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Rapid Fermentation of Beer Using an Immobilized Yeast Multistage Bioreactor System

Control of Minor Products of Carbohydrate Metabolism

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ABSTRACT

The characteristics of yeast carbohydrate metabolism in a multistage bioreactor system for rapid beer fermentation were investigated. The yield of biomass in a continuous stirred-tank reactor (CSTR) was approx 10%, whereas the yield in an immobilized yeast packed-bed reactor (PBR) was below 1.0%. The overall ethanol productivity per extract consumed was slightly higher than for conventional beer fermentation owing to the restricted growth of immobilized yeast. The organic acid composition was different from a conventional fermentation broth, i.e., higher amount of succinate and lower amount of acetate. Virtually no acetate was produced in the CSTR. Succinate was produced in both the CSTR and PBR, but the amount produced per extract consumed was approximately two times larger in the CSTR. A

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large amount of α-acetolactate was produced in an early stage of a continuous operation of the PBR. However, once the immobilized yeast growth had stabilized, the productivity was lowered to approx 0.07 mg/(10 mg extract). Control of the amino acid composition of the wort fed to the PBR was not effective in repressing the production of α-acetolactate in the PBR. A strategy for controlling minor byproducts of carbohydrate metabolism in a multistage beer fermentation system was discussed.

Index Entries: Immobilization; bioreactor; yeast; carbohydrate metabolism; organic acid; a-acetolactate; diacetyl; beer brewing.

INTRODUCTION

The flavor of beer is a result of yeast metabolism, and brewery fermentation is carried out under controlled conditions to achieve the desired flavor of the product. Obviously, the major objective of fermentation is to produce ethanol anaerobically. However, it is more important to control the concentration of byproducts of yeast metabolism. Of primary concern are nitrogenous metabolism and carbohydrate metabolism, because most flavored substances are produced in a small amount as byproducts of these pathways (*1*).

In the conventional process, wort is aerated before and/or after inoculating the yeast to accelerate growth. Thus, fermentation and yeast growth proceed simultaneously. As a consequence, fermentation is slightly aerobic at the beginning of a process and becomes anaerobic later. The flavor of the beer therefore results from a mixture of aerobic and anaerobic metabolic products, and also a mixture of the metabolites produced during the growth and stationary phases. A number of investigations have been carried out to control the formation of byproducts in conventional batch fermentation (*2–4*).

The minor metabolic byproducts of carbohydrate metabolism are glycerol, organic acids (e.g., acetate, lactate, succinate, malate, citrate, and pyruvate), and acetohydroxy acids (e.g., α-acetolactate and α-acetohydroxybutyrate). Organic acids are important because they give sourness to a beer. This is especially important for acetate, which seems to be involved in giving a beer body. Under anaerobic conditions, overproduction of glycerol occurs to maintain the redox balance of the cell, and the enzymes involved in the TCA cycle are generally at a low level of activity. In order to synthesize organic acids essential for growth, however, a limited turnover of the TCA cycle is assumed (*5*), in which succinate is the end product of the cycle (Fig. 1). In the case of *E. coli*, acetate production is assumed to be an overflow phenomenon (*6*), and similar phenomena have been reported for *Saccharomyces cerevisiae* when the cultivation is switched from aerobic to anaerobic (*7*).

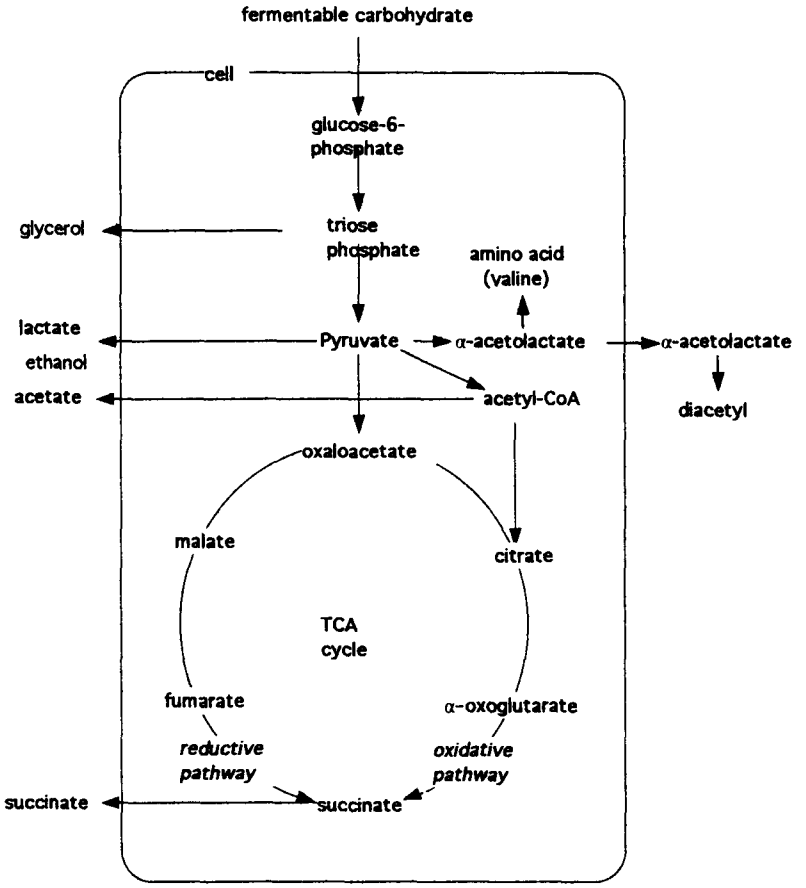
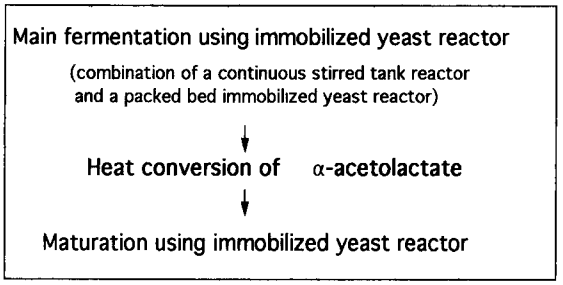


Fig. 1. Metabolic scheme of organic acid and α-acetolactate excretion by the yeast.

Acetohydroxy acids are not flavored substances, but can be easily and spontaneously converted to diacetyl or 2,3-pentanedione (*8*). Diacetyl gives an off flavor to a beer at a low concentration (>0.06–0.08 mg/L). α-Acetolactate is formed from pyruvate during glycolysis and is partly used for biosynthesis of amino acid (valine), the remainder being secreted by the yeast during fermentation (Fig. 2). The problems of α-acetolactate production using an immobilized yeast reactor have been reported (*9,10*). Control of α-acetolactate by reducing the dissolved oxygen level of the wort (*11*) or by immobilizing the yeast cells in a double-layered gel fiber (*12*) has been reported.

The drawback of using an immobilized yeast bioreactor of a packed-bed type is that because of limited transfer of nutrients and oxygen, it tends to be anaerobic in the reactor and cell growth is limited (*10,13*). Furthermore, a change in the metabolism of the immobilized cells has been reported because of complex microenvironmental conditions resulting



Multi-stage rapid fermentation system

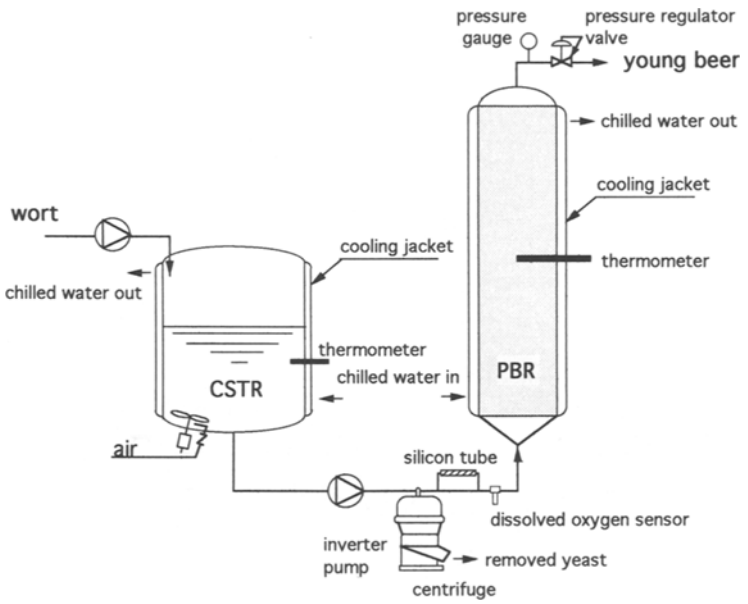


Fig. 2. Schematic diagram of the multistage fermentation process.

from cell immobilization (*14*). The basic concept of a multistage bioreactor system is to achieve a flavor profile similar to a conventional beer through a combination of a continuous stirred-tank reactor (CSTR) and a packed-bed-type plug flow reactor as reported in the previous article (*15*). The CSTR is responsible for yeast growth, and the PBR is responsible for ethanol production without yeast growth. The system is comprised of the main fermentation stage using an immobilized yeast reactor, heat conversion process of α-acetolactate, and maturation process using a second immobilized yeast reactor. The main focus of this investigation is the first process, and the objective of the current study is to investigate a control strategy for minor products of carbohydrate metabolism using the multistage bioreactor system.

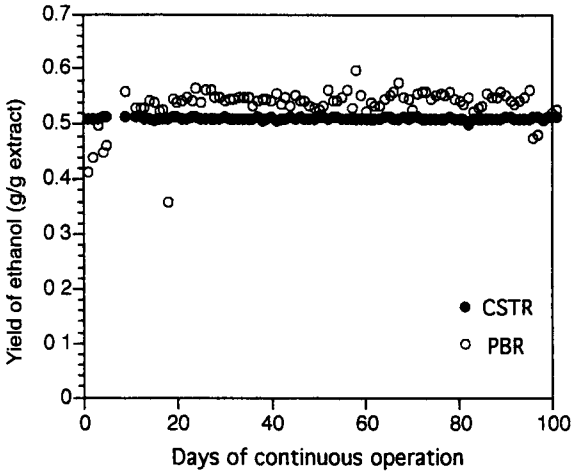


Fig. 3. Ethanol yield in the CSTR and the PBR.

MATERIALS AND METHODS

Microorganisms and Media

Saccharomyces cerevisiae SMA (culture collection of Kirin Brewery Co., Ltd., Japan) was used for all experiments. This strain is a typical bottom-fermenting brewer's yeast for pilsner-type beer. Typical brewer's wort (11% extract, malt:adjunct = 5:2 [w/w]) was used for the brewery fermentation.

Conventional Batch Fermentation

A conventional batch fermentation was carried out using a 200-L fermentation vessel at a constant temperature of 8°C, inoculation of the yeast being 0.5 g wet wt/(1000 mL wort) (pressed yeast water content 80%). The wort was saturated with dissolved oxygen by aeration before inoculation of the yeast.

Multistage Continuous Fermentation

A schematic diagram of the main fermentation process, which consists of two stages, is shown in Fig. 3. The first stage is a CSTR equipped with marine impeller at the bottom. An air sparger was installed at the base of the agitation impeller. Wort was supplied along the inner wall of the reactor to prevent foaming. Wort was fed continuously after passing through the continuous sterilizer (at 70–80°C, 20–30 min). The reactor temperature was controlled at 13°C. The air-sparging rate was 0.017 vvm. The reactor was controlled to maintain the apparent extract concentration at 8.0%. Yeast cells in the wort were continuously removed after the CSTR

by centrifugation to below 1.0×10^6 cells/mL in order to eliminate any effect of these suspended cells on the fermentation in the PBR.

The second stage is an immobilized yeast packed-bed reactor (PBR). A tubular-type reactor (1.9-L vol, diameter-to-length ratio 1:4) with cooling jackets was used for the PBR. Porous spherical glass beads (3-mm diameter Bioceramics®, Kirin, Japan) were used as a carrier material. The central pore size was 10–20 μm, with a bulk density of 0.35 g/cm³ and surface area of 3.13 m²/g. The void volume of the reactor was 40% (v/v). The PBR was operated at 8°C. The pressure was controlled at 0.01 kg/cm² to avoid microbial contamination. The flow rate (80–90 mL/h) was controlled to maintain a desirable residual apparent extract concentration (1.8–2.5%).

Analytical

Acetate, lactate, succinate, and malate were measured by HPLC methods (model LC-9A, Shimadzu, Japan). A sample was previously degassed by shaking for 30 min at room temperature and then filtered (0.45 μm). Pyruvate and citrate were measured enzymatically using an automatic analyzer (type CL-7000, Hitachi, Japan).

α-Acetolactate and diacetyl were measured as diacetyl by gas chromatography (Model GC9A Head Space Analysis System, Shimadzu Co., Ltd., Japan, fitted with an electron capture detector). The pretreatment was carried out by removal of the yeast from the broth using centrifuge (0°C, 3000 rpm, 10 min) and then heating at 60°C for 90 min under aerobic conditions to convert α-acetolactate to diacetyl.

The alcoholic content and the extract of beer were determined by the European Brewery Convention method (*16*). One hundred grams of beer were distilled, the alcoholic content was determined from the specific gravity of the distillate diluted to 100 g, and the extract was determined from the specific gravity of the residue diluted to 100 g. The major component of the extract is maltose.

RESULTS AND DISCUSSION

Ethanol Production and Yield of Biomass

One of the characteristics of brewer's yeast (*Saccharomyces cerevisiae*) is its weak respiratory capacity compared to baker's yeast (*Saccharomyces cerevisiae*) possibly because brewer's yeast has been selected for hundreds of years under anaerobic conditions. In conventional fermentation, the yield of biomass is approx 0.05 g dry weight/(g extract) (*17*).

	Extract	→	ethanol	+	CO_2	+	biomass
	2.0065 g		1 g		0.9565 g		0.11 g
Molar ratio:	1	:	2	:	2		
Molar ratio:	1			:			0.0548

(Apparent molecular weight of extract and biomass is assumed to be 180.)

Under glucose-limiting aerobic conditions, the biomass yield of baker's yeast is approx 0.50 g dry wt/g extract. Probably because of weak mitochondria function, the maximum biomass yield of brewer's yeast was only approx 0.1 g dry wt/g extract even under aerobic glucose-limited conditions (data not shown). The biomass yield in the CSTR with aeration was at the maximal achievable level of approx 0.1 g dry wt/g extract, whereas the yield in the PBR was minimal, being below 0.01 g dry wt/g extract or almost zero. Accordingly, the overall yield of biomass in the system, based on initial extract concentration, can be controlled by altering the extract consumption ratio between the CSTR and PBR. In other words, the yield of biomass can be increased by increasing extract consumption in the CSTR and decreased by increasing extract consumption in the PBR.

Because of the reduced biomass yield in the PBR, ethanol production was approx 0.06 g ethanol/g extract higher than in the CSTR (Fig. 3). This is one of the advantages of the multistage reactor system in that the ethanol content of the product can be easily controlled by changing the extract consumption ratio between the CSTR and PBR.

The unbalanced flux of carbohydrate metabolism between the CSTR and the PBR may directly affect the formation of minor byproducts of carbohydrate metabolism. An overall balance of organic acid composition and α-acetolactate production is discussed in the following sections.

Organic Acid Production

In a conventional process, approx 50% of total organic acids are derived from the wort, the remaining 50% being produced by the yeast during fermentation, acetate and succinate being the major organic acids produced by yeast (Fig. 4). Although there was little difference in the total organic acid concentration between the conventionally fermented beer and the bioreactor beer, the latter tended to contain a lower concentration of acetate and a higher concentration of lactate and succinate. The contribution of each reactor to the production of individual organic acids and to the total amount of organic acids was analyzed. (Fig. 5). Approximately 38% of total organic acids was produced in the PBR, and 62% was produced in the CSTR. This ratio reflects the extract consumption ratio between the two reactors, 2.6 g/100 g wort being consumed in the CSTR, and 4.4 g/100 g wort being consumed in the PBR. The productivity of total organic acids was virtually the same in both reactors. However, there were characteristic differences in the composition of organic acids in each reactor (Fig. 6). Acetate was solely produced in the PBR with lactate and succinate being produced in both reactors. However, the productivity of these organic acids per extract consumed was higher in the CSTR. The production of malate and citrate was relatively low, most being derived from the wort. A slight amount of malate was produced only in the PBR and citrate only in the CSTR. Succinate, conversely, can only be produced by the yeast since it is not present in the wort. The biosynthetic

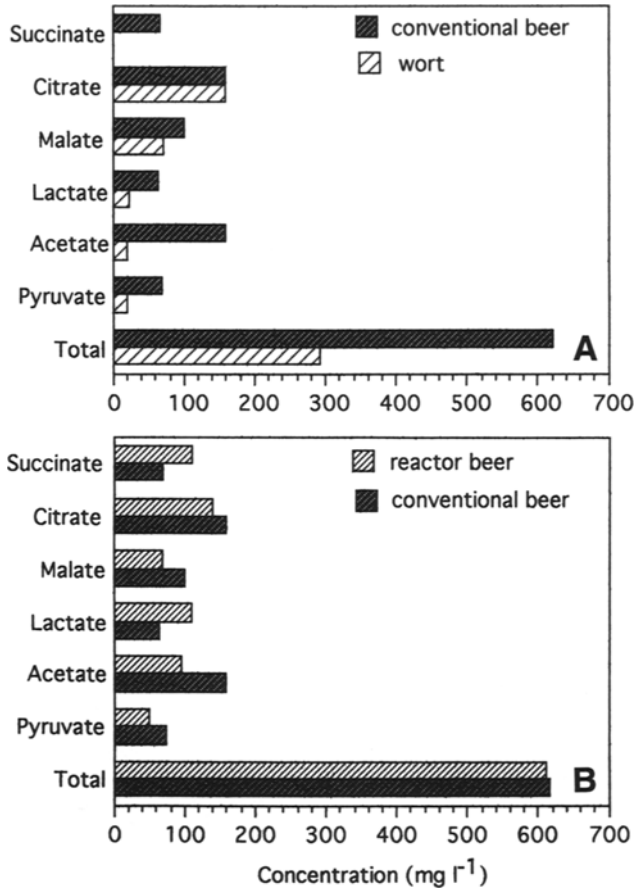


Fig. 4. (**A**) Organic acid composition of a conventional beer. (**B**) Comparison of organic acid composition of conventional beer and the reactor beer.

profile for organic acids is thus different between the two reactors, presumably reflecting the different phase of the yeast.

Production of Organic Acid by Immobilized Yeast Under Nitrogen Starvation

Because the overall productivity of acetate in the multistage reactor was slightly lower than that of a conventional fermentation process, the effect of a single carbohydrate source on the metabolic flux of acetate was investigated by feeding maltose solution (8 g/100 g water, pH 4.3) into the PBR. Acetate and succinate were produced in an approximately equimolar ratio at the start of fermentation. However, the production ratio of acetate (A/S) gradually increased up to 6.5 (Fig. 6). The average A/S ratio of the CSTR was 0, whereas for the PBR, it was 1.2–1.3, and for a conventional process, it was 4.3.

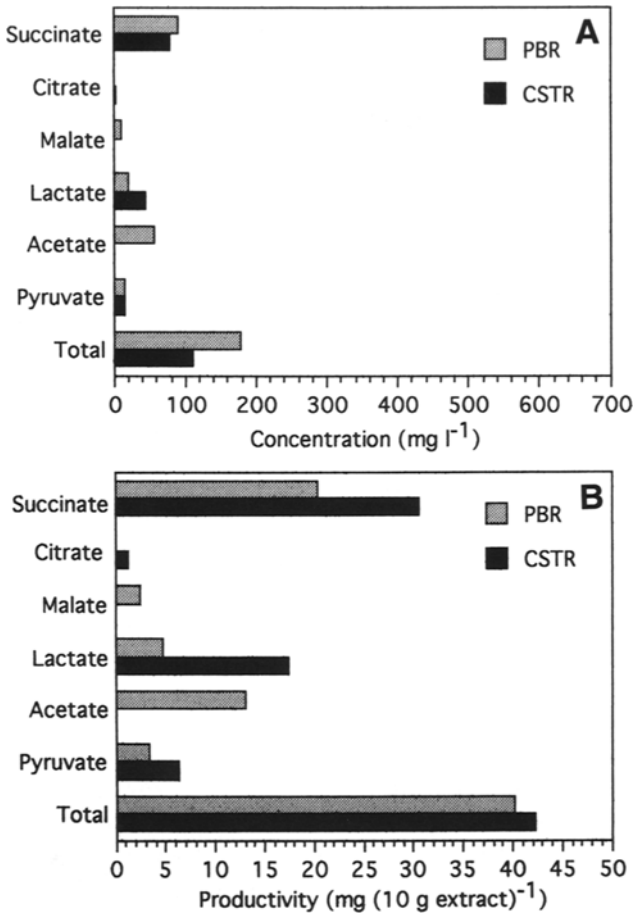


Fig. 5. (**A**) Production of organic acid in the CSTR and PBR. (**B**) Productivity of organic acid per unit extract consumed in the CSTR and PBR.

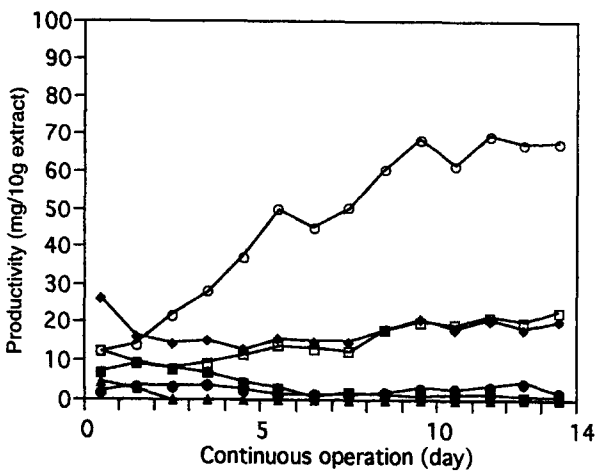


Fig. 6. Organic acid productivity under nitrogen starvation. ○ Acetate, □ lactate, ■ pyruvate, ● citrate, ▲ malate, ◆ succinate.

This indicates that the productivity of acetate in the PBR can be increased by maintaining the immobilized yeast in the stationary phase. However, the productivity of other organic acids remained almost constant.

Control Strategy for Organic Acid Composition in the Multistage Process

Lower concentration of acetate and higher concentration of succinate are characteristics of bioreactor beer. It is quite characteristic for the multistage system that little or no acetate is produced in the CSTR. In a conventional process, more than 70% of acetate in the product is produced in the initial 3 d of a 7-d main fermentation. Probably the yeast in the CSTR can be regarded as being in a respiro-fermentative metabolic condition. Generally, acetate is known to be excreted by yeast under fermentative conditions through hydrolysis of acetyl-CoA. However, when the TCA cycle turns over, acetate may not be excreted as an overflow, the TCA cycle being a bottleneck of metabolic flux. Assuming that sufficient glycolytic flux and the TCA bottleneck (e.g., anaerobic conditions) are necessary for acetate excretion, little or no excretion of acetate in the CSTR is agreeable.

Two strategies for increasing acetate concentration of the reactor beer, therefore, can be possible. One is to increase anaerobicity in the CSTR to tighten the TCA bottleneck, and another is to increase the extract consumption ratio in the PBR. The former way may introduce the difficulty of controlling the CSTR. The latter way introduces an insufficient consumption of amino acid. The former way, however, seems to be better for improving acetate concentration of the product when considering the quality.

Succinate is regarded as an end product of anaerobic growth conditions, excretion of succinate being an end product of a limited turnover of the TCA. The interesting thing is that high productivity of succinate per extract is observed in both the CSTR and the PBR (Fig. 5B), although the yeast in the PBR is in a nongrowth phase. Furthermore, malate is mainly excreted in the PBR, whereas citrate is mainly excreted in the CSTR. Two pathways for succinate excretion under fermentative conditions are proposed (5). One is an oxidative pathway, and the other is a reductive pathway (Fig. 1). Considering that the yeast in the CSTR is in a respiro-fermentative metabolic condition, succinate in the CSTR may be produced oxidatively, because succinate production under aerobic conditions is through an oxidative pathway. Excretion of malate and succinate in the PBR is agreeable if the succinate is produced through a reductive pathway by the yeast in the PBR. At present, a high concentration of succinate in the process is uncontrollable, except when using a yeast having a low succinate excretion property.

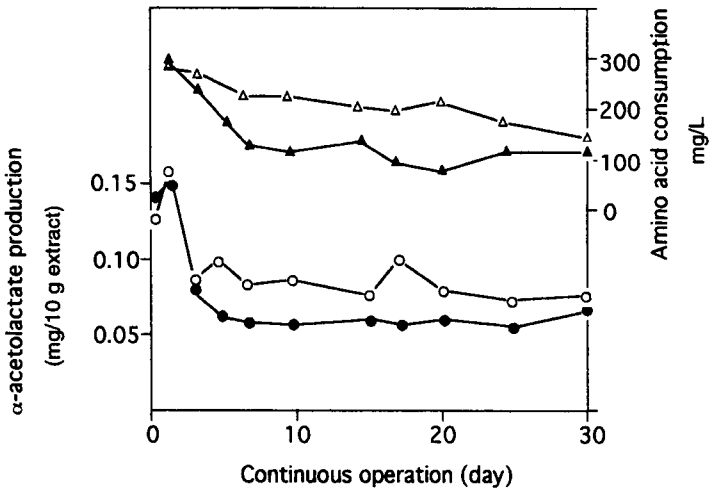


Fig. 7. Effect of dissolved oxygen concentration on α-acetolactate production in the PBR. ○ △ Aerobic conditions (D.O. = 6.0 mg/L). ●, ▲ Anaerobic conditions (D.O. < 0.1 mg/L).

Effect of Dissolved Oxygen Level in Wort on α-Acetolactate Formation

According to Onaka et al. (*11*), production of α-acetolactate in the PBR could be depressed by decreasing the dissolved oxygen level in the PBR, that is, the dissolved oxygen level is a major factor in controlling the α-acetolactate productivity in the PBR. Results of a continuous operation with a different dissolved oxygen level showed (Fig. 7), however, that the difference in α-acetolactate productivity became smaller in a long continuous run. This is probably because the production of α-acetolactate is not directly dependent on the dissolved oxygen content of wort itself, but rather on the physiological state of a cell. Even when the deoxygenated wort was continuously fed to the reactor (Fig. 7), a high level of α-acetolactate was produced during the early stages. This was because the immobilized cell was not in the stationary phase. α-Acetolactate production with time decreased with decreasing amino acid uptake. α-Acetolactate productivity after stabilization was 0.07–0.09 mg/10 g extract consumed regardless of dissolved oxygen concentration.

Effect of Amino Acid Composition of Wort on α-Acetolactate Formation

α-Acetolactate is an intermediate of valine biosynthesis. Therefore, if the end product (i.e., valine) is consumed by a cell, it can inhibit the production of α-acetolactate. In the case of conventional batch fermentation,

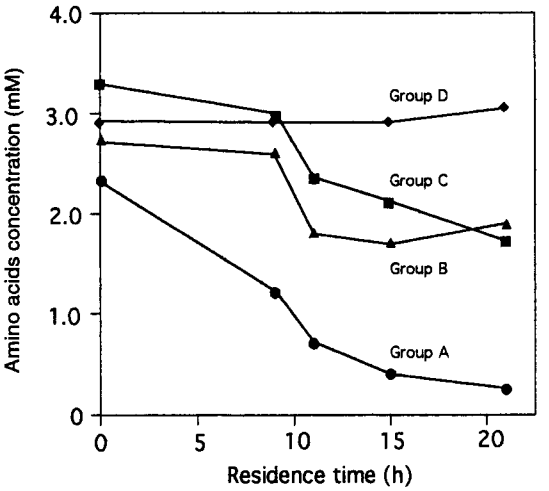


Fig. 8. Correlation between amino acids composition and residence time during the CSTR. Temperature = 13°C, aeration = 20 mL/min, volume = 2 L. Group A = Thr, Ser, Met, Leu, Lys. Group B = Ile, NH3, His, Try, Arg. Group C = Asp, Glu, Gly, Ala, Val, Tyr, Phe. Group D = Pro.

easily assimilable amino acids are consumed during the early stage of fermentation, and when valine begins to be assimilated by the yeast, α-acetolactate production will be inhibited (*18*).

The effect of amino acid composition of the wort, fed to the PBR, was examined. Figure 8 shows the amino acid composition vs residence time in the CSTR and shows that easily assimilable amino acids (Group A) were almost totally consumed in the CSTR. Therefore, the wort composition fed to the PBR was ideal for the yeast to assimilate valine (Group C, less-assimilable amino acids). It was found, however, that α-acetolactate production in the PBR was not inhibited during continuous operation of the process, even when the residence time of the CSTR was as long as 20 h (Fig. 11). The reason for this may be that amino acid uptake is repressed in the PBR. However, this does not explain the overproduction of α-acetolactate during the early stages of a continuous operation, where valine assimilation occurred.

Effect of Temperature on α-Acetolactate Formation

The productivity of α-acetolactate was examined by changing the temperature from 0 to 10°C under aerobic conditions (7–8 mg/L of dissolved oxygen) (Fig. 9). Little difference in α-acetolactate productivity was observed (0.06–0.08 mg/[g extract]) over this temperature range. Another strain of yeast had a tendency to produce more α-acetolactate per extract consumed with increasing temperature (data not shown).

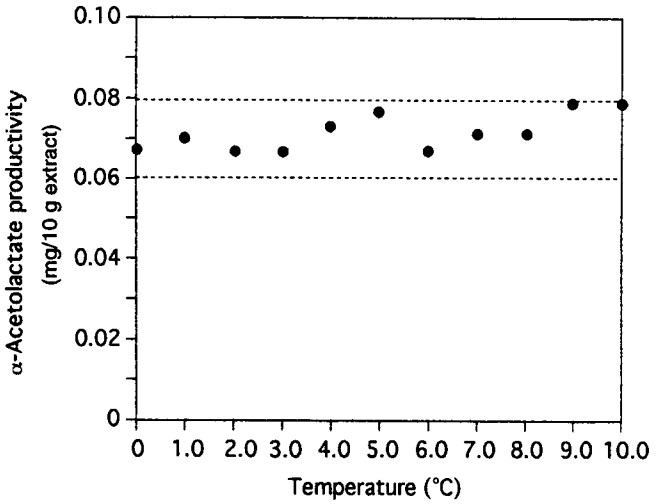


Fig. 9. α-Acetolactate productivity of the immobilized yeast under various operational temperatures.

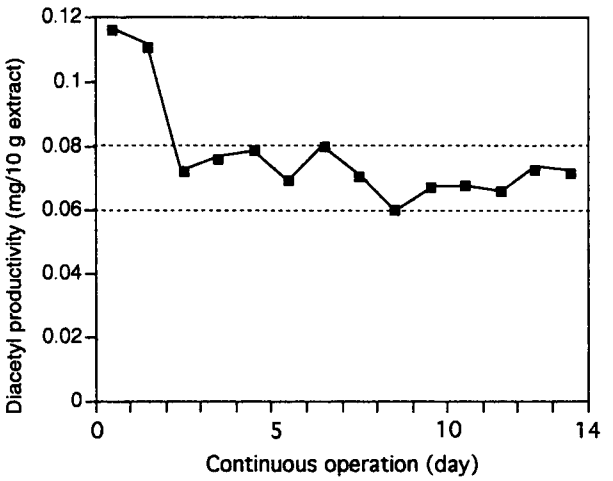


Fig. 10. α-Acetolactate productivity under nitrogen starvation.

α-Acetolactate Formation Under Nitrogen Starvation

Maltose solution (8 g/100 mL, pH 4.3) was fed continuously to the PBR to measure α-acetolactate production with growth restriction. As Fig. 10 indicates, constant production of α-acetolactate was observed (0.06–0.08 mg/[10 g extract]). This result indicates that α-acetolactate could be produced even if no amino acid was consumed by the yeast.

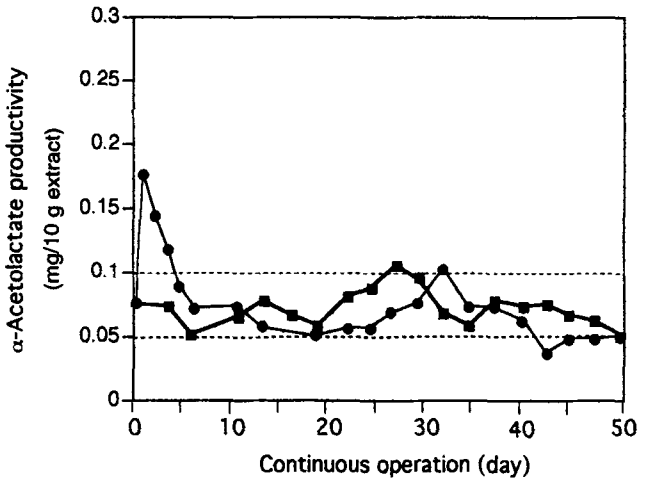


Fig. 11. α-Acetolactate productivity in the CSTR and PBR under continuous operation. Dissolved oxygen content at the inlet of the PBR was below 0.04 mg/L. ● α-Acetolactate productivity in the PBR. ■ α-Acetolactate productivity in the CSTR.

α-Acetolactate Formation in the CSTR and the PBR

Little difference was noted between the two reactors with α-acetolactate production in both the CSTR and PBR being 0.05–0.10 mg/(10 g extract), although the CSTR was operated with aeration, whereas the PBR was not aerated (dissolved oxygen $<<0.1$ mg/L). During the early stages of continuous operation of the PBR (stabilization process), with continously fed aerobic wort, amino acid consumption gradually decreased with time, with a concomitant gradual decrease in α-acetolactate production.

Control Strategy for Repression of Diacetyl Production

One of the drawbacks of producing beer using an immobilized yeast reactor is high concentration of α-acetolactate (*19*). Several reports suggest, however, that the α-acetolactate concentration of beer produced using an immobilized yeast reactor was below the threshold taste level (*20*).

In previous sections, factors that may affect the productivity of α-acetolactate were examined. Because α-acetolactate is considered to be formed as an intermediate in the metabolic pathway of valine synthesis from pyruvate, its formation can be regarded as a result of yeast growth, especially when valine is not assimilated by the yeast, it being the end product, and may inhibit the biosynthetic pathway of valine.

Assuming these schemes, two strategies for repressing α-acetolactate formation might be possible. One is to maintain the yeast in stationary phase, where synthesis of amino acid is repressed. Another is to increase the relative concentration of valine, aiming at increasing valine assimilation and substantially inhibiting valine biosynthesis.

In the PBR, factors that will accelerate growth (e.g., dissolved oxygen concentration, temperature) were not the essential factors that control α-acetolactate formation. In a continuous operation of the reactor, regardless of these factors, cells stabilized in time, i.e., the productivity of α-acetolactate gradually decreased in time. Furthermore, as long as the assimilation of carbohydrate occurs, even if the cells are in stationary phase, production of α-acetolactate seems to occur (Fig. 10).

Overproduction of α-acetolactate was usually observed in a start-up process of the PBR (Fig. 7). Although the relative concentration of valine was increased in the CSTR, inhibition of α-acetolactate production was negligible in the start-up process.

When the system was operated by maintaining a balanced carbohydrate metabolism and biosynthesis, i.e., the stabilized phase of the PBR operation, overproduction of α-acetolactate was prevented. The most important factor in the process is the rapid stabilization of the immobilized cells. However, the mechanism of the start-up process is still unclear. Under stabilized conditions, it is important not to break the microenvironment of the immobilized cells through disturbance of, for example, the flow rate, and so forth.

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