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Note

Coupling of Fermentation and Esterification: Microbial Esterification of Decanoic Acid with Ethanol Produced *via* Fermentation

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Two different kinds of bioprocess, ethanol fermentation and subsequent microbial esterification, were coupled using *Issatchenkia terricola* IFO 0933 in an interface bioreactor. The strain produced ethyl decanoate (Et-DA) by esterification of exogenous decanoic acid (DA) with ethanol produced *via* fermentation. The efficiency of the new coupling system depended on the concentration of glucose in a carrier and DA in an organic phase (decane) in an agar plate interface bioreactor. Optimum glucose content and DA concentration were 4% and 29 mM, respectively.

Key words: ethanol fermentation; microbial esterification; coupling system; interface bioreactor

In general, microbial transformation and fermentation are recognized to be different bioprocesses from each other. While the former is generally applied to one step enzymatic reactions with living or resting cells, multiple steps of enzymatic reaction are used in fermentation with living cells.

As to the coupling of these two different bioprocesses, some useful nucleotides, such as uridine triphosphate (UTP)[1] and guanosine-5′-monophosphate (GMP)[2] are produced *via* the coupling of microbial transformation and ATP regeneration. Isoamyl acetate is produced *via* the coupling of transacetylation from acetyl coenzyme A (acetyl-CoA) to isoamyl alcohol and acetyl-CoA production *via* metabolism of saccharides.[3] Ethyl hexanoate (ethyl caproate) is also produced by both the coupling of esterification and ethanol fermentation, and the coupling of transacylation and hexanoyl-CoA production.[4]

We have constructed a new acetylation system without any acetyl donor, which we referred to as a double coupling system. In this system, microbial transacetylation by alcohol acetyltransferase and acetyl-CoA formation *via* metabolism of glucose are effectively coupled.[5] For example, using *Pichia kluyveri* IFO 1165, (*RS*)-citronellol is optically resolved in high optical and chemical yield.[6] In this study, we disclose a new coupling system in which an exogenous carboxylic acid is esterified by ethanol, which is produced by fermentation in the interface bioreactor.[7] In this system, these two different bioprocesses are effectively coupled in the intact cells of an yeast, *Issatchenkia terricola* IFO 0933, to afford Et-DA without addition of ethanol (Fig. 1).

First, we screened for microorganisms having a coupling activity of ethanol fermentation and esterification with the agar plate interface bioreactor. A nutrient agar plate consisted of 5.0 g of peptone, 3.0 g of malt extract, 3.0 g of yeast extract, 10.0–100.0 g of glucose, 1.0 g of $MgSO_4 \cdot 7H_2O$, 15.0 g of agar powder, and 1.0 liter of distilled water (pH 6.0) was prepared in a glass petri dish (surface

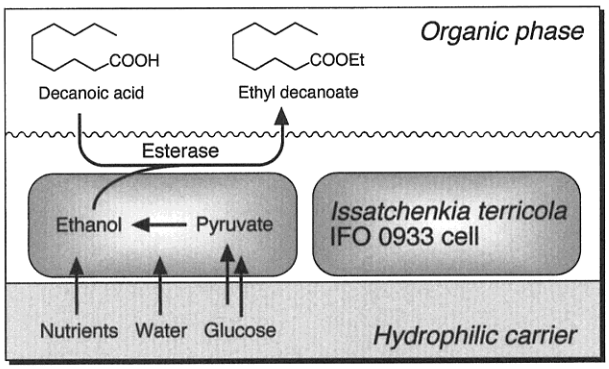

Fig. 1. Principle of the New Coupling System of Ethanol Fermentation and Microbial Esterification in the Interface Bioreactor.

Decanoic acid in the organic phase is esterified by ethanol produced *via* fermentation of glucose by an esterase.

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Abbreviations: DA, decanoic acid; Et-DA, ethyl decanoate; Et-HA, ethyl hexanoate; UTP, uridine triphosphate; GMP, guanosine-5′-monophosphate; ATP, adenosine triphosphate; acetyl-CoA, acetyl coenzyme A; hexanoyl-CoA, hexanoyl coenzyme A

area, 38.5 cm^2; volume, 25 ml). Three hundred μl of a cell suspension (1 loopful/ml-medium) was spread on the agar plate, and excess moisture was removed by allowing the plate to stand at room temperature. After precultivation for 1 day, 8 ml of a 58 mM solution of DA in decane was added to the surface of the plate, and incubation was done at 30°C by allowing the dish to stand for 10 days. After the incubation, the decane layer was analyzed with gas chromatography: the column contained Thermon-3000/Chromosorb W (diameter, 2.6 mm; length, 3 m); the column temperature was increased from 100 to 240°C at a rate of 7°C; the injector and detector temperature were 245 and 250°C, respectively; the carrier gas was N_2 (60 ml/min).

As shown in Table 1, 6 strains produced Et-DA without addition of exogenous ethanol. Especially, *Issatchenkia terricola* IFO 0933 accumulated Et-DA in high level, *i.e.*, 18.4 mM Et-DA in 64% conversion yield. The excess consumption of DA by *I. terricola* may be the results of *via* β-oxidation and diffusion into the agar plate. *Saccharomyces cerevisiae* IFO 0565 and *Zygosaccharomyces bailii* IFO 0488 consumed a large quantity of DA without production of Et-DA.

Time course of Et-DA production by *I. terricola* is shown in Fig. 2. For 2 days after the start of the incubation, a time lag of Et-DA production was observed because of the strong toxicity of DA.[8] However after the 4th day, Et-DA production and DA consumption proceeded in parallel. Although this strain could also esterify DA with exogenous ethanol (0.5%, w/v) in the interface bioreactor, the productivity of Et-DA was lower than that with the endogenous ethanol (9.1 mM *vs.* 18.4 mM). It is favorable that ethanol is produced at an adequate rate *via* fermentation without inhibitory action.

As for the microbial production of esters in fermentation, ethyl hexanoate is produced by both the esterification of hexanoic acid with endogenous ethanol and the transacylation of hexanoyl-CoA with endogenous ethanol in *Saccharomyces*[4] and *Aspergillus*.[9] The former reaction is catalyzed by esterase and the latter one is catalyzed by alcohol acyltransferase. However, it was reported that exogenous hexanoic acid was esterified with ethanol produced *via* fermentation by the aid of esterase without transacylation.[10] Thus, *I. terricola* IFO 0933 may produce Et-DA by the coupling of the esterase-catalyzed esterification and ethanol fermentation.

The coupling of esterification and ethanol fermentation was affected by glucose content in the carrier and DA concentration in the organic phase. The maximal productivity of Et-DA was gained at 4 wt% glucose content. High glucose content (over 5 wt%) led to the decrease of Et-DA productivity. DA concentration in the organic phase also affected on Et-DA production as shown in Fig. 3. The higher the DA concentration, the lower the Et-DA production because of the appearance of toxicity of DA.[8]

Table 1. Screening for Microorganisms Having a Coupling Activity of Ethanol Fermentation and Esterification

Strain	Product (mM)	
	DA	Et-DA
Issatchenkia terricola IFO 0933	29.3	18.4
Saccharomyces cerevisiae IFO 0565	32.3	2.8
Kluyveromyces phaffii IFO 1672	52.4	2.1
Zygosaccharomyces bailii IFO 0488	29.8	1.6
Saccharomyces cerevisiae IFO 0224	49.3	1.2
Candida krusei IFO 1395	51.1	0.9
Issatchenkia orientalis IFO 1279	50.4	0.7
Kloeckera apiculata IFO 0175	53.7	ND

ND, not detected.

Each strain was inoculated on a nutrient agar plate. After 1-day precultivation, 8 ml of a 58 mM solution of decanoic acid in decane was added, and incubation was done at 30°C by allowing the plate to stand for 10 days.

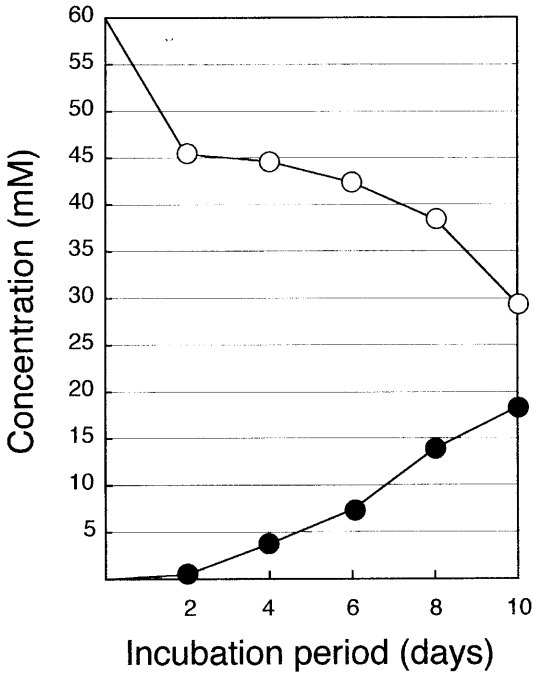

Fig. 2. Time Course of Ethyl Decanoate Production in the Fermentation-Esterification Coupling System.

Symbols: ○, decanoic acid concentration; ●, ethyl decanoate concentration. *I. terricola* IFO 0933 was inoculated on the nutrient agar plate prepared in a glass petri dish. After 1-day precultivation, 8 ml of a 58 mM solution of decanoic acid in decane was added, and incubation was performed at 30°C by allowing the dish to stand.

In conclusion, the new coupling system of ethanol fermentation and microbial esterification with *I. terricola* IFO 0933 afforded Et-DA. Other combinations of microbial transformation, such as oxidoreduction and fermentation, will be also possible, and it is expected that novel synthetic strategies will be constructed with diverse functions of many living microorganisms.

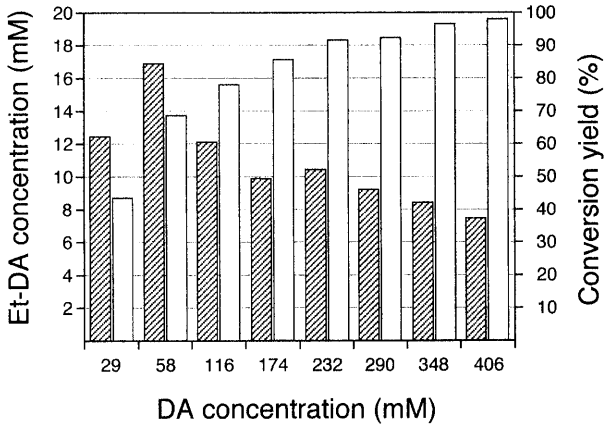

Fig. 3. Effect of Decanoic Acid Concentration on the Fermentation-Esterification Coupling System.

▨, Ethyl decanoate [Et-DA] concentration; □, conversion yield. *I. terricola* IFO 0933 was inoculated on the nutrient agar plate. After 1-day precultivation, 8 ml of a 29–406 mM decanoic acid (DA) in decane was added, and incubation was performed at 30°C by allowing the dish to stand for 10 days.

Acknowledgments

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