วิธีการผลิตไพรูเวตและการนำไพรูเวตไปประยุกต์ใช้ในกระบวนการผลิตฟีนิลแอซิทิลคาร์บินอล

Routes of Pyruvate Production and Application of Pyruvate for (*R*)-phenylacetylcarbinol Production

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บทคัดย่อ

ไพรูเวตเป็นสารที่ผลิตได้จากกระบวนการย่อยสลายกลูโคสในกระบวนการไกลโคไลซิส ซึ่งสารดังกล่าวนี้ถูกนำมาปรับใช้อย่างแพร่หลายไม่ว่าจะใช้เป็นส่วนผสมในอาหาร ยา และสารเคมีในอุตสาหกรรม ไพรูเวตผลิตได้ทั้งจากการสังเคราะห์ทางเคมีและกระบวนการทางชีวภาพ อย่างไรก็ตาม การผลิตสารไพรูเวตได้มุ่งเป้าไปยังวิธีการทางเทคโนโลยีชีวภาพ ในบทความวิจัยดังกล่าวนี้ ผู้เขียนได้รวบรวมวิธีการผลิตไพรูเวตด้วยวิธีทางชีวภาพผ่านกระบวนการหมักด้วยจุลินทรีย์ที่ทำการย่อยสลายกลูโคสหรือแหล่งคาร์บอนที่มีต้นทุนต่ำในขั้นตอนเมแทบอลิซึมในสภาวะที่มีออกซิเจน รวมถึงการนำไพรูเวตไปประยุกต์ใช้เป็นสารตั้งต้นในกระบวนไบโอทรานสฟอร์เมชั่นเพื่อผลิตสารฟีนิลแอซิทิลคาร์บินอลโดยการเร่งปฏิกิริยาด้วยเอนไซม์ไพรูเวตดีคาร์บอกซีเลสที่พบได้ในจุลินทรีย์ที่ผลิตเอทานอล ฟีนิลแอซิทิลคาร์บินอลถูกนำมาใช้เป็นสารตั้งต้นในการผลิตยาเอฟิดรินและซูโดเอฟีดรินที่ออกฤทธิ์บรรเทาอาการหอบหืดและไข้หวัด

คำสำคัญ : ไพรูเวต, เทคโนโลยีชีวภาพ / ไบโอทรานสฟอร์เมชั่น / การหมัก / ฟีนิลแอซิทิลคาร์บินอล

ABSTRACT

Pyruvate is the output of the anaerobic metabolism of glucose known as glycolysis. It has been widely applied in food, pharmaceutical, and agrochemical industries. Pyruvate can be produced by both systems of chemical and biological. However, pyruvate production is more focused in biotechnological systems. In this review, I summarize biotechnological production methods of pyruvate which can use direct fermentation based on microorganisms during the oxidative metabolism of glucose or cheap carbon source. In addition, the use of pyruvate for application to (*R*)-phenylacetylcarbinol (PAC) in further biotransformation stage involving feeding of substrate pyruvate, catalyzed by pyruvate decarboxylase (PDC) present in ethanol producing microorganisms is also reviewed. PAC is the chiral precursor for synthesis of the pharmaceuticals ephedrine and pseudoephedrine used to relieve symptoms of congestion associated with asthma and influenza.

Keywords : Pyruvate / Biotechnological / Biotransformation / Fermentation / (*R*)-phenylacetylcarbinol

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Introduction

Pyruvate is a key precursor metabolite for the biosynthesis of both aerobic and anaerobic conditions, which is also an important biosynthetic precursor metabolite. It is currently manufactured for use as a dietary supplement, a weight-control supplement, anutraceutical, and an antioxidant. Pyruvate is produced by numerous routes such as chemical and fermentation processes. Biotechnological production of pyruvate has become a fast-growing market and fermentation processes have been widely established in pyruvate production. The searches of more biocatalysts process for pyruvate production has been focused in both efficient and cost-effective (Xu *et al*., 2008).

(R)-phenylacetylcarbinol (PAC) is a precursor for the commercial production of ephedrine and pseudoephedrine, which are used primarily as bronchial dilators and nasal decongestants (Suresh *et al*., 2009). In South-East Asia, pharmaceutical preparations are increasingly used for the manufacturing of methamphetamine. Since the increased in demand of ephedrine and pseudoephedrine, price is subsequently increased ten folds. The price per kg of ephedrine and pseudoephedrine are 1,139.76 and 47.49 USD, respectively. Shin and Rogers (1995) divide the PAC production process into two stages as followings; (1) fermentative yeast growth which involve the accumulation of yeast cells containing pyruvate decarboxylase (PDC), and (2) biotransformation stage involve the feeding of substrate pyruvate and benzaldehyde to produce PAC together with some benzyl alcohol production. Moreover, substrate preferred of PDC is pyruvate (Schutz *et al*., 2005). In addition, it can be noted that the high yield PAC from biotransformation process is required for the high production of pyruvate from hexoses in the live yeast cells process (Oliver *et al*., 1999).

The present review focuses on the fermentative production of pyruvate based on microorganisms. The application of pyruvate as a substrate for PAC production in biotransformation process catalyzed PDC enzyme stabilization is reviewed also.

Biotechnological production of pyruvate

Pyruvate plays an important metabolite in the central metabolism of living cells. It's currently manufactured for use as a dietary supplement, weight-control supplement, nutraceutical, and antioxidant. It's also used as a promising raw material for the synthesis of pharmaceutical precursors such as:

- *L*-tyrosine: *L*-tyrosine is a nonessential organic amino acid that is a building block of protein. It is a precursor of the neurotransmitter dopamine, as well as a precursor to the adrenal hormones norepinephrine and epinephrine. The body can make *L*-tyrosine from the amino acid phenylalanine. *L*-tyrosine rich foods include animal meat, wheat products, oatmeal and seafood (Wang *et al*., 2007).

- *N*-acetyl-*D*-neuraminic acid: organic acid from *N*-acetylmonosamine and pyruvate.

- (*R*)-phenylacetylcarbinol (PAC): the chiral precursor for the production of the pharmaceuticals ephedrine and pseudoephedrine.

Biotechnological production of pyruvate can use direct fermentation using a cheap carbon source, immobilized enzymes, and whole-cells catalysts.

1. Fermentative production of pyruvate

Glycolysis is a primitive set of reactions used in both pro- and eukaryotes which glucose (6-C molecules) is converted into an intermediary molecule called pyruvic acid (3-C molecules) (Fig. 1). Different fermentation pathways and products of anaerobic metabolism play essential roles in surviving prolonged periods under anoxia (Li *et al*., 2001). Many researchers have attempted to identify microorganisms with high pyruvate-producing ability, it is still difficult to obtain strains that can accumulate large amounts of pyruvate by conventional mutation and selection approaches since pyruvate is locate data vital junction of cells metabolism. *Escherichia coli* are used in commercial fermentative pyruvate production. However, construction of *E. coli* for pyruvate production during the oxidative metabolism of glucose requires the minimization of further transformation of pyruvate for biomass production and the elimination of major nonessential pathways that consume pyruvate (Zelic *et al*., 2003). There are many ways for pyruvate production using cheap carbon source. Agustina *et al*. (2009) used deadstock dried longan as a carbon source for bioconversion of sugars to pyruvate. Moreover, Natikarn and Leksawasdi (2009) also used fermentation media with carbon source from ground solid corn waste that was predigested by amylase enzymes prior to mixing with molasses.

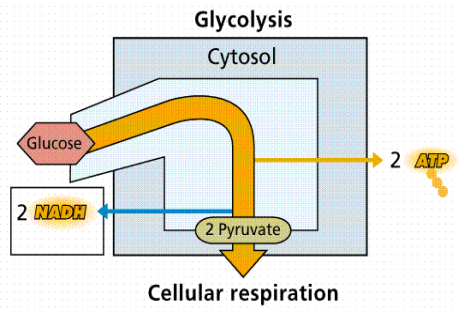

Figure 1: Synthetic pathways for production of pyruvate (Farabee *et al*., 2010)

2. Whole-cells catalysts for pyruvate production

Pyruvate is produced from a substrate using a series of enzymes in microbial cells, whereas in the latter, pyruvate is synthesized from a substrate by a single enzyme. In the whole-cell method, pyruvate is produced by conversion of the substrate with biocatalysts separated from a growth medium such as glucose, glycerol, citruspeel extract,and 1,2-propanediol, fumarate (Mu, 2004). Pyruvate can be produced from lactate through

biocatalysis. Whole cells biocatalysts could catalyze lactate to pyruvate under relatively mild conditions (Gao *et al*., 2011). In addition, the production of pyruvate at a high concentration from the cheaper lactate substrate is a valuable process in an industrial scale. Various lactate-utilizing microbial strains from soil were screened for pyruvate production from lactate by whole cells. Among them, *Acinetobacter sp.* showed the highest biotransformation efficiency from lactate to pyruvate in the optimized dry-cell concentration, pH, and temperature of 6 g/L, pH 7.0-7.5, and 30 $^{\circ}$C, respectively. Under the optimized reaction conditions, l-lactate at concentrations of 200 and 500 mM were almost totally stoichiometrically converted into pyruvate in 8 and 12 h, respectively. About 60% of 800 mM of l-lactate was transformed into pyruvate in 24 h. This reduced conversion rate is probably due to the high substrate inhibition in biotransformation (Ma *et al*., 2003).

Whole cells of *Pseudomonas stutzeri* SDM catalyze the cheap DL-lactate as the substrate under optimal conditions. This process could be produced pyruvate at a high concentration (48.4 g l^{-1}) and a high yield (98%). The bioconversion system provides a promising alternative for the green production of pyruvate. Fed-batch fermentation was applied to the production of pyruvate by using *E. coli* YYC202 strain. A final pyruvate concentration higher than 62 g/L, a space-time yield of up to 42 g/L/d and pyruvate/glucose molar yield of 1.11 mol/mol were achieved at the optimal process conditions. Experimental evidence was gathered that pyruvate export is active (Zelic *et al*., 2003).

3. Pyruvate production form immobilized enzymes

Enzymatic synthesis of chemicals has become a valuable tool for synthesis chemists. Different enzymes such as formaldehyde dehydrogenase, pyruvatesynthase, glycolate oxidase,tartrate dehydratase, and D-amino acid oxidase have been employed in the enzymatic synthesis of pyruvate. Buto *et al.* (1994) have evaluated D-amino acid oxidase for the production of pyruvic acid. Under optimum operation conditions, over 90% of the substrate is converted into pyruvate with a maximum yield of 0.23 g of pyruvic acid/day/enzyme unit. In 2001, Miyazaki has developed a new enzymatic synthesis process to produce pyruvic acid from acetaldehyde and CO_2 using PDC. The maximum pyruvate yield of 81% was obtained in 500 mM $NaHCO_3$–Na_2CO_3 buffer. Although the concentration of pyruvate was not high, this reaction might become a recommendable, environmentally safe CO_2 immobilization procedure for pyruvate production because CO_2 is considered to be a greenhouse gas.

The biotransformation step of pyruvate by using PDC

The chiral pharmaceuticals ephedrine and pseudoephedrine are currently produced commercially a biotransformation of benzaldehyde by fermenting baker's yeast followed by chemical conversions. (Fig. 2)

3.1 In ethanol fermentation, PDC decarboxylates pyruvate to acetaldehyde (Fig. 2a) using thiamine pyrophosphate (TPP) and Mg2+ as cofactors.

3.2 As a side reaction, PDC can ligate TPP-bound 'active acetaldehyde' to added benzaldehyde resulting in PAC (Fig. 2b).

3.3 Furthermore PDC can convert pyruvate and acetaldehyde to acetoin.

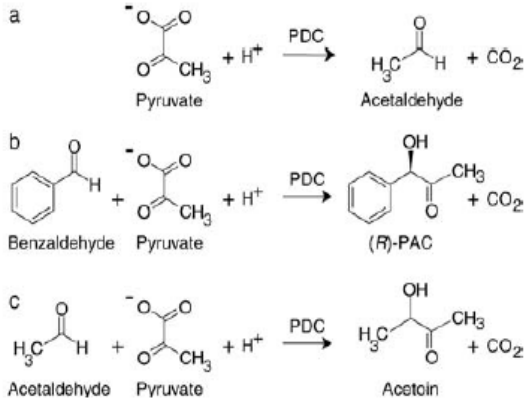

Figure 2: Reactions catalyzed by PDC. a Pyruvate decarboxylation, b Biotransformation of benzaldehyde and pyruvate into PAC, c By-product acetoin formation from acetaldehyde and pyruvate (Rosche *et al.,* 2002)

The role of pyruvate in enhancing pyruvate decarboxylase stability for PAC production

PAC production at a low buffer concentration with pH control by the addition of acid and the effects of glycerol as a potential additive on enzyme stabilization during catalysis was evaluated.

1. Factor of pH for increasing final PAC levels

Control of pH was identified as a crucial factor for increasing final PAC levels. pH increases were restricted by high concentrations (2.5 M) of MOPS (3-(N-morpholino)-propane sulfonic acid) buffer, which had the additional benefit of stabilizing PDC. For example, partially purified *C.utilis* PDC produced 51.2 g/l PAC in 21 h in a simple batch biotransformation in 2.5 M MOPS buffer.

From the table 1, the enzyme was very stable in 20 mM MOPS buffer in the absence of benzaldehyde (half-life 138 h). PDC was completely inactivated in less than 30 min when exposed to a benzaldehyde emulsion of 400 mM in the same buffer concentration (Fig. 3). The same degree of inactivation was observed in the presence of 2 M glycerol (Fig. 4), while 2.5 M MOPS slightly enhanced PDC stability resulting in a half-life of 40 min in the presence of 400 mM benzaldehyde. So that, inclusion of 2 M glycerol did not protect PDC from inactivation by benzaldehyde, initial rates in 20 mM MOPS buffer increased with increasing glycerol concentrations as illustrated. Moreover, at 2 M glycerol the initial activity was 50% higher compared to the activity in the absence of glycerol. Hence glycerol increased

the activity more efficiently than MOPS, while high MOPS concentrations slightly enhanced PDC stability in the presence of 400 mM benzaldehyde (Rosche *et al*., 2003).

Table 1: Effect of benzaldehyde emulsion on *Candida utilis* PDC stability

	Benzaldehyde (mM)	Residual activity after 30 min (%)	Half-life (h)
20 mM MOPS (control)	0	100	138
20 mM MOPS	400	0	< 0.25
20 mM MOPS, 2 M glycerol	345	0	< 0.25
20 mM MOPS, 2 M glycerol	400	0	< 0.25
2.5 M MOPS	400	58	0.7

(Source: Rosche *et al*., 2005)

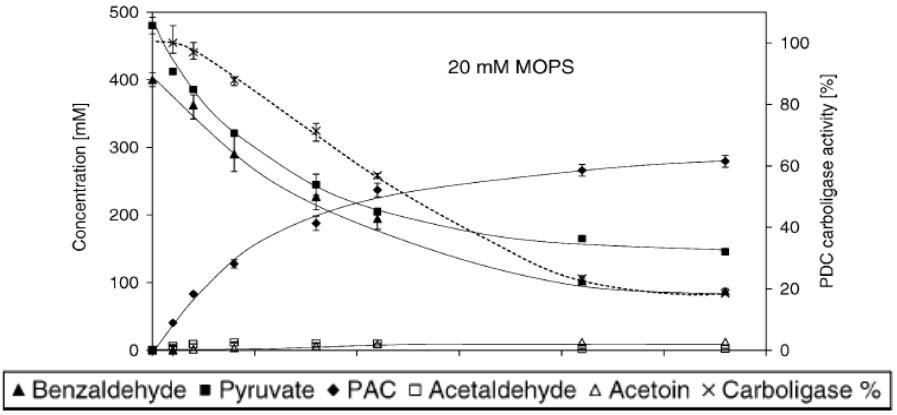

Figure 3: PAC, acetaldehyde, and acetoin production by partially purified *C. utilis PDC.* Actual concentrations are plotted without taking into account dilution by acid addition. Error bars indicate lowest and highest results of 2–3 measurements (Rosche *et al*., 2005).

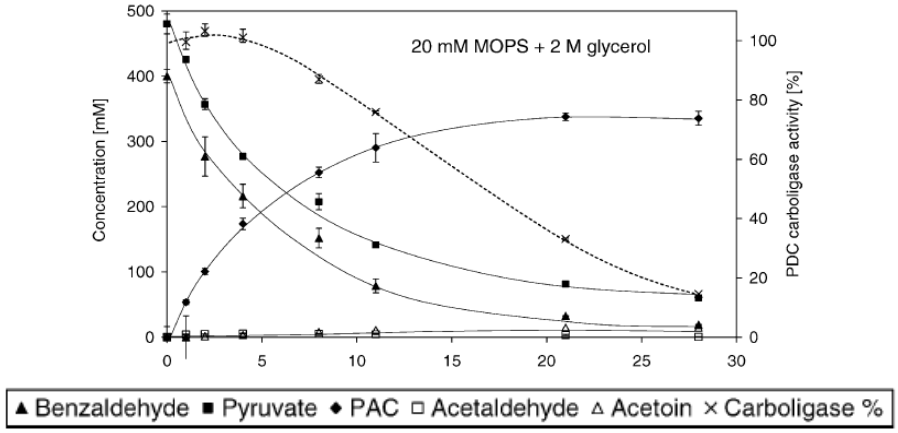

Figure 4: PAC production by partially purified *C. utilis PDC* in the presence of glycerol. Actual concentrations are plotted without taking into account dilution by acid addition. Error bars indicate lowest and highest results of 2–3 measurements (Rosche *et al*., 2005).

The presence of 20 mM MOPS and 2M glycerol with pH controlled at 7 by acetic acid addition. Under these conditions was produced in 21 h and by-product concentrations were low acetaldehyde and acetoin. Molar balances closed within 1% based on initial pyruvate and benzaldehyde concentrations and the molar yields of PAC on consumed substrates were 99% for benzaldehyde and 92% for pyruvate.

PAC production from pyruvate and benzaldehyde was enhanced with pH control. Furthermore biotransformation in 20 mM MOPS with pH control and addition of 2 M glycerol resulted in a specific PAC production profile that superimposes PAC formation in 2.5 M MOPS buffer. Both conditions were also final reached to 6.1 mg PAC/U PDC. PAC concentrations, initial productivity and yield were similar even though the enzyme had been more stable in 2.5 M MOPS (Fig. 5) (Rosche *et al*., 2005).

To enhance PDC stability towards benzaldehyde as followed:

-PDC was strongly and irreversibly deactivated by 400mM benzaldehyde emulsions.

-PDC was more stable towards a benzaldehyde emulsion when pyruvate was present.

-The enzyme in catalytic action was more stable than the resting enzyme.

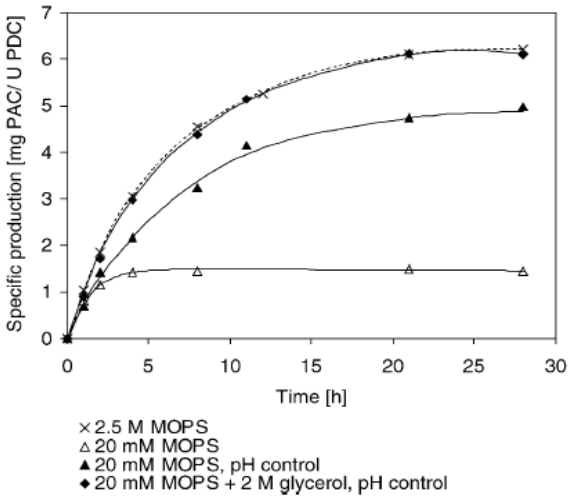

Figure 5: Effect of buffer concentration, pH control and glycerol on specific PAC production by *C. utilis* PDC. The 2.5M MOPS and 20mM MOPS without pH control (this study) and with pH control (Rosche *et al*., 2005).

2. Three possible causes for the decreased PDC inactivation

Stabilization against benzaldehyde possibly could occur as shown in Figure 6 for example;

-Direct effect of pyruvate e.g. the binding of pyruvate to PDC and conformational change of the protein, thus protecting essential sites.

-Protection during catalytic action of PDC

-Effects of products: stabilizing influence of reaction products PAC, CO_2, acetaldehyde and/or acetoin.

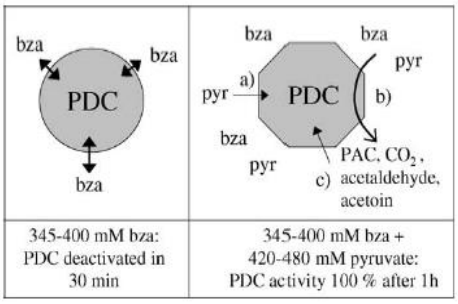

Figure 6: Scheme of PDC inactivation by benzaldehyde (left) and stabilization during catalytic action in the presence of pyruvate (right) (Rosche *et al*., 2005).

Improved PAC production with *C. utilis* pyruvate decarboxylase

(*R*)-Phenylacetylcarbinol is the chiral precursor for synthesis of the pharmaceuticals ephedrine and pseudoephedrine used to relieve symptoms of congestion associated with asthma, colds and influenza.

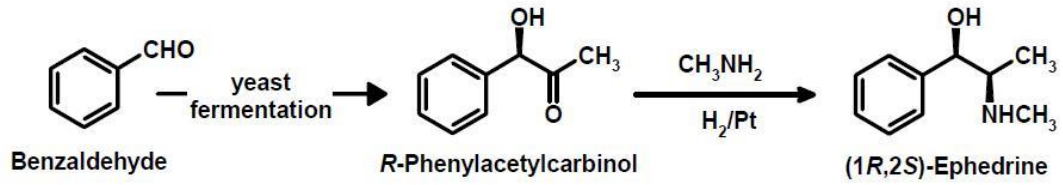

Figure 7: Synthesis of the pharmaceuticals ephedrine and pseudoephedrine (Satianegara, 2006)

PAC production was further improved in an aqueous/benzaldehyde emulsion system. This might be due to the process which was limited by loss of PDC activity due to soluble benzaldehyde in the aqueous phase (up to 100 mM). Moreover, higher temperature operation was effected on specific rate of PAC and by-product concentration (Hussain, 2009).

1. Two-phase organic/aqueous phase system

A reaction time deactivation constant of 2.64 × 10-3 h-1 and a benzaldehyde deactivation coefficient of 1.98 × 10-4 mM / h were determined for benzaldehyde concentration levels up to 200 mM. For this reason, the two-phase batch process was further improved for increased PAC production in an aqueous / benzaldehyde emulsion system (Fig. 8) (Rosche *et al*., 2002). The facilitating higher initial benzaldehyde and final PAC levels were partitioned away from the enzyme in an organic phase and thereby reducing enzyme deactivation (Sandford *et al*., 2005).

In which both benzaldehyde and PAC would selectively partition into the organic phase while pyruvate and PDC would be maintained in the aqueous phase, thereby minimizing the loss of PDC activity.

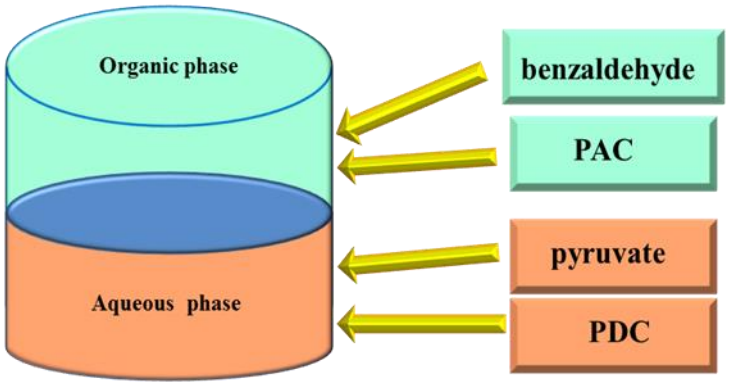

Fig. 8: Organic / aqueous two-phase system for PAC production (Rosche *et al*., 2002)

2. Organic solvents: octanol

Following the screening of a range of organic solvents, octanol was selected in view of its high partition coefficients for benzaldehyde and PAC, and its apparent minimal effects on PDC deactivation. As reported by Rosche *et al*. (2002) and Sandford *et al*. (2005), PAC higher than 100 g/l was achieved in the octanol phase. These experiments were carried out at 4°C. An aqueous phase system that higher PAC yields were achieved at lower temperatures due to reduced by-product acetoin production.

2.1 Decreasing organic to aqueous phase volume ratio

The PDC activity decreased with time in each case, although the rates of decrease were almost identical for the three different phase volume ratios (Fig. 9).

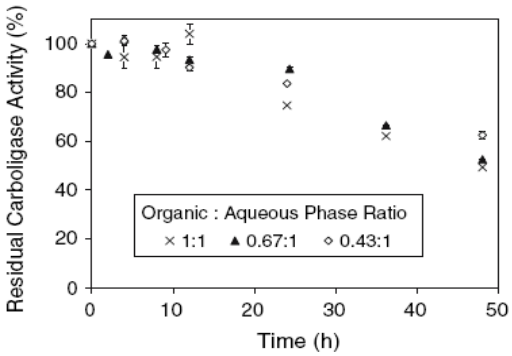

Figure 9: Effect of organic to aqueous phase volume ratio on enzyme stability during catalysis (Gunawan *et al*., 2007)

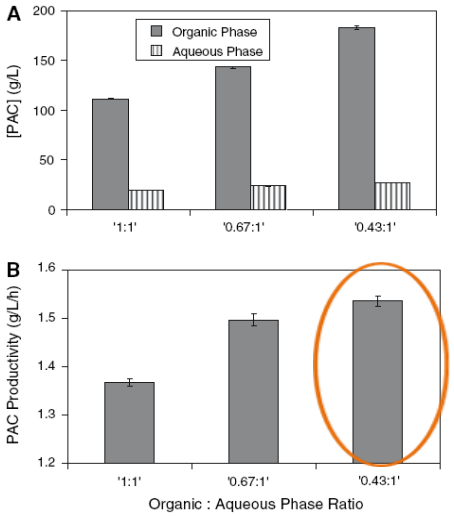

Figure 10: Effect of decreasing the organic to aqueous phase volume ratio in aqueous/octanol benzaldehyde emulsion system at 4°C, initial pH 6.5 (Gunawan *et al*., 2007).

After the reaction had proceeded for 48 h, the organic phase PAC concentration increased with decreased phase volume ratios. This was accompanied by a smaller increase of the aqueous phase PAC concentration (Fig. 10). Productivity also increased with the reduced phase volume ratios with the value for 0.43:1 being approximately 10% higher compared to that at 1:1.

From the results it can be seen that PAC concentrations continued to increase in both phases over 48 h, although with a declining overall rate. A final organic phase concentration of approximately 1,200 mM (180 g/l) PAC was achieved. A higher PAC concentration may have been possible if the reaction time had been extended as residual pyruvate, benzaldehyde and PDC activity remained. Acetoin was the major by-product with similar concentrations of 20-30 mM evident in both phases. Benzaldehyde concentrations in the aqueous phase remained relatively low throughout the biotransformation (less than 30 mM) and this would have been important in reducing the extent of PDC deactivation.

2.2 The effect of increasing temperature

To investigate the effect of increasing temperature from 4°C to 20°C, a maximum organic phase PAC concentration of approximately 1,400 mM (210 g/l) was achieved under the conditions of 20°C. The concentration of acetoin in both phases at 48 h was approximately 2-fold higher at the higher temperature with a value close to 70 mM in the organic phase and 40 mM in the aqueous phase. Similar acetaldehyde concentrations at 48 h of 12-15 mM (organic phase) and 3-4 mM (aqueous phase) were evident at both temperatures (Fig. 11 and 12). The highest organic phase PAC concentration (212 g/l), the highest PAC productivity (4 g/l/h) and the highest specific PAC production (29 mg/U initial carboligase activity) were all achieved at the lowest phase ratio (0.43:1).The concentration of acetoin in both phases at 48 h was approximately 2-fold higher at the higher temperature with a value close to 70 mM in the organic phase and 40 mM in the aqueous phase. Similar acetaldehyde concentrations at 48 h of 12–15 mM (organic phase) and 3–4 mM (aqueous phase) were evident at both temperatures (Gunawan *et al*., 2007).

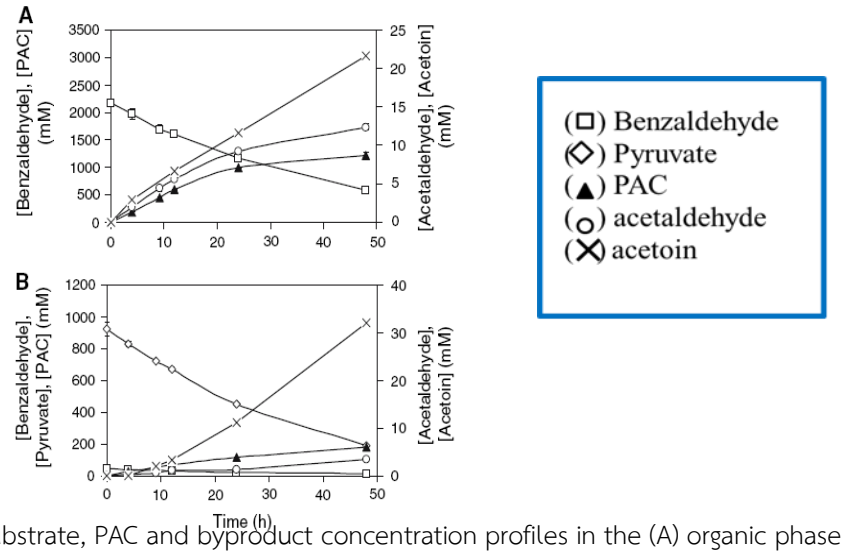

Figure 11: Substrate, PAC and byproduct concentration profiles in the (A) organic phase and (B) aqueous phase for an organic:aqueous phase volume ratio of 0.43:1 at 4°C, initial pH 6.5 (Gunawan *et al*., 2007).

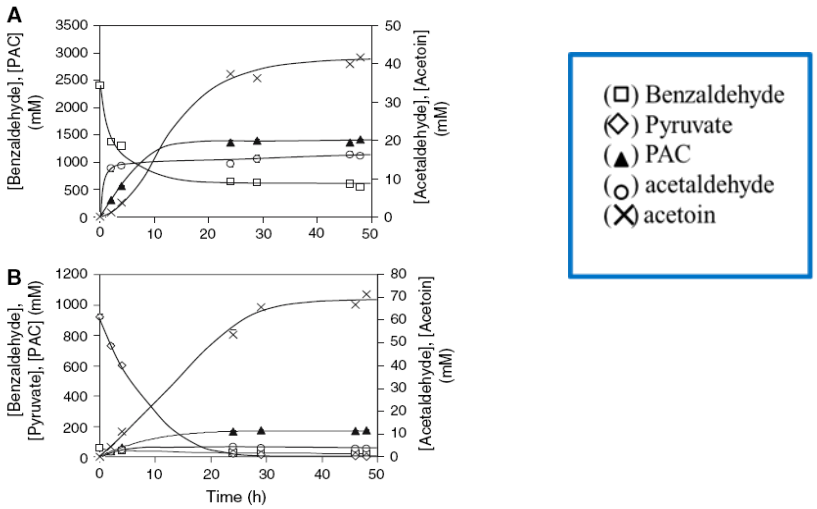

Figure 12: Substrate, PAC and by-product concentration profiles in the (A) organic phase and (B) aqueous phase at organic : aqueous phase volume ratio of 0.43 : 1 at 20 °C, initial pH 6.5 (Gunawan *et al*., 2007).

PAC yields

-PAC yields on benzaldehyde consumed indicating negligible conversion of benzaldehyde to by-products such as benzyl alcohol.

-PAC yields on pyruvate consumed were less than theoretical due to production of acetoin and acetaldehyde.

-PAC yields were more affected by the increase in temperature than by any change in phase volume ratios.

Conclusions

In this review, the production of pyruvate by fermentation and biocatalysis, are highlighted. Biocatalysis fermentation are the preferred method for pyruvate production because of the simple composition of the reaction mixture, high substrate conversion rate, and convenience of recovery. The main applications of pyruvate are promising developments in PAC production through biotransformation process involve feeding substrate pyruvate and benzaldehyde. Researchers have attempted to find the most optimum for PAC production in biocatalytic processes. In comparison biotransformation methods, a lower organic to aqueous phase ratio has been shown to be beneficial for PAC production at both 4 and 20°C. The lowest phase ratio evaluated of 0.43:1, resulted in the highest PAC concentrations and productivities. The lowest organic to aqueous phase ratio may have the additional advantages of facilitating PAC recovery from smaller organic phase volumes with relative high substrate of pyruvate containing in aqueous phase.

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