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Dynamics of Mixed-Cultures of *Gluconobacter suboxydans* and *Saccharomyces uvarum*

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Abstract

A mixed-culture of *Gluconobacter suboxydans* IFO 3172 and *Saccharomyces uvarum* IFO 0751 was performed in a synthetic medium. The optimal inoculum ratio of *G. suboxydans* and *S. uvarum* for mixed-culture fermentation was 150 : 1. The optimum pH, incubation temperature and aeration rate for mixed-culture fermentation were 5.0, 30°C and 2.25vvm, respectively. As a result of batch pure- and mixed-culture fermentation, specific growth rate in pure-culture of both strain was lower than that in mixed-culture. The yield of cell mass from *S. uvarum* exclusively decreased. The growth rate of the mixed-culture was very similar to the pure-culture in the beginning of culture, but it has been decreased after 16hrs. In the mean time, *S. uvarum* in mixed-culture fermentation could grow due to fructose converted, but it could not grow in pure-culture fermentation. Thus, the relationship was a sort of commensalism. The kinetic parameters calculated through steady-state results during continuous fermentations are as follows: μ_{max1}=0.118(h^{-1}), Ks_1=0.330(g/L), μ_{max2}=0.162(h^{-1}), Ks_2=0.038(g/L). The yield of bacterial cell mass was relatively constant, but yield of yeast cell mass was gradually decreased.

Key words: *mixed-culture, commensalism*

INTRODUCTION

The dynamics of mixed-cultures are important considerations in some commercial fermentations and many food fermentations, such as cheese manufacture, depending on multiple interacting species. In recent years, the properties of mixed-cultures have been attracted increasing attention. This interest has been stimulated by various events: (i) the interaction of specific organisms into environments for purposes such as pest and pathogen control, enhancing soil fertility and plant growth, pollution treatment, and mineralization of crop residues in the field(1); (ii) the realization that the establishment and virulence phases of many diseases of man and animals are the consequence of mixed infections(2); and (iii) the exploitation of defined mixed-cultures for a range of biotechnological purposes(3-13).

The major interactions between two organisms in a mixed-culture are competition, neutralism, mutualism, commensalism, amensalism, and prey-predator interactions. Among the major interactions, commensalism is an interaction in which one population is positively affected by the presence of the other(14), but the second population is not affected by the presence of the first population. Two common mechanisms are as follows: (i) the second population produces a required nutrient or growth factor for the first population; (ii) the second population removes a substance from the media that is toxic to the first population.

The chemostat type of continuous culture is probably the best experimental system for studying the population interactions(15). Chemostat culture enables environmental factors, either singly or in combination, to be altered systematically, and defined perturbations to be imposed on the mixed-culture. Both of these manipulations can reveal the salient features of the mechanism(s) of species interaction.

In this paper, we report the commensalism between *G. suboxydans* and *S. uvarum* in batch and continuous mixed-culture fermentations.

MATERIALS AND METHODS

Microorganisms

Gluconobacter suboxydans IFO 3172 and *Saccharomyces uvarum* IFO 0751 were used in this study. Stock

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cultures were maintained at -70°C in medium with 20% glycerol. Working cultures were transferred monthly and maintained on agar plate at 4°C.

Media

Medium A was used for pure-culture of *G. suboxydans* and Medium B was used for pure-culture of *S. uvarum*. For mixed-culture experiment, medium C was used(Table 1)(16,17).

Fermentation conditions

Table 2 summarizes the culture conditions for each pure- and mixed-culture fermentations in batch and continuous system. Batch fermentations were performed in 50ml of medium in 250-ml Erlenmyer flasks. Incubation was at 30°C with shaking of 120rpm. Initial pH was approximately 5.0; pH was not controlled during the fermentation. Samples(5ml) were taken from the medium every 2h and stored at -70°C for analysis.

Batch fermentations with pH control were performed in 500ml of medium in 1-L jar fermenter(model C-30, New Brunswick Scientific Co., New Brunswick, NJ).

Table 1. Composition of culture media

Component	Medium		
	A	B	C
Mannitol	10g	-	10g
Fructose	-	10g	-
$(NH_4)_2SO_4$	1g	1g	1g
Solution A	5ml	5ml	5ml
Solution B	5ml	5ml	5ml
Alanine	0.2g	-	0.2g
Cysteine	0.2g	-	0.2g
Histidine	0.2g	-	0.2g
Isoleucine	0.2g	-	0.2g
Proline	0.2g	-	0.2g
Valine	0.2g	-	0.2g
p-Aminobenzoic acid	1mg	-	1mg
Biotin	0.04mg	0.04mg	0.04mg
Calcium pantothenate	1mg	1mg	1mg
Inositol	40mg	40mg	40mg
Nicotinic acid	1mg	-	1mg
Pyridoxine · HCl	2mg	2mg	2mg
Thiamine · HCl	10mg	10mg	10mg
$CaCl_2 \cdot 4H_2O$	-	50mg	50mg
$ZnCl_2$	-	5μl	5μl
$CuSO_4 \cdot 5H_2O$	-	0.5mg	0.5mg

Solution A(For one liter): KH_2PO_4 100g, K_2HPO_4 100g
Solution B(For one liter): NaCl 2g, $FeSO_4 \cdot 7H_2O$ 2g, $MgSO_4 \cdot 7H_2O$ 40g, $MnSO_4 \cdot 4H_2O$ 2g, Conc. HCl 2ml

Table 2. Culture conditions

Condition	Fermentation system	
	Flask	1-L jar fermenter
Total volume(L)	0.25	1.0
Working volume(L)	0.05	0.5
Inoculum(v/v, %)	B : 2.0 Y : 0.5	B : 20 Y : 5.0
Agitation(rpm)	120	300
Aeration(vvm)	-	2.25
Temperature(°C)	30	30
pH	5.0	5.0

B: *Gluconobacter suboxydans*
Y: *Saccharomyces uvarum*

The pH was maintained at 5.0±0.2 by automatic addition of 3N H_2SO_4 and 3N NaOH. Typical pH-controlled batch fermentations were run up to 48h. Every 2 to 4h, 10ml samples were taken and stored at -70°C for analysis.

Continuous fermentations were also performed in a model C-30 bench-top fermenter(New Brunswick). Temperature was kept at 30°C, agitation rate at 300rpm, and pH at 5.0±0.2 by automatic addition of 3N H_2SO_4 and 3N NaOH by pH controller(model pH-40, New Brunswick). Working volume was about 500ml. Fermentations were started as batch fermentations, and feeding of medium(0.25% mannitol-containing medium C) for continuous fermentation was started when the limiting substrate concentration approached to zero. Flow rate was controlled by means of a Masterflex microprocessor pumpdrive peristaltic pump(Cole-Parmer Instrument Co., Chicago, IL). Every 4h, 10ml samples were taken and stored at -70°C for analysis.

Analytical methods

Dry cell weight was calculated from a standard curve by determining the amount of protein of cell mass using Lowry method(18).

The total cell numbers were measured using Petroff-Hausser(Hausser Scientific Co.) for *G. suboxydans* and Haemacytometer(Superior Co.) for *S. uvarum*, respectively.

The concentration of mannitol was determined by polarimeter(model DIP-140, JASCO)(19). Fructose concentration was determined by a modified dinitrosalicylic acid(DNS) method(20). The color tests were made with 2ml of DNS reagent consisted of 1% dinitrosalicylic acid, 0.2% phenol, 0.05% sodium sulfite, and 1% sodium hy-

droxide. The mixtures were heated for 15min in a boiling water bath and then cooled down for 3min under running tap water(18°C). After 10ml of distilled water were added to each tube, the solution was thoroughly mixed, and the absorbance at 575nm was measured. The color obtained was stable for 1h. The fructose concentration was calculated from a standard curve of known fructose solution vs. absorbance at 575nm.

RESULTS AND DISCUSSION

Determination of inoculum ratio

Mixed-culture fermentations were performed under different inoculum ratio of *G. suboxydans* to *S. uvarum*, and optimal inoculum ratio was 150 : 1 for mixed-culture fermentations as shown in Table 3.

Optimum conditions for mixed-culture fermentations

To define the optimum conditions for mixed-culture fermentations, initial pH, incubation temperature, and aeration rate were determined. When specific conditions were varied to determine the optimum value, all other conditions were kept at the following standard values: inoculum ratio of bacteria to yeast, 150 : 1; initial pH, 5.0; incubation temperature, 30°C; aeration rate, 1vvm. The optimum pH, incubation temperature and aeration rate for mixed-culture fermentations were 5.0, 30°C and 2.25vvm, respectively(Fig. 1).

Mixed-culture vs pure-culture

Once optimum conditions for mixed-culture fermentations had been determined, the fermentations of pure- and mixed-culture were performed. Fig. 2 shows the results of a pure-culture controlled at pH 5.0. Mannitol was consumed within 48h, and maximum cell number was also reached at that time. It also showed that mannitol is a limiting substrate. In contrast, batch fermentation of pure yeast culture under similar conditions

Table 3. Effect of inoculum ratio of *G. suboxydans* to *S. uvarum* in mixed-culture fermentations

Inoculum ratio (*G. suboxydans* : *S. uvarum*)	Cell growth(A_{610}) (after 24h cultivation)
100 : 0	0.714
180 : 1	1.081
150 : 1	1.355
120 : 1	1.205
90 : 1	1.184
60 : 1	1.104
30 : 1	1.173
0 : 100	0.494

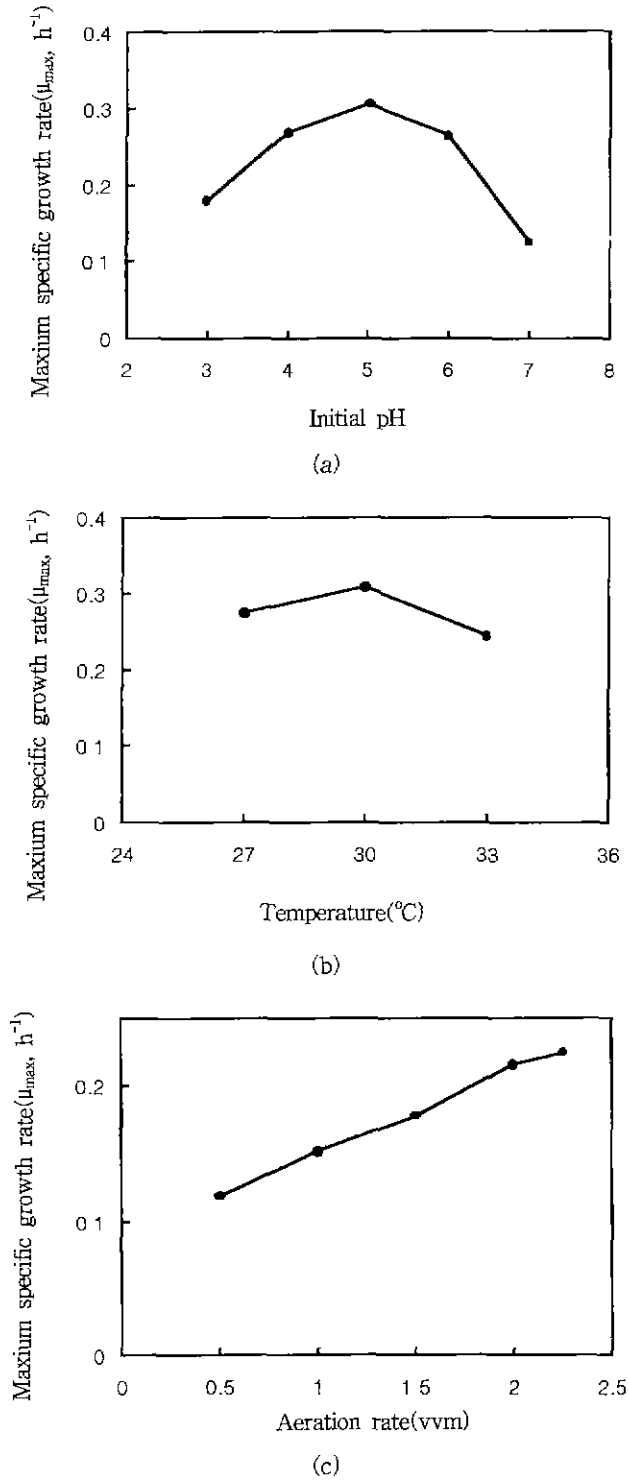

Fig. 1. Optimum conditions on growth of *G. suboxydans* and *S. uvarum* in mixed-culture fermentations. (a) Effect of initial pH; (b) Effect of incubation temperature; (c) Effect of aeration rate

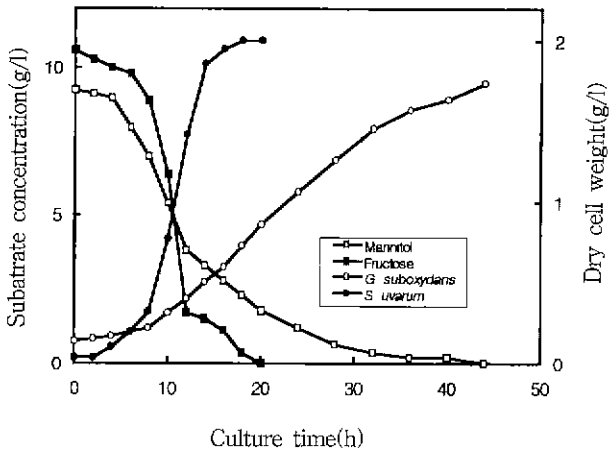

Fig. 2. Typical pure-culture fermentation of *G. suboxydans* and *S. uvarum*.

took 20h and fructose(consumed by *S. uvarum*) was depleted after 20h, showing fructose is a limiting substrate as shown in Fig. 2.

The growth curve of two organisms in mixed-culture is quite different from that in pure-culture(Fig. 3). Fructose was rapidly consumed, and the fructose concentration in the medium was below the limit of detection of the modified DNS method by the time of the next sampling. The growth of *S. uvarum* using fructose which was converted by *G. suboxydans* could continue for further fermentation.

Table 4 gives the results of 48h fermentations by different fermentations. The specific growth rate of two organisms in pure-culture fermentations was lower than that in mixed-culture fermentations. Yields($Y_{x/s}$) of yeast cell mass from fructose were significantly low in mixed-culture, but the yield of bacterial cell from mannitol were the same for the two systems.

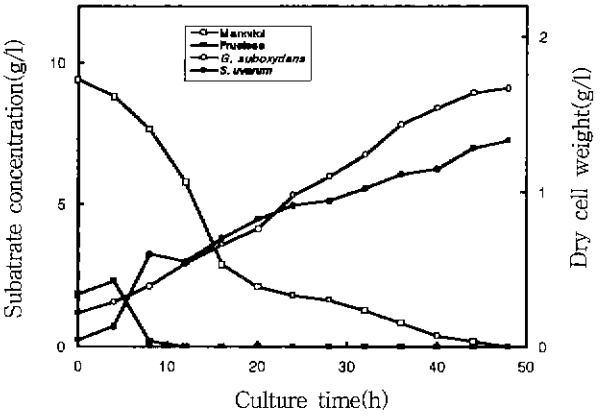

Fig. 3. Typical mixed-culture fermentation of *G. suboxydans* and *S. uvarum*.

Table 4. Growth results in batch pure- and mixed-culture fermentations

Parameter	Pure-culture		Mixed-culture	
	G. suboxydans	*S. uvarum*	*G. suboxydans*	*S. uvarum*
μ_{max}	0.097	0.276	0.083	0.222
$Y_{x/s}$	0.194	0.181	0.194	0.161
q_s	0.525	1.322	0.472	1.571

μ_{max}; Maximum specific growth rate(h^{-1})
$Y_{x/s}$; Cell mass yield(g cell produced/g substrate consumed)
q_s; Specific carbohydrate uptake rate(g substrate/g cell · h)

Commensalism was achieved by growing mannitol-limited *G. suboxydans* and fructose-limited *Saccharomyces uvarum*, with the fructose needed for the growth of the yeast being supplied by the bacteria. In the absence of fructose, *S. uvarum* in pure-culture did not growth, but *S. uvarum* in mixed-culture with *G. suboxydans* could grow well.

Continuous mixed-culture fermentations

Interactions between two organisms were tested for their characteristics in continuous mixed-culture fermentations, in which oscillations of cells and substrate levels could be avoided. Feeding of fresh medium was started after about 48h of batch cultivation, when the mannitol concentration was close to zero. The medium for continuous fermentations was medium C, with 0.25% mannitol instead of the 1% mannitol as the limiting substrate. Feeding was started at the lowest dilution rate ($0.0474h^{-1}$), and kept at steady state for four volume change in the vessel, and then increased to the next higher dilution rate. Fig. 4 shows steady-state behavior of continuous mixed-culture fermentation. The kinetic parameters calculated from steady-state results using Lin-

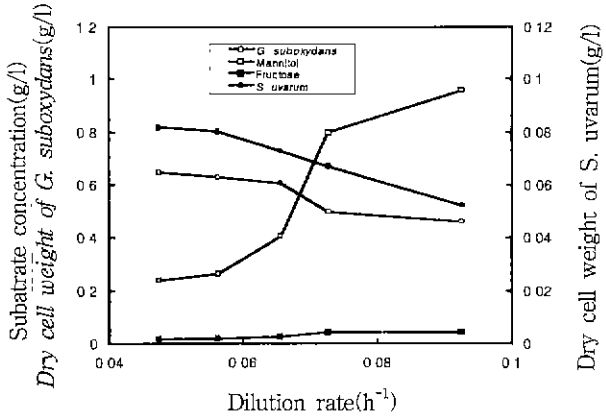

Fig. 4. Steady-state behavior of mixed-culture fermentation.

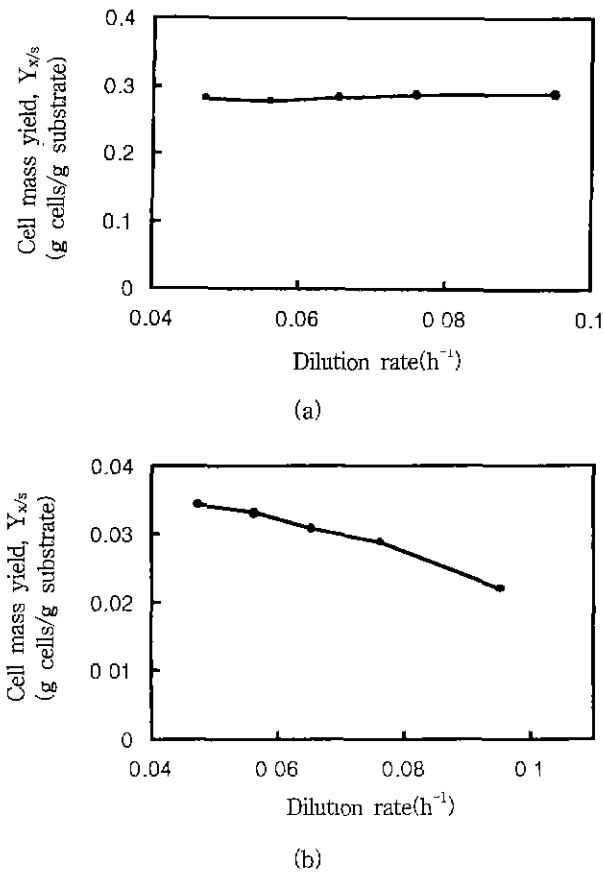

Fig. 5. Effect of dilution rate on growth yield in continuous mixed-culture fermentation.
(a) *G. suboxydans*, (b) *S. uvarum*

eweaver-Burk equation were as follows: μ_{max1}=0.118 (h^{-1}), K_{S1}=0.330(g/L), μ_{max2}=0.162(h^{-1}), K_{S2}=0.038(g/L).

The residual mannitol concentration in the medium increased with increasing dilution rate. Yield of bacterial cell mass from mannitol was relatively constant, but yield of yeast cell mass from fructose was gradually decreased over the dilution rates tested(Fig. 5).

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