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Short Communication

Screening of 50 Microbial Strains for Production of Ethanol and (*R*)-phenylacetylcarbinol

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ABSTRACT

The ethanol production capability of 50 microbial strains was determined under shaking condition. Yeast malt medium was used as carbon and complex nitrogen sources for conversion of reducing sugars into ethanol. In order to compare the capability of these wild-type micro-organisms which were readily available in Thailand for the production of ethanol and PAC, the corresponding carboligase activity of PDC of each microbial strain was determined. The level of ethanol production and ethanol yield in term of grams of ethanol produced per gram of sugar utilized being produced under the investigated condition could be ranked in the descending order as following; *Candida shehatae* 5843 (4.21 ± 0.39 g/l and 0.47 ± 0.05 g/g), *C. maltosa* 5165 (3.69 ± 0.25 g/l and 0.39 ± 0.03 g/g), *C. lusitaniae* 5156 (3.52 ± 0.19 g/l and 0.51 ± 0.03 g/g), and *C. krusei* 5624 (3.51 ± 0.04 g/l and 0.42 ± 0.00 g/g). Moreover, five microbial strains were identified in the microbial screening for PAC production: *C. tropicalis* 5350 (19.8 ± 3.36 mM), *C. tropicalis* 5144 (9.85 ± 1.42 mM), *C. fennica* 5618 (8.41 ± 0.95 mM), *C. tropicalis* 5306 (8.39 ± 0.69 mM), and *C. utilis* 5198 (8.29 ± 1.51 mM). Ranking of each microbial strain by taking into account the production level of dried biomass, ethanol, PAC, PDC, and remnant cells viability after cultivation resulted in two promising microbial candidates, namely, *C. tropicalis* 5350 and 5306 with corresponding score of 70.02% and 58.03%, respectively.

Keywords: *(R)*-phenylacetylcarbinol, ethanol, pyruvate decarboxylase, microbes, screening

1. INTRODUCTION

The production and utilization of bioethanol has attracted worldwide attention as a strategy for reducing global warming and improving global energy security. Most ethanol is produced by yeast fermentation of sugar, in a process that is traditionally batch. During production of ethanol, pyruvate decarboxylase (PDC) catalyzes the nonoxidative decarboxylation of pyruvate to acetaldehyde, which is subsequently reduced

to ethanol [12]. The ethanol containing supernatant can be distilled and filtered to obtain high purity grade ethanol while the remnant microbial biomass with PDC enzyme is subsequently utilized for the biotransformation of pyruvate and benzaldehyde to produce *(R)*-phenylacetylcarbinol (PAC) [1].

PAC is the precursor for the commercial production of ephedrine and pseudoephedrine, which are used primarily as bronchial dilators and nasal decongestants. In ethanol fermentation, PDC decarboxylates pyruvate to acetaldehyde (Figure 1a) using thiamine pyrophosphate (TPP) and Mg^{2+} as cofactors. As a side reaction, PDC can ligate TPP-bound 'active acetaldehyde' to added benzaldehyde resulting in PAC (Figure 1b) [11]. Current commercial PAC production processes involve the use of fermenting yeast to produce sufficient biomass for the associated accumulation of the intracellular pyruvate and the induction of PDC synthesis in a fed-batch process. Different species of yeasts, fungi and bacteria such as *S. cerevisiae, Rhizopus javanicus* and *Zymomonas mobilis* were used for biotransformation purposes [5]. Strains belonging to the genera *Saccharomyces* and *Candida* had been found to be more efficient PAC producers at commercial scale and the former was generally chosen due to relatively safe handling and production performance. Extensive studies for extracted PDC from yeasts *S. cerevisiae, C. utilis*, and the bacterium *Z. mobilis*, revealed that *C. utilis* PDC was the best choice in term of activity and stability in comparison to *Z. mobilis* PDC due to the low affinity of the latter to benzaldehyde as well as significant substrate inhibition [12].

In the present study, we have investigated PDC carboligase activities from 50 ethanol producing microbial strains and demonstrated their potential for ethanol and PAC production. The ranking of the best microbial strain was also performed by taking into account the production level of dried biomass, ethanol, PAC, PDC, and remnant cells viability after cultivation.

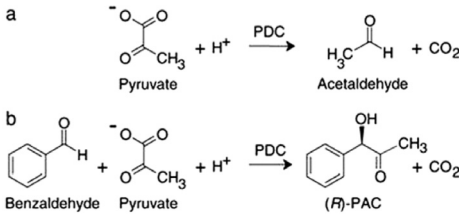

Figure 1. Reactions catalyzed by pyruvate decarboxylase (PDC). a) Pyruvate decarboxylation, b) Biotransformation of benzaldehyde and pyruvate into PAC [15].

2. MATERIALS AND METHODS

2.1 Inoculum Media Preparation

Nutrient broth for *Escherichia coli* and *Klebsiella* sp. consisted of (in one litre): 3.0 g beef extract and 5.0 g peptone. Yeast medium for *Candida* spp., *Saccharomyces* spp., and *Kluyveromyces* spp. composed of (in one litre): 10.0 g glucose, 3.0 g yeast extract, 5.0 g malt extract and 5.0 g peptone. *Zymomonas* medium for *Z. mobilis* consisted of (in one litre): 20.0 g glucose, 10.0 g yeast extract and 10.0 g peptone. The media was sterilized at 121°C, 15 psi for 15 min with a portable pressure sterilizer (All American, Model No.1925x, Wisconsin, United States).

2.2 Microorganisms

The following 46 microbial strains were ordered from Thailand Institute of Scientific and Technological Research (TISTR); *Candida famata* 5098, *C. fennica* 5618, *C. guilliermondii* 5808, *C. krusei* (5259, 5264, 5271, 5288, 5296, 5301, 5303, and 5624), *C. lusitaniae* 5156, *C. magnoliae* 5664, *C. maltosa* 5165, *C. oleophila* 5687, *C. parapsilosis* 5008, *C. pelliculosa* 5809, *C. pseudointermedia* 5069, *C. pseudotropicalis* 5336, *C. pulcherrima* (5120 and 5810), *C. shehatae* 5843, *C.* sp. 5285, *C. tropicalis* (5144, 5306,

5350, and 5615), *C. utilis* (5001, 5032, 5043, 5046, 5198, and 5352), *Escherichia coli* (361 and 1261), *Klebsiella sp.* 1383, *Kluyveromyces marxianus* (709700 and 5695), *Saccharomyces ellipsoideus* (5194 and 5199), *S. cerevisiae* (5020, 5339, and 5606), and *Zymomonas mobilis* (405, 548, and 550).

Moreover, 4 strains of yeast were obtained from the Culture Collection of the School of Biotechnology and Biomolecular Sciences, University of New South Wales, Sydney, Australia; *C. utilis* (strain 709400 and 709700), *S. cureanes* 709800, and *S. cerevisiae* 706900.

2.3 Preparation of Microbial Inocula

The frozen stock of 0.8 ml for each microbial strain was thawed and added to 10 ml of corresponding cultivation media in 50 ml centrifuge tube for the later cultivation in 100 ml scale. The cultivation of each inoculum was carried out at temperature 30°C on a rotary shaker at 200 rpm for 24 h.

2.4 Cultivation of Microbial Culture in 100 ml Scale

The prepared microbial inocula in the previous step were added to the following 100 ml cultivation media in 250 ml Erlenmeyer flask. The cultivation of each microbial strain was carried out at 30°C on a rotary shaker at 200 rpm for 24 h.

2.5 Analytical Determination

For the measurement of dried biomass concentration, 10 ml of culture broth was centrifuged for 15 min at 2,822 × g. Wet biomass was washed twice with distilled water and dried at 105°C to constant weight for analysis of dried biomass concentration. The determination of sugars (glucose) and alcohol (ethanol) was performed with high performance liquid chromatography (HPLC) using Aminex® HPX-87H column (BioRad, Hercules, California, United States of America). PAC was quantified by HPLC. PDC carboligase activity was measured as formation of PAC in 30 min at 25°C from 40 mM benzaldehyde and 100 mM pyruvate in carboligase buffer. One unit (U) carboligase activity was defined as the amount of enzyme that produces 1 mmol PAC from pyruvate and benzaldehyde per min at pH 6.4 and 25°C in a carboligase assay specified by Rosche *et al.* [11].

Cells viability measurement was determined using microscope with hemocytometer for cells counting [2].

2.6 Hypothesis Testing

All treatments were carried out with 5 replicates with the calculated mean values. The reliabilities of the mean values among the samples were identified and assessed for significant difference based on the Tukey Honestly Significant Difference (HSD) procedure. The statistical analysis was employed by SPSS for Windows®, with Statistical significance at $p \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1 Ethanol Production and Glucose Consumption

The ethanol production level was dependent on each microbial strain. *C. shehatae* 5843 showed the maximum ethanol production of 4.21 ± 0.39 g/l and ethanol yield of 0.47 ± 0.05 g ethanol produced per g sugar utilized and productivity of 0.088 ± 0.001 g/l/h under the specified fermentation conditions of pH 6.0, 30°C, agitation speed of 200 rpm, and cultivation period of 48 h. This was compared to the optimized process parameters study for ethanol production from *C. shehatae* at pH 5.0, 30°C, agitation speed of 140 rpm, and cultivation period of 48 h where the ethanol yield of 0.31 g ethanol produced per g sugar utilized and ethanol

productivity of 0.076 g/l/h were obtained [4].

This was compared to *E. coli* 361 exhibited the minimum of 0.09 ± 0.01g/l and ethanol yield of 0.01 ± 0.00 g/g. The glucose consumption after 48 h for *E. coli* 361 and *Klebsiella sp.*1383 were the highest (with less than 0.25 g/l of residual glucose).

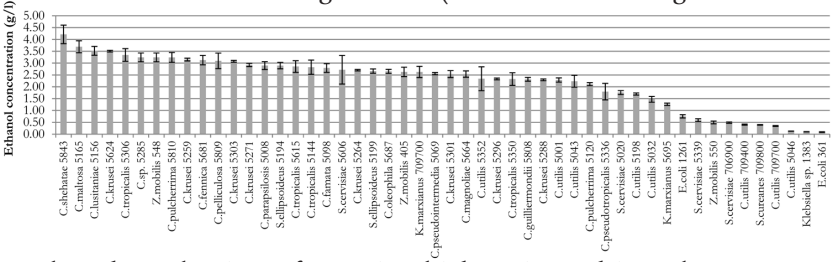

Figure 2. Ethanol production of 50 microbial strains cultivated at 30°C on a rotary shaker at 200 rpm for 24 h.

These microbial strains also generated the small amount of ethanol concentration (less than 0.10 g/l) and had the ethanol yield of 0.01 ± 0.00 g ethanol per g sugar utilized. According to Najafpour *et al*. [8], bacteria produced by-product such as acetone, glycerol, acetate, and lactate which resulted in the decreased production of ethanol from glucose. Moreover, the major disadvantages associated with using *E. coli* cultures were a narrow and neutral pH growth range (6.0-8.0) and less hardy cultures compared to yeast [9]. In contrast, *C. lusitaniae* 5156 and *C.* sp. 5285 were the potential yeast strains capable of producing ethanol from glucose with high ethanol concentration and ethanol yield of 3.52 ± 0.19 g/l, 0.51 ± 0.03 g ethanol per g sugar utilized as well as 3.24 ± 0.19 g/l, 0.50 ± 0.03 g ethanol per g sugar utilized, respectively. *C. lusitaniae* showed good results with a corresponding yield of 0.34 g ethanol per g sugar utilized. The maximum ethanol production level of both yeast strains suggested that these yeasts might be able to utilize glucose obtained from hydrolysis process of cellulosic biomass for biofuels production. In addition, some yeast strains, such as *S. rouxii*, could also consume ethanol as an alternative carbon source for growth resulting in the mitigation of ethanol concentration level [13].

3.2 PDC Carboligase Activity and PAC Formation

PAC formation from benzaldehyde and pyruvate was detected in all tested microbial cells extracts of 50 strains. The high activity was found in *C. tropicalis* 5350 and 5144 at 2.18 ± 0.83 and 5144 at 2.18 ± 0.83 and 1.08 ± 0.35 U/ml with high PAC production of 19.83 ± 3.36 and 9.85 ± 1.42 mM, respectively. Similar results were observed in the study of Rogers *et al.* [10] who reported the highest carboligase activities from *C. utilis*, *C. tropicalis*, and *C. albicans* which provided high level of PAC formation than the other yeast strains. Moreover, Kulkurni and Shukla [7] also suggested that PAC productivity was dependent on the nature of each microorganism and its metabolic status. These two strains of *Candida* were also produced high level of dried biomass concentrations of 4.59 ± 0.09 g/l and 3.77 ± 0.41 g/l, respectively. This result correlated PAC formation to biomass production level for cells that underwent fermentative metabolic pathway. According to Hussain [6], growth quality is one of the responsible factors for a better PAC production.

Some microbial strains such as *C. tropicalis*

5615 showed the highest dried biomass concentration of 4.61 ± 0.10 g/l with the lowest PDC activity (0.06 ± 0.01 U/ml) and PAC production (0.55 ± 0.06 mM)(Figure 2). It was possible that physiological conditions of this yeast had influenced the PAC formation level [3]. Evidently, the provided growth conditions for these yeast strains might still be sufficiently aerobic and thus prevented the formation of PDC. The result of ethanol production level from *C. tropicalis* 5615 showed moderate production level at 2.86 ± 0.25 g/l.

The quantitation of cells viability for all microbial strains indicated the presence of live cells at a higher level than 87% after 24 h cultivation. The highest cells viability was observed with *C. krusei* 5296 (99.31 ± 0.36 %) which was not differ significantly ($p > 0.05$) from the other 46 strains. Interestingly, the viable cells and dried biomass yield of *C. tropicalis* 5615, *C. tropicalis* 5350, *S. cervisiae* 5339, and *C. tropicalis* 5144 were high but ethanol production was relatively low. This might suggest that these yeasts utilized sugars under aerobic condition for biomass production. All fifty yeast strains seemed to have adequate nutrients and oxygen for cells proliferation as evident from high cells viability after cultivation. The ranking of studied microbes using the scores from the results of dried biomass, ethanol, PAC, PDC, and remnant cells viability were indicated in Table 1. The scores were calculated as proportion percentages of all factors combined. The maximum score was 70.02 ± 4.36 % for *C. tropicalis* 5350 which was followed by 5306 (58.03 ± 0.80 %). The microbial strains with high scores were subsequently selected for further experiments such as evaluation of cells strains, as well as to studies on the best method of PDC crude extract preparation.

3. CONCLUSIONS

Four microbial strains (*Candida shehatae* 5843, *C. maltosa* 5165, *C. lusitaniae* 5156, and *C. krusei* 5624) were able to produce appreciable amount of ethanol concentration. In terms of biomass production, *C. tropicalis* 5615 and 5350 offered the highest dried biomass concentration level. The highest carboligase activities (0.39 ± 0.06 U/ml) and PAC production (19.83±3.36 mM) was observed for *C. tropicalis* 5350. However, the most interesting candidate were *C. tropicalis* 5350 and 5306 after taking into account the production level of dried biomass, ethanol, PAC, PDC, and remnant cells viability with corresponding score of 70.02% and 58.03%, respectively.

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Table 1. The ranking of first 10 potential microbial strains by taking into account the production level of dried biomass, ethanol, PAC, PDC, and cell viability.

No.	Microbial strain	Dried biomass (score 100)	Ethanol (score 100)	PAC (score 100)	PDC (score 100)	Viable cell (score 100)	Total (score 500)	% Total
1	*C.tropicalis* 5350	84.81 ± 1.58	42.04 ± 4.86	65.36 ± 11.08	65.36 ± 11.08	92.52 ± 1.82	350.09 ± 21.79	70.02 ± 4.36
2	*C.tropicalis* 5306	78.23 ± 3.37	60.46 ± 4.83	27.65 ± 2.27	27.65 ± 2.27	96.18 ± 1.88	290.17 ± 3.98	58.03 ± 0.80
3	*C.tropicalis* 5144	69.69 ± 7.60	51.14 ± 5.47	32.48 ± 4.67	32.48 ± 4.67	95.26 ± 1.44	281.05 ± 17.38	56.21 ± 3.48
4	*C.fennica* 5618	59.45 ± 2.06	56.54 ± 3.56	27.71 ± 3.13	27.71 ± 3.13	97.62 ± 0.51	269.03 ± 9.92	53.81 ± 1.98
5	*C.lusitaniae* 5156	56.71 ± 1.08	63.64 ± 3.38	20.84 ± 0.73	20.84 ± 0.73	96.42 ± 0.25	258.46 ± 3.72	51.69 ± 0.74
6	*C.pulcherrima* 5810	66.10 ± 0.89	58.60 ± 3.75	16.80 ± 1.28	16.80 ± 1.28	88.55 ± 3.17	246.84 ± 6.60	49.37 ± 1.32
7	*C.shehatae* 5843	34.20 ± 2.21	76.19 ± 7.05	17.29 ± 0.40	17.29 ± 0.40	98.18 ± 0.35	243.16 ± 7.71	48.63 ± 1.54
8	*Z.mobilis* 548	56.78 ± 1.14	58.62 ± 3.37	13.53 ± 1.07	13.53 ± 1.07	97.50 ± 2.50	239.96 ± 4.06	47.99 ± 0.81
9	*C.magnoliae* 5664	60.26 ± 2.11	45.84 ± 2.43	17.94 ± 0.88	17.94 ± 0.88	97.78 ± 1.09	239.77 ± 1.69	47.95 ± 0.34
10	*C.tropicalis* 5615	85.21 ± 1.78	51.65 ± 4.46	1.80 ± 0.18	1.80 ± 0.18	97.59 ± 0.15	239.96 ± 4.06	47.61 ± 1.10

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