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Glucose metabolism and bioreduction of 2-butanone by *Candida utilis* studied by means of ion-exchange chromatography

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Abstract

The metabolism of glucose during cell growth was important for the production yield of the yeast mediated bioreduction of ketones. Thus, the choice of the very simple acyclic ketone 2-butanone as the substrate for *Candida utilis* can easily explain the effect of glucose metabolism on the enantioselective bioreduction mechanism of ketone. The glucose metabolism was extended by adding the sorbitol (polyol) pathway to the Embden–Meyerhoff pathway due to the existence of a NADP-specific polyol dehydrogenase in *C. utilis*. The metabolite, glycerol, was produced via the sorbitol pathway which could suppress the production of ethanol by the Embden–Meyerhoff pathway. The reaction mechanism of bioreduction showed that ethanol, an energy source of the bioreduction, must be oxidized to acetic acid to activate the oxidoreductase. The formation of acetic acid, therefore, can serve as a mark for the success of bioreduction. On the other hand, the unconsumed glucose and the less added 2-butanone indicated a negative effect for the bioreduction. Overall, the microbial biotransformation of 2-butanone to *S*-form 2-butanol studied by ion-exchange chromatography was successful.

Keywords: *Candida utilis*; Glucose; Butanone; Ketones

1. Introduction

The Aminex HPX-87H HPLC column was developed about 19 years ago. The material packed in this column is sulfonated divinylbenzene–styrene copolymer resin in the hydrogen form which is essentially a strong cation exchanger. Since over 200 small organic molecules important in biology and industry have been surveyed by this column, it is the most widely used column of this type. The study of HPLC elution behavior of alcohols, aldehydes, ketones, organic acids and carbohydrates with this column was an example of general method development [1]. There were also clinical applications such as the rapid profiling of plasma and urinary organic acids [2] and the measurement of selected blood citric acid cycle [3]. HPLC with this column was also used for the identification of both aerobic [4,5] and anaerobic species [6,7] of clinical bacteria, either alone or combined with another analytical method. All of the reviewed studies make us confident that this column is suitable for metabolite analysis and for monitoring biotransformation processes [8–12].

In the agricultural, pharmaceutical, and food industries, optically active alcohols are particularly valuable as the 'building block' molecules for synthesizing new chiral compounds which may be useful in drug design, fruit and beverage flavours, and insecticides [13]. Instead of being used as a solvent or fuel in the chemical industry [14], 2-butanol could be important for the asymmetric synthesis of many optically active compounds by its unsymmetrical molecular structure. The methods for the stereoselective formation of chiral 2-butanol can

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be proceeded by either organometallic catalysts or biocatalysts. However, we preferred to use the bioreduction process of 2-butanone. The selection of 2-butanone as the starting material was due to its cheap price, purity, and easy obtainability.

Fermenting baker's yeast (*Saccharomyces cerevisiae*) can reduce various prochiral carbonyl compounds to corresponding chiral secondary alcohols with high enantioselectivity [15]. Macleod and his coworkers [16] first studied systematically the asymmetric bioreduction of acyclic ketones with baker's yeast. These kinds of biotransformations perhaps are the most widely applicable reactions in synthetic organic chemistry [17]. Instead of using baker's yeast to perform the enantioselective bioreduction of various ketones, *Candida utilis* possesses some more advantages than the baker's yeast for the reduction of ketones [18]. As we proceeded with the microbial bioreductions, glucose was usually the only carbon source for growing the yeast. The metabolism of glucose during cell growth was, thus, important to the yeast mediated biotransformation of ketones. The production yield of the bioreduction process can be controlled if we understand the relationship between the glucose metabolism and the bioreduction mechanism of ketone.

Thus, in this paper, we used the very simple acyclic ketone 2-butanone as the substrate for *C. utilis* to survey the effect of glucose metabolism on the enantioselective bioreduction of ketone with the assistance of HPLC. The results of this paper indicate again the application potential of ion-exchange chromatography.

2. Experimental

2.1. Materials

2-Butanone, 2-butanol, absolute ethanol, peptone, D-(−)-sorbitol, KH_2PO_4, H_2SO_4, K_2HPO_4, $(NH_4)_2SO_4$, $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, and $FeSO_4 \cdot 7H_2O$ of reagent grade were all purchased from Merck (Darmstadt, Germany). Reagent-grade glyceric acid, malt extract, NADH, citric acid, isocitric acid, malic acid, succinic acid, 2,3-butanediol were bought from Sigma (St. Louis. MO, USA). Reagent-grade acetoin was obtained from Aldrich (Milwaukee, WI, USA). Diethyl ether was supplied by BDH (Poole, UK) in chromatographic grade. Yeast extract and agar for culture media were from DIFCO (Detroit, MI, USA). Freeze dried *C. utilis* CCRC 21690 was bought from the Culture Collection Research Center of Food Industry Research and Development Institute (Hsinchu, Taiwan). Distilled water was further purified by deionization and filtration before use.

2.2. Instrumentation

HPLC was performed with a Shimadzu (Kyoto, Japan) LC-9A dual-piston, solvent-delivery module with a high sensitivity filter unit, a Rheodyne (Cotati, CA, USA) model 7125 injection valve with a 20-μl sampling loop, a Shimadzu CTO-6A column oven, and a Shimadzu RID-6A refractive index detector. The prepacked Aminex HPX-87H column (Bio-Rad, CA, USA) was 300 mm×7.8 mm I.D. with sulfonated divinylbenzene–styrene copolymer resin of 9 μm particle size. The data system was a 80486-CPU level personal computer equipped with an ink-jet printer (DJ500C, Hewlett–Packard, NY, USA). The two-channel chromatography data processing software was developed by Chem-Lab (Taipei, Taiwan).

2.3. Cell growth

Freeze dried *C. utilis* was dissolved in a 0.3–0.5 ml of a medium containing 3.0 g yeast extract, 3.0 g malt extract, 5.0 g peptone, 10.0 g D-(+)-glucose and 1.0 l distilled water. An inoculation of the cell suspension was made onto agar slants prepared by adding 2% (w/v) agar into the above medium. The incubation temperature for agar slants was at 30°C. Yeast grown on agar slants was inoculated into 100 ml of the following synthetic growth medium: 1.5 g KH_2PO_4, 2.9 g K_2HPO_4, 1.3 g $(NH_4)_2SO_4$, 1.8 g $MgSO_4 \cdot 7H_2O$, 0.0175 g $CaCl_2 \cdot 2H_2O$, 0.1 ml $FeSO_4$ (1.25%, w/v), 6.0 g D-(+)-glucose and 1.0 l distilled water. The inoculated growth medium was incubated at 30°C in an orbital shaking incubator for two days at 150 rpm. Yeast grown in the 100 ml media was subsequently transferred to a 2-l capacity stirred tank fermenter (Rikakial, M-100, Tokyo, Japan) containing 1 liter of the same synthetic medium for further growth. The culture was agitated at a stirring rate

150 rpm and air was bubbled through the system at a flow-rate 0.5 l min^{-1} (Rikakial, FM-110, Tokyo, Japan). The fermenter temperature was controlled at 30°C but the pH of the culture was left uncontrolled. Generally, cell concentration reached a maximum value in about two days. The yeast was then used directly for the bioreduction of 2-butanone.

2.4. Bioreduction

2-Butanone is water soluble. Thus, a measured 2-butanone can be transferred directly into the fermenter. However, we mixed 2-butanone with 10 ml absolute ethanol, an energy source of the bioreduction, to reduce the number of transferring. About 0.1 g coenzyme NADH was then also added externally to assist the reaction [19]. At this moment, the air flow was stopped to avoid the stripping of highly volatile 2-butanone (b.p. 80°C) out of the fermenter. The bioreduction of 2-butanone was performed in a batch type reaction with a stirring rate of 150 rpm and a reaction temperature of 30°C. During the bioreduction, the pH of the cell culture was monitored but left uncontrolled around a value of 2.3. The total reaction period was three days in this case.

2.5. Cell metabolites and product analysis

Cell culture (5 ml) was removed from the fermenter and filtered through a 0.45 μm microporous membrane (Gelman Science, Ann Arbor, MI, USA). Yeast retained on the membrane was washed with distilled water and dried at 65°C for 24 h. The dry cell mass of yeast was estimated by comparing with the mass of the original membrane.

The cell metabolites, 2-butanone and 2-butanol in the filtered cell culture can be analyzed by HPLC either with suitable dilution or without dilution. The dilution was performed with the mobile phase of 0.005 *M* H_2SO_4 solution. The mobile phase was eluted isocratically at a rate of 0.6 ml min^{-1}. The column temperature was maintained at 45°C. Standard addition method was extensively used for qualitative determination of unknown substances. Quantitative measurement of various substances in the cell culture was performed by using the external calibration curve method.

2.6. Estimation of optical purity

A 100-ml quantity of the final reaction culture was used for the extraction of 2-butanol and other substances. The same volume of cell growth culture was also extracted, just before the reaction started, to serve as the background measurement. The extraction was performed by adding an equal volume of diethyl ether. After extraction, the diethyl ether layer was dried with anhydrous magnesium sulfate and filtered through a 0.45 μm microporous membrane. The optical purity of the extracted cell culture was measured roughly by a polarimeter (Atago, Polax-D, Tokyo, Japan). The measured optical rotation of the solution only indicates the enantiomeric excess of the *R*-form or *S*-form of 2-butanol.

3. Results and discussion

3.1. Glucose metabolism in Candida utilis

C. utilis can grow in a very simple growth medium in which the only carbon source and the only nitrogen source are glucose and ammonium sulfate, respectively. The highest dry cell mass was 6.36 mg ml^{-1} and the final pH of the cell culture was 2.4 in one of our experimental results [18]. Since the pH of cell culture at the beginning was 5.9, the release of acid compounds such as citric acid, succinic acid, malic acid and acetic acid during cell growth was responsible for the decrease of pH. The two large peaks in the chromatogram of Fig. 1A were citric acid and ethanol. Thus, the chromatographic results indicated that the major metabolic pathway of glucose with *C. utilis* should be via the Embden–Meyerhoff pathway to produce two molecules of pyruvate. One of the pyruvate molecules underwent fermentation by *C. utilis* to give ethanol [20]. The other pyruvate molecule was converted, under aerobic conditions, into acetyl SCoA which was further broken down by the citric acid cycle. There were also very small amounts of glycerol, acetic acid, acetoin and 2,3-butandiol in the cell culture. The appearance of these side-reaction products was due to some other minor metabolic pathways of glucose which have been explained by Oura [21]. The accumulation of glyceric acid in the cell

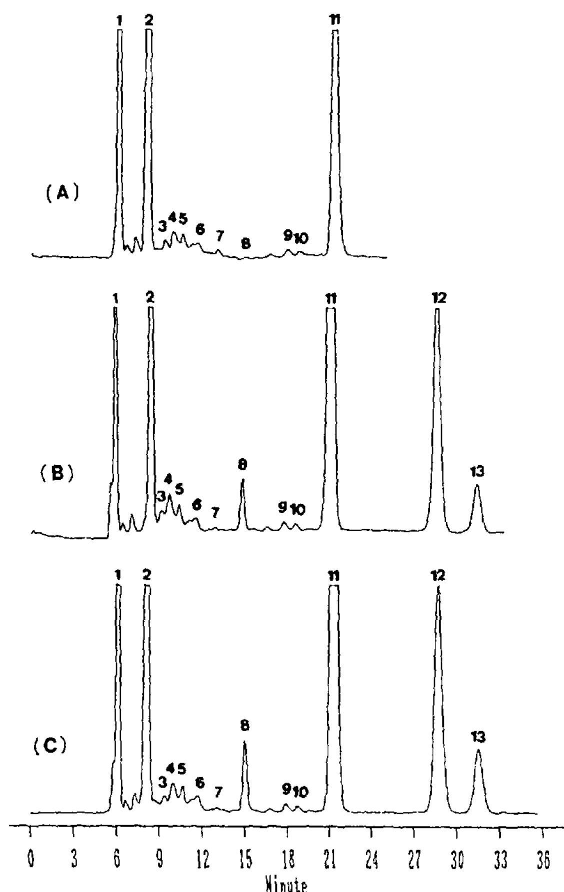

Fig. 1. The metabolites of glucose in the growth culture of *C. utilis* and the subsequent bioreduction culture with 5 ml 2-butanone. Chromatogram (A): the cell culture just before adding any 2-butanone for bioreduction. Chromatogram (B): the bioreduction culture for a reaction period of two days. Chromatogram (C): the bioreduction culture for a reaction period of three days. Peaks: 1=solvent front; 2=citric acid; 3=malic acid; 4=sorbitol; 5=glyceric acid; 6=succinic acid; 7=glycerol; 8=acetic acid; 9=acetoin; 10=2,3-butanediol; 11=ethanol; 12=2-butanone; 13=2-butanol.

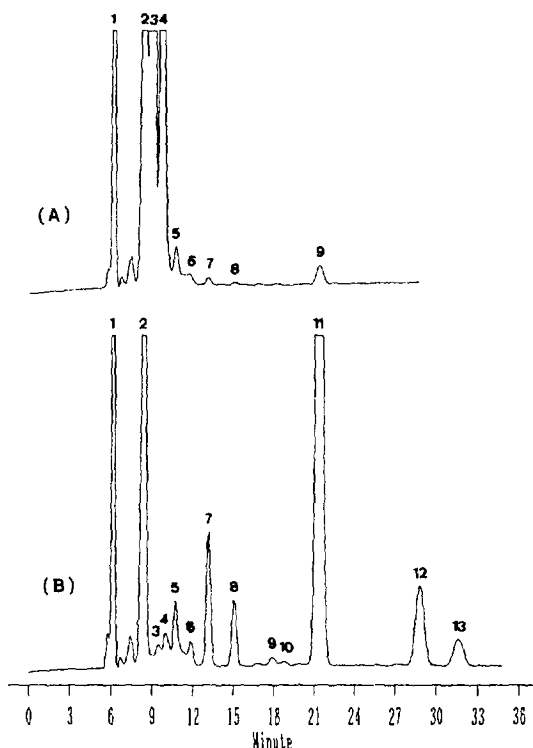

Fig. 2. The effect of sorbitol and glycerol on the bioreduction of 2-butanone with *C. utilis*. Chromatogram (A): the cell culture just before adding any 2-butanone for bioreduction. Peaks: 1=solvent front; 2=citric acid; 3=glucose; 4=sorbitol; 5=glyceric acid; 6=succinic acid; 7=glycerol; 8=acetic acid; 9=ethanol. Chromatogram (B): the bioreduction culture for a reaction period of three days. Peaks: 1=solvent front; 2=citric acid; 3=malic acid; 4=sorbitol; 5=glyceric acid; 6=succinic acid; 7=glycerol; 8=acetic acid; 9=acetoin; 10=2,3-butanediol; 11=ethanol; 12=2-butanone; 13=2-butanol.

culture indicated the existence of a two-step oxidation of glycerol with alcohol dehydrogenase. These two sequential reactions are reversible that was suggested by Mayes [22].

Sometimes sorbitol was present in the cell culture as shown in the chromatogram of Fig. 2A. The appearance of sorbitol indicated an unusual metabolism of glucose with *C. utilis*. The reversible production of sorbitol could be catalyzed by a NADP-specific polyol dehydrogenase on the aldehyde group of glucose. The NADP-specific polyol dehydrogenase, an aldose reductase, was found in the cell of *C. utilis* [23]. The sorbitol can then be transferred to fructose, fructose 1-phosphate, and finally converted to glyceric acid through either the dihdroxyacetone-phosphate or the glyceraldehyde. The conversion of glucose to sorbitol and the subsequent reactions was called the sorbitol (polyol) pathway [22]. This pathway was thus strongly supported by the chromatograms in Fig. 2 where a large peak of sorbitol and two more obvious peaks of glycerol and glyceric acid were identified in the cell culture. As the sorbitol (polyol) pathway was proceeded by *C. utilis*, the normal glycolysis and fermentation of glucose

were repressed to leave a large amount of unconsumed glucose and a very small amount of ethanol in the cell culture. We, therefore, were assured that the glucose metabolism of *C. utilis* could be extended to a more complete scheme by adding the sorbitol pathway to the Embden–Meyerhoff pathway. There were still several other unidentified minor components in the cell culture which indicated the possibility of existence of other minor glucose metabolic pathways.

3.2. Batch type bioreduction of 2-butanone

The bioreduction of 2-butanone started after growing the yeast for two days. The cell mass of yeast at this time was usually at saturation point. The bioreduction of 2-butanone with *C. utilis* was then continuously performed for three more days. All glucose was consumed after the three-day long bioreduction period. The biotransformation product 2-butanol and an increased amount of acetic acid were found in the cell culture. Other components existed in the cell culture before the bioreduction started were still remained. Quantitative studies for the amount of acetic acid and 2-butanol revealed that the increase of acetic acid was proportional to the increase of 2-butanol. This phenomenon was shown by the chromatograms (B) and (C) in Fig. 1. Thus, a series of experiments were designed to check the mol ratio of 2-butanol to acetic acid. The average mol ratio of 2-butanol to acetic acid obtained from two repeating experiments performed by adding 5 ml 2-butanone, 10 ml ethanol and 0.1 mg NADH was 0.90 ± 0.05. However, the mol ratio of 2-butanol to acetic acid for the third run of the same experiment was only about 0.49. We checked the third run chromatogram in Fig. 2B and found that the accumulation of glycerol and glyceric acid was more than before the bioreduction reaction started. The formation of glycerol and glyceric acid should be from the unconsumed glucose and the sorbitol which were indicated in the chromatogram of Fig. 2A. The production of glycerol from glucose may lead to a less production of ethanol and the consumption of more NADH. Therefore, the decrease of ethanol and NADH in the cell culture affected the bioreduction of 2-butanone negatively.

Another series of experiments was performed by keeping the experimental conditions the same as before but increasing the volume of 2-butanone to 10 ml. The average mol ratio of 2-butanol to acetic acid calculated from three repeating reaction runs was 0.98 ± 0.12 which was quite close to the ideal mol ratio, 1.0. Although glucose and sorbitol were also found in the cell growth culture at the beginning of the bioreduction, low concentrations of glycerol were formed and higher concentrations of ethanol were produced at the end of the bioreduction. Thus, an increase of 2-butanone in the cell culture could repress the formation of glycerol. This part of experimental results were shown by the chromatograms of Fig. 3.

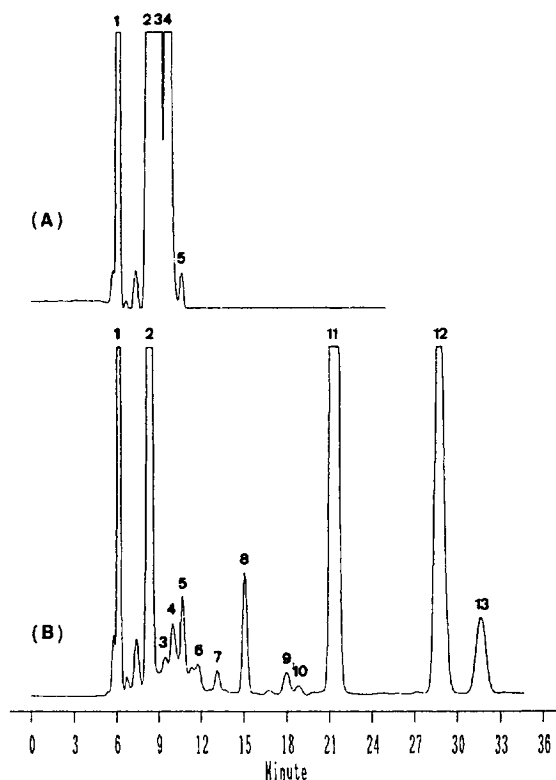

Fig. 3. The effect of 2-butanone on glucose metabolism of *C. utilis*. Chromatogram (A): the cell culture just before adding any 2-butanone for bioreduction. Peaks: 1=solvent front; 2=citric acid; 3=glucose; 4=sorbitol; 5=glyceric acid; Chromatogram (B): the bioreduction culture for a reaction period of three days. Peaks: 1=solvent front; 2=citric acid; 3=malic acid; 4=sorbitol; 5=glyceric acid; 6=succinic acid; 7=glycerol; 8=acetic acid; 9=acetoin; 10=2,3-butanediol; 11=ethanol; 12=2-butanone; 13=2-butanol.

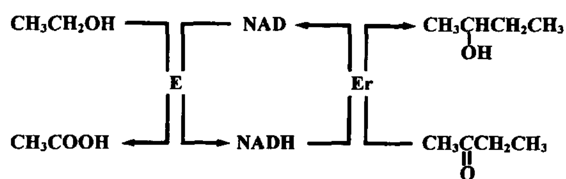

Fig. 4. The outline of the reaction mechanism for the bioreduction of 2-butanone to 2-butanol with *C. utilis*.

According to the above discussions, the reaction mechanism for the bioreduction of 2-butanone to 2-butanol was outlined in Fig. 4 [24,25]. In this reaction mechanism, E and Er represent enzymes used for the ethanol oxidation to acetic acid and for the 2-butanone reduction to 2-butanol respectively. Thus, there are two possible reasons that may violate the mol ratio of 2-butanol to acetic acid from the ideal value 1.0. The first reason is due to the competition for NADH by other reactions such as the glycerol formation from glucose. That is to say that the NADH generated by the ethanol oxidation to acetic acid may not be used by the 2-butanone bioreduction to produce the 2-butanol. The second reason is that some of the acetic acid can be produced from the unconsumed glucose during the bioreduction process. As far as we know, however, the first reason should be the most important one.

The optical activity measurement of the extracted cell culture was estimated by a polarimeter to be $+0.15°$. The measured optical rotation roughly indicated that the bioreduction product of 2-butanol was rich in *S*-form. The result obtained here for the enantioselective bioreduction of 2-butanone to 2-butanol by *C. utilis* was coincident with previous results [16,17].

3.3. Effect of ethanol

The energy source for the bioreduction was ethanol which was the fermentation product of glucose by *C. utilis* as well. The bioreduction mechanism suggested that the amount of ethanol should be at least as much as the 2-butanone in moles in order to reduce all the added 2-butanone. Thus, the dissolved oxygen in the medium for the bioreduction process should be at a low level. The low oxygen level of the cell culture would affect the growth of yeast most and would not affect the bioreduction process. Many times the ethanol produced from fermentation of yeast was not enough for the bioreduction process, therefore, we still need to add more ethanol into the culture. More ethanol in the cell culture probably inhibits the formation of sorbitol, and thus can assist the bioreduction of 2-butanone. However, too much ethanol may also inhibit the activity of cell or oxido-reductase for the bioreduction of 2-butanone. Our experimental works on the optimization of ethanol for the bioreduction were rough. Analysis of ethanol, 2-butanol and the productivity number (PN) of the bioreduction process [18] indicated only that the externally added ethanol should not be less than 10 ml per 1.1 l reaction culture.

4. Conclusions

We found that the bioreduction mechanism was closely related to the metabolism of glucose. The useful glucose metabolism for the bioreduction of 2-butanone should be the Embden–Meyerhoff pathway to form the ethanol. The disadvantageous glucose metabolism for the bioreduction should be the sorbitol (polyol) pathway for the production of sorbitol, glycerol, and glyceric acid. The bioreduction mechanism showed that as long as the 2-butanone was reduced by the oxido-reductase to give the 2-butanol, correspondingly, the ethanol was oxidized to form acetic acid and a conjugation of the oxidation–reduction of coenzyme NADH occurred. In this case, acetic acid can serve as the mark for the proceeding of the bioreduction. The supply of NADH was, thus, also important for the bioreduction. Overall, the HPLC technique of the ion-exchange chromatography was successfully applied to the biotransformation of 2-butanone to 2-butanol.

Acknowledgments

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