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Rapid Fermentation of Beer Using an Immobilized Yeast Multistage Bioreactor System

Balance Control of Extract and Amino Acid Uptake

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ABSTRACT

A multistage bioreactor system for rapid beer fermentation was developed. The main fermentation process, which conventionally requires 7 d, could be shortened to 2 d by this system. The concentration of esters and higher alcohols are major factors in brewery fermentation, their production being closely related to the yeast growth phase. Yeast metabolism was successfully subdivided into a growth and a restricted phase through a combination of a continuous stirred-tank reactor (CSTR) and an immobilized yeast packed-bed reactor (PBR). Production of higher alcohols was high in the CSTR because of its association with the level of biosynthetic activity *de novo*. A small amount was also produced in the PBR, however, possibly a result of an overflow in carbohydrate metabolism. Ester formation mainly occurred in the PBR, a linear increase in the level of ester being observed

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with flow through the PBR. The reactor system control strategy was to maximize the level of both higher alcohol and ester formation. The CSTR/PBR control range, based on extract consumption, was varied between 1:1 and 1:2. A ratio of 1:1 tended to create a flat beer, whereas a ratio 1:2 gave a beer of richer quality. Amino acid uptake by the yeast directly contributed to a reduction in the wort pH, whereas no relation could be observed between the level of organic acid production and pH.

Index Entries: Immobilized microorganisms; bioreactor; yeast; amino acid metabolism; higher alcohol; ester; beer brewing.

INTRODUCTION

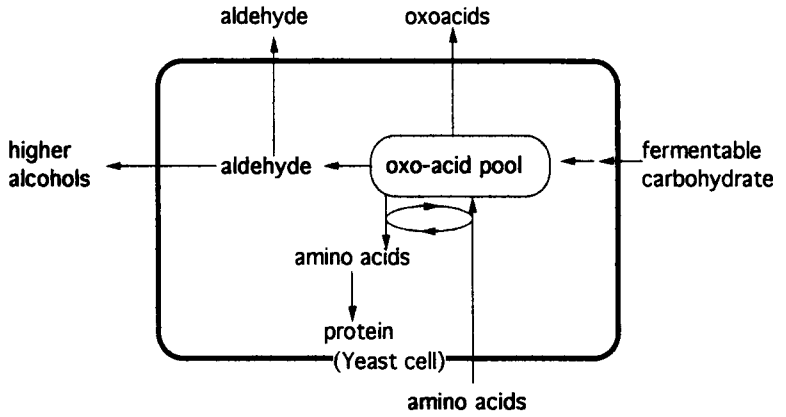
The advantage of using an immobilized yeast bioreactor for alcohol fermentation is that the fermentation time can be reduced because of the greatly elevated concentration of cells, i.e., living catalyst. In addition, the fermentor itself is of smaller dimensions, requiring a small space for installation. Brewing industries have to invest a large amount of capital into a plant building, because it usually takes approx 1 mo to produce the beer and several fermentation vessels are necessary. It is therefore of economic importance for brewing engineers to attempt to speed up the brewing process and also reduce plant investment costs.

An immobilized yeast bioreactor is extremely effective when it is used for simple alcohol production, and many investigations have been conducted in the field (*1*). For fermentation of products, such as beer, wine, sake, and soy sauce, however, because the fermented broth is in itself the final product, many problems regarding flavor and taste have yet to be solved, and its application for brewing on a commercial scale is still to be achieved.

Beer brewing using a bioreactor must be analyzed in order to guarantee a product with all of the qualities of a beer brewed by conventional means. Beer produced using an immobilized yeast plug flow reactor was reported (*2,3*). However, the beer had an unbalanced flavor possibly because of limited uptake of nitrogenous substrates by the yeast. Amino acid metabolism is important in beer fermentation not only to sustain growth, but also for biosynthesis of higher alcohol and ester, which are essential components that give character to a beer.

Higher alcohol formation is linked to amino acid biosynthesis, and the absorption of amino acid from the wort may promote the formation of the corresponding fusel alcohol (Fig. 1) (*4,5*). The principal factors that increase higher alcohol production in brewery fermentations are (*4,6*):

1. An elevated level of amino acids in the wort;
2. Anaerobic conditions;
3. High temperature;



Alcohol	Aldehyde	Oxo-acid	Amino acid
Ethanol	Acetaldehyde	Pyruvic acid	Alanine
Propanol	Propionaldehyde	α-Oxobutyric acid	α-Aminobutyric
Isobutanol	Isobutyraldehyde	α-Oxoisobutyric acid	Valine
Isoamylalcohol	Isovaleraldehyde	α-Oxoisocaproic acid	Leucine

Fig. 1. Metabolic scheme of higher alcohol formation.

4. Continuous agitation;
5. A large amount of yeast growth; and
6. High ethanol concentration.

For an immobilized yeast bioreactor, where yeast growth is negligible, conditions are not favorable for high production levels of fusel alcohols. Introduction of a CSTR in a two-stage bioreactor fermentation would therefore be expected to elevate higher alcohol production (*7*), since in a CSTR, high cell growth occurs.

It has been proposed that ester biosynthesis in yeast occurs via alcoholysis of acyl CoA compounds (*4,8–15*). Figure 2 shows the metabolic interrelationship of intracellular compounds leading to ester biosynthesis. The principal factors that increase ester production in brewery fermentations are (*4*):

1. Restricted yeast growth through insufficient oxygen supply;
2. Any factors that increase the intracellular pool of acetyl CoA; and
3. (Higher) alcohol supply.

Many reports are available relating to the control of ester formation during brewery fermentation in a conventional batch process (*16–19*). Few reports are available, however, for rapid processes using an immobilized yeast reactor (*20–22*).

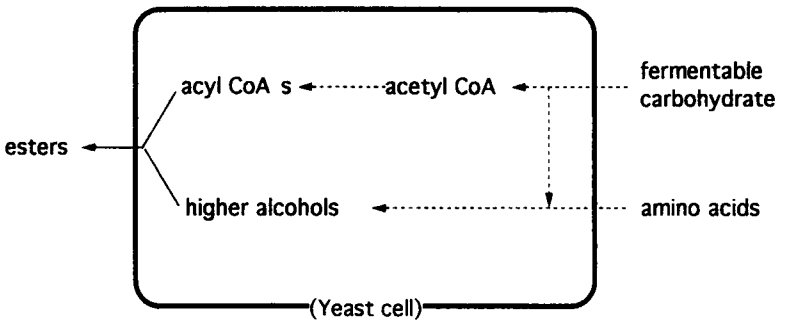


Fig. 2. Metabolic scheme of ester formation.

A multistage fermentation system was developed to produce a balanced flavor pilsner-type beer rapidly, 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 is to characterize higher alcohol and ester production in the system, these components being essential for flavor control of beer fermentation.

MATERIALS AND METHODS

Microorganisms and Media

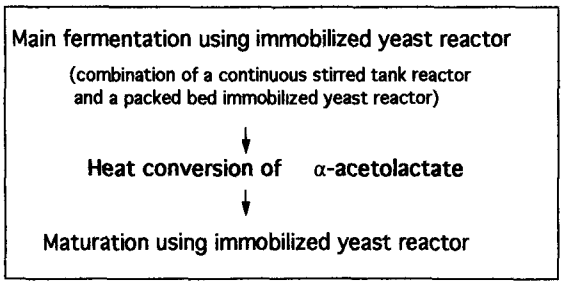
Saccharomyces cerevisiae SMA (culture collection of Kirin Brewery Co., Ltd. Japan) was used for all experiments. This strain is a typical bottom-fermenting brewer's yeast for pilsner-type beer. Typical brewer's wort (11% extract, malt:adjunct = 5:2 [w/w]) was used for the brewery fermentation.

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)/L of wort (pressed yeast, water content 80%). The wort was saturated with dissolved oxygen by aeration before yeast innoculation.

Multistage Continuous Fermentation

A schematic diagram of the main fermentation process, which consists of two stages, is shown in Fig. 3. The first stage was 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 with 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, and the air-sparging rate was 0.017 vvm. The reactor was controlled to maintain the apparent extract concen-



Multi-stage rapid fermentation system

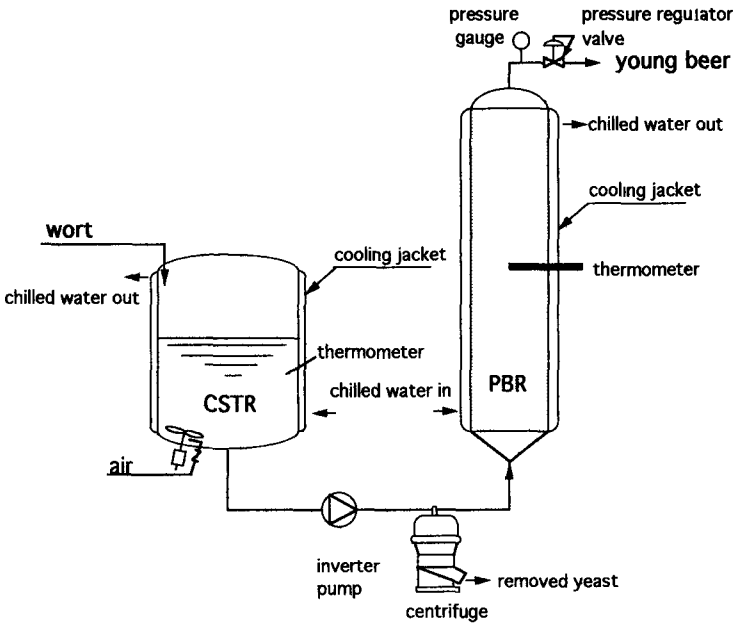


Fig. 3. Schematic diagram of the multistage fermentation process.

tration 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 was an immobilized yeast PBR consisting of a cylindroconical-type reactor (1.9-L vol, diameter-to-length ratio 1:4) with cooling jackets. Porous spherical glass beads (3-mm diameter, Bioceramics®, Kirin, Japan) were used as a carrier material. The central pore size was 10–20 μ, with a bulk density of 0.35 g/cm^3, and surface area of 3.13 m^2/g. The void volume of the reactor was 40% (v/v). The PBR was operated at 8°C, and the pressure was controlled at 0.01 kg/cm^2 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 ester and higher alcohols in the fermented broth were determined by gas chromatography (Model GC-14A Head Space analysis system, Shimadzu, Japan, fitted with FID). The pretreatment was carried out by heating 10 mL of sample for 45 min at 45°C in a 20-mL vial with silicon rubber stopper (*11*). Amino acid was analyzed using an amino acid analyzer (Model MLC-203, ATO, Japan).

Apparent extract (*23*), that is, the dehumidified solid content of wort or fermented wort as a percentage by weight, was determined from the specific gravity of a sample, which was determined densitometrically using an automatic beer analyzer (SCABA, Servo Chem AB, Stockholm, Sweden). The major component of the extract was maltose.

RESULTS AND DISCUSSION

Control of Carbohydrate and Nitrogenous Substance Uptake

A time-course of apparent extract and total nitrogen concentration in a conventional batch process is shown in Fig. 4A. Extract consumption (shown as apparent extract decrease) and amino acid assimilation (shown as total nitrogen decrease) occurred simultaneously during the initial 5 d (growth phase), but during the subsequent 3 d, assimilation of amino acid ceased, whereas extract consumption continued at the same rate (stationary phase). The formation of higher alcohols, such as *n*-propanol, mainly occurred in the initial phase, whereas ester formation commenced during late initial phase and continued into the stationary phase (Fig. 4B). This is because higher alcohol formation is associated with yeast growth, and ester formation is associated with restricted yeast growth. Control of yeast growth therefore has a direct influence on beer flavor. It is necessary to balance the overall consumption of amino acid and extract uptake in order to produce a beer having the same character as would have been achieved by a conventional fermentation.

The pattern of extract consumption and amino acid assimilation by yeast in the multistage fermentation process is shown in Fig. 5. The first stage of the system (CSTR) corresponds to the initial phase of a conventional process. In this stage, amino acids were mainly assimilated by the yeast as a result of growth. The second stage of the system (PBR) corresponds to the latter phase of a conventional process, in which fermentable extract was mainly consumed by yeast in the stationary phase. Approximately 200 mg/L (per 10 g/L extract consumed) were assimilated in the CSTR, with <20 mg/L being assimilated in the PBR. A 10-fold difference in amino acid consumption per extract could therefore be realized using the multistage bioreactor system. The average amino acid consumption per extract for the conventional fermentation was approx 100 mg/L.

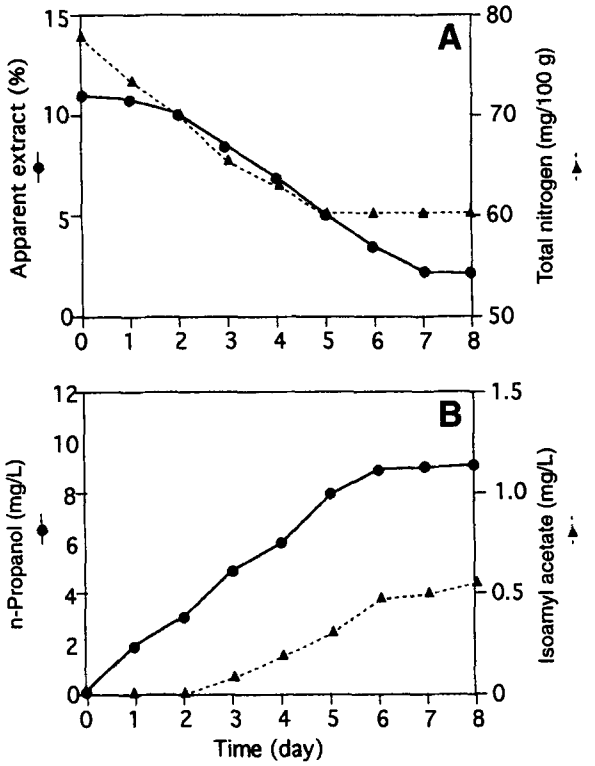


Fig. 4. (**A**) Sugar consumption and nitrogen assimilation in a conventional batch process. (**B**) Higher alcohol and ester formation in a conventional batch process.

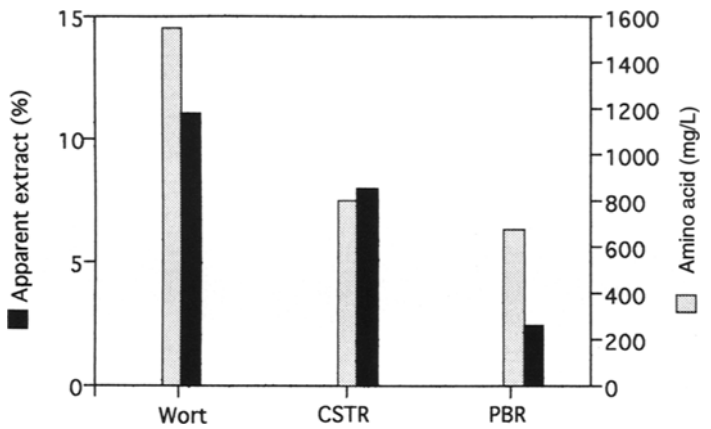


Fig. 5. Sugar consumption and amino acid assimilation in the multistage bioreactor system.

The process time of the first stage is accelerated by elevated fermentation temperature, continuous agitation, and continuous aeration. The process time of the second stage is accelerated by a high concentration of immobilized yeast cells. The overall fermentation time was 2 d.

The best strategy for controlling a multistage bioreactor system is to attempt to achieve the same ratio of overall extract consumption to amino acid consumption as for a conventional process. The extract uptake in the CSTR can be calculated from the following equation;

$$X\,AU_{CSTR} + (1 - X)\,AU_{PBR} = AU_{CONV} \quad (1)$$

where AU_{CSTR} = amino acid uptake/10 g/L extract consumed in the CSTR, AU_{PBR} = amino acid uptake/10 g/L extract consumed in the PBR, AU_{CONV} = amino acid uptake/10 g/L extract consumed in a conventional process, and X = (extract consumption in the CSTR)/(total extract consumption in the overall system).

From Eq. (1), 44% of total extract consumption should occur in the CSTR in order to realize the same level of amino acid consumption in the multistage system as would occur in a conventional fermentation.

Effect of Amino Acid Uptake on Beer pH

The object of the CSTR step is to form flavored substances, such as higher alcohols, but in addition it should effect a reduction in pH of the beer. Figure 6A shows the relationship between amino acid concentration and pH in the CSTR. Little change in pH was observed in the PBR because of low amino acid consumption. Other factors that may possibly affect pH could be formation of organic acids and carbon dioxide. However, no correlation was observed for these factors in either the CSTR or PBR. This was despite the fact that organic acid excretion occurred in the PBR stage (Fig. 6B). Beer pH contributes to the refreshing taste of beer, and this aspect must therefore be considered in the control strategy.

Higher Alcohol Formation in the System

The results of experimental runs to determine the productivity of higher alcohols (*n*-propanol, isobutanol, and isoamylalcohol) per extract consumed in the CSTR and PBR are shown in Fig. 7. Larger amounts of higher alcohols were produced per unit extract consumed in the CSTR than in the PBR. Average productivity of *n*-propanol, isobutanol, and isoamylalcohol in the CSTR was 2.2, 0.9, and 0.6 mg/L, respectively, whereas in the PBR, the value was 0.8, 6.0, and 3.5 mg/L, respectively.

The biosynthesis of higher alcohols is generally considered to be related to amino acid metabolism (*see* Fig. 1). The carbon skeleton of an amino acid synthesized in vivo is mainly derived from the same amino acid absorbed by the yeast (Ehrlich pathway). Amino acids are first deaminated to form the corresponding oxo-acid and then the amino acids are biosyn-

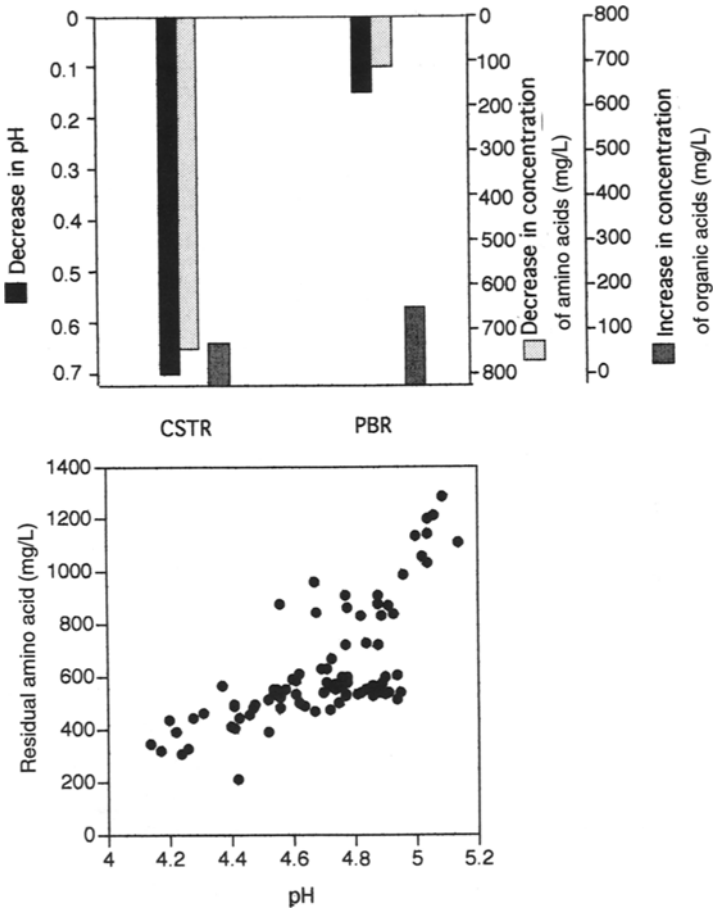


Fig. 6. (**A**) Change of pH, amino acid, and organic acid concentration. (**B**) Relationship between amino acid uptake and pH reduction in the CSTR. Initial concentration of amino acid was 1550 mg/L. Initial pH was 5.5.

thesized in the yeast. The oxo-acid pool is thought to be mainly derived from wort amino acid uptake and also partly from carbohydrate metabolism. Because higher alcohols are formed from the oxo-acid pool, yeast growth is essential for higher alcohol formation. In the multistage system, owing to elevated amino acid assimilation, greater productivity of higher alcohols could be achieved in the CSTR than in the PBR. This is the principal problem of beer brewed using a PBR only, that is, a low content of higher alcohols.

Productivity of *n*-propanol in the CSTR was higher than for a typical conventional process. However, the productivity of other higher alcohols was still lower than for a conventional fermentation. This may probably be because the rate of uptake of valine and leucine was lower in the CSTR, being the corresponding amino acid to isobutanol and isoamylalcohol,

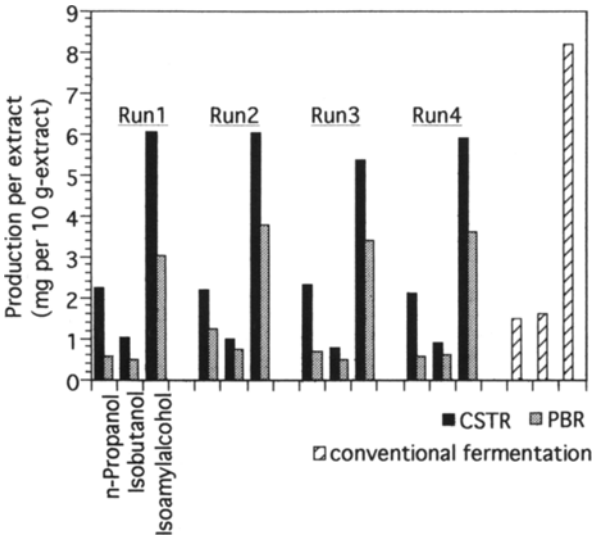


Fig. 7. Higher alcohol formation in the CSTR and the PBR.

respectively. Valine and leucine are not so readily assimilated when other amino acids are present in the wort (*4*). This result indicates that the total amount of *n*-propanol produced can be under as good control as a conventional method through a combination of the CSTR and PBR. However, the final concentration of isobutanol and isoamylalcohol is not as high as would be achieved conventionally. Nevertheless, progress has been made in higher alcohol production as compared with a simple process using a PBR only.

Figure 8 shows the result of continuous run using the multistage process. The level of *n*-propanol produced was the same as a conventional method, but less isobutanol and isoamylalcohol were produced as previously predicted. Possible control strategies to increase the level of higher alcohol in the final product is either to increase the operating temperature and/or aeration to increase amino acid metabolic flux or increase the extract consumption ratio of the CSTR. However the latter method is undesirable from the point of view of fermentation efficiency.

Although a fall-off of fusel alcohol production is observed toward the later phase of a conventional batch fermentation (*see* Fig. 4B), a small amount of higher alcohol formation occurred in the PBR possibly as a result of overflow in carbohydrate metabolism. The productivity of *n*-propanol, isobutanol, and isoamylalcohol per unit extract consumed in the PBR was almost the same as for the case where amino acid metabolism was restricted through nitrogen starvation.

Because low consumption of amino acid in the PBR was observed, biosynthesis of higher alcohols via carbohydrate flux was examined by passing maltose solution (8%) through the PBR. The productivity of *n*-propanol, isobutanol, and isoamylalcohol per 10 g/L maltose consumed is shown in Fig. 9. Isoamylalcohol production decreased slightly with time.

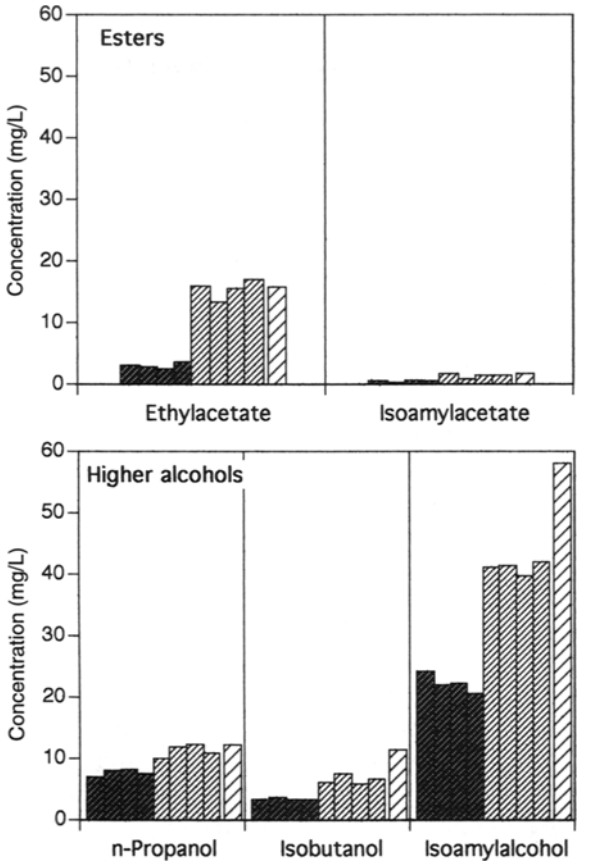


Fig. 8. Flavor profile of the reactor beer. ▩ Production in the CSTR, ▨ production in the CSTR + PBR, ▨ production in the conventional process.

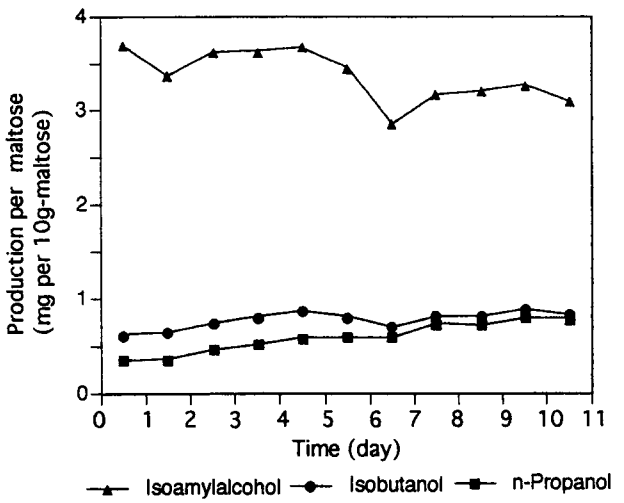


Fig. 9. Production of higher alcohols under nitrogen starvation.

However, production of the other higher alcohols increased slightly or remained virtually constant. The final productivity of *n*-propanol, isobutanol, and isoamylalcohol per maltose consumed was 0.9, 1.0, and 4.1 mg/L, respectively. This result indicates that even if amino acids are not assimilated by the yeast, an oxo-acid pool can be produced through carbohydrate metabolism, although lower than when amino acid assimilation occurs.

Ester Formation in the System

The average productivity of ethylacetate and isoamylacetate in the CSTR was 0.8 and 0.08 mg/L/extract consumed respectively, whereas in the PBR, it was 2.4 and 0.2 mg/L, respectively. The average productivity of ethylacetate and isoamylacetate in a conventional method was 2.2 and 0.15 mg/L, respectively. These results indicate that ester productivity in the CSTR was lower than a conventional method, whereas in the PBR, it was higher. The same final concentration of ester can therefore be achieved as compared to a conventional process through the combination of the CSTR and the PBR. The expected concentration of ester calculated from Eq. (1) for an extract consumption ratio of CSTR:PBR = 44:56 is 15.3 and 1.3 mg/L for ethylacetate and isoamylacetate, respectively.

The results for continuous operation of the multistage process are shown in Fig. 8. Comparable levels of esters were achieved as compared a conventional process. Using the calculated ratio of CSTR:PBR derived from Eq. (1), approx 75% of total ester production occurred in the PBR, the remaining 25% occurring in the CSTR.

Ester synthesis during beer fermentation can be influenced by several factors, such as the lipid content of the wort, dissolved oxygen level of the wort, the yeast strain, and so on (*4*). This is because acetate-ester production (e.g., ethylacetate, isoamylacetate) catalyzed by alcohol-acetyl transferase, being a cell-wall-bound enzyme, is inhibited by unsaturated fatty acids (*11–13*). Any factors that may increase the level of unsaturated fatty acids, therefore, may repress the synthesis of esters. The factors mentioned above effect an increase in unsaturated fatty acids in a cell. High production of esters in the PBR and low production in the CSTR may be therefore result from the difference in aerobicity.

Figure 10 shows the productivity of esters with time under growth restriction through nitrogen starvation. A gradual decrease in ester productivity was observed, since a small amount of metabolic turnover is probably necessary to maintain esterase activity. By contrast in the PBR, relatively constant ester productivity was observed over a long period (data not shown), and a continued increase in the level of ester was observed from inlet to outlet of the reactor (Fig. 12). In the conventional process (Fig. 4B), ester formation occurs in the later phase, where the yeast changes its metabolic pattern from aerobic to anaerobic and consequently ceases nitrogen assimilation, whereas the esterase activity is maintained.

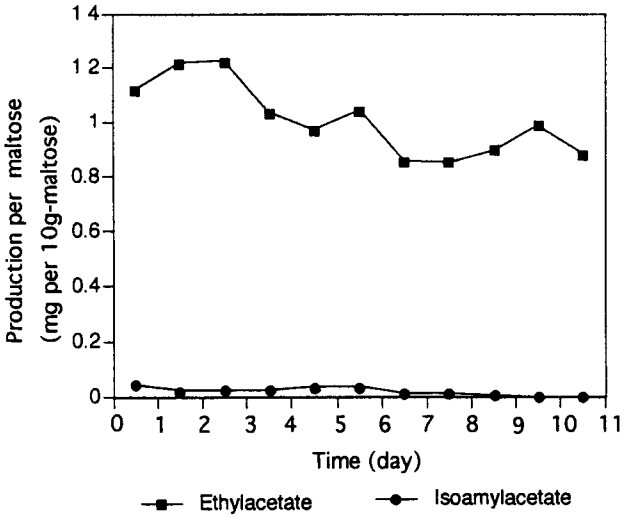


Fig. 10. Production of acetate esters under nitrogen starvation.

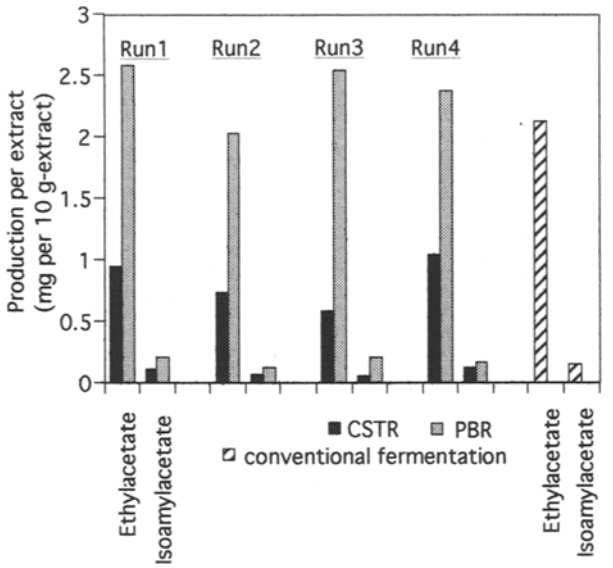


Fig. 11. Ester formation in the CSTR and the PBR.

The probable reason for constant and high ester formation in the PBR is that the low level of amino acid uptake ([15–20 mg/L]/[10 g/L extract]) extract), i.e., slight growth phase, could be maintained in the PBR under continuous operation. A possible strategy for increasing ester production is to increase extract consumption in the PBR by keeping the yeast in a slight growth phase or to decrease aerobicity in the CSTR.

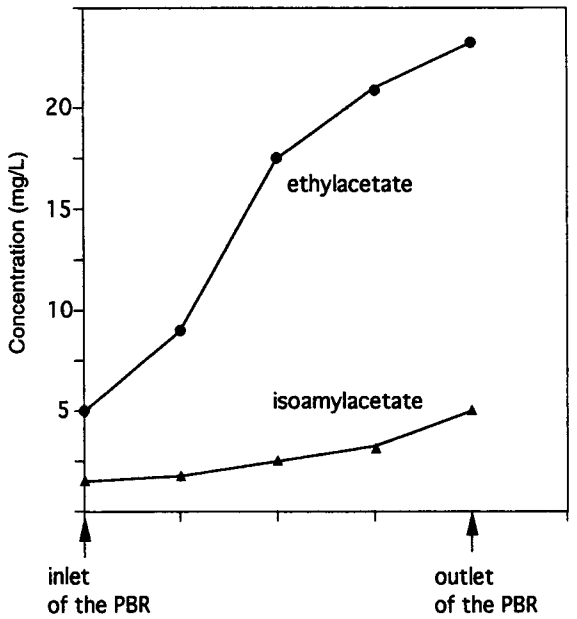


Fig. 12. Ester formation during fermentation in the PBR.

SUMMARY

An immobilized yeast bioreactor is effective for accelerating fermentation. It usually takes 7 d to assimilate 8% extract (from 11 to 3%) in a conventional process, that is, 1% extract consumption/d, whereas using an immobilized yeast reactor, this process could be accelerated sixfold, that is, 6% extract consumed/d.

The balance of flavored substances, such as esters and higher alcohols, however, was not comparable with a conventional beer. In order to solve this problem, the concept of "balanced control of extract and amino acid uptake" was introduced into the process involving an immobilized yeast reactor, this is, to maintain a constant ratio of amino acid uptake to extract uptake in the process as would occur in a conventional fermentation.

The CSTR was installed before the immobilized yeast reactor in order to achieve this balance control. The CSTR was operated at higher temperature and with aeration to improve yeast growth, and hence, amino acid uptake.

The total productivity of higher alcohols and ester could be well balanced through a combination of these two types of reactors. Productivity of higher alcohols was higher in the CSTR, whereas ester productivity was higher in the PBR. The extract consumption balance between the two reactors could be set to the desired level by maintaining a constant consumption ratio of amino acids and extract. This method proved effective for achieving a balanced production of flavored substances. Moreover, this system was also effective in reducing the pH value to a desired level.

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