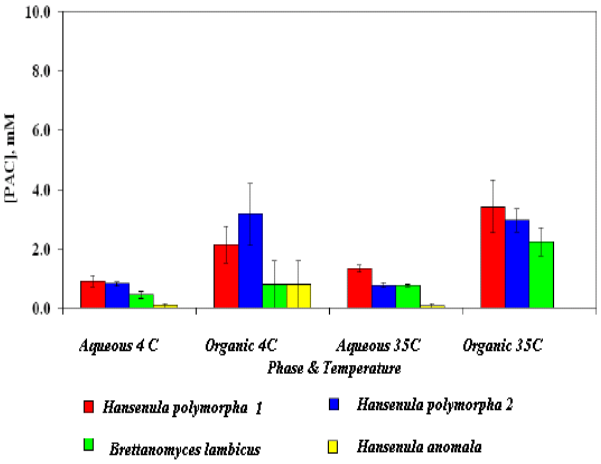



Fig. 6: PAC concentration in two phase biotransformation system after 24 h.

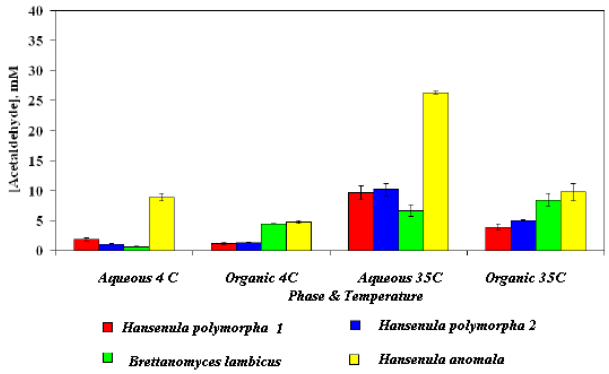



Fig. 7: Acetaldehyde concentration in two phase biotransformation system after 24 h.

## Conclusion

In conclusion, the screening of yeasts capable of utilizing sugars in a mixture between dried ephedra extract and molasses in 1:1 were carried out and resulted in three microbial strains, namely, *Hansenula polymorpha* and *Hansenula anomala*. The production of PAC in a stand still condition using biphasic system and whole cell biocatalyst obtained from the fermentation of dried ephedra extract – molasses mixture was relatively small in the current study with higher level of acetaldehyde being produced. Further study aimed at increasing the level of PAC in the present system is currently under investigation by our research group.

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