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Ethanol, Acetic acid, Acetaldehyde 기질에서의 *Rhodotorula* sp. Y-55의 증식 특징

†이 인 · 김 상 해 · *주 우 홍 · **전 홍 기 · ***권 오 창

인제대학교 기초과학연구소,* 창원대학교 생물학과,** 부산대학교 미생물학과,*** 동아대학교 원예학과

Growth Characteristics of *Rhodotorula* sp. Y-55 on Ethanol, Acetic acid, and Acetaldehyde Substrates

Yeehn Yeeh†, Sang Hae Kim, Woo Hong Joo*, Hong Ki Jun**, and Oh Chang Kwon***

Institute of Basic Sciences, Inje University, Kimhae, Kyungnam 621-749, Korea
*Department of Biology, Changwon National University, Changwon, Kyungnam 641-773, Korea
** Department of Microbiology, Pusan National University, Pusan 609-735, Korea
*** Department of Horticulture, Dong-A University, Pusan 604-714, Korea

ABSTRACT

The growth characteristics of *Rhodotorula* sp. Y-55 were examined on minimal medium containing ethanol, acetic acid or acetadehyde as a sole carbon source by batch culture. The increased concentration of substrate reduced overall growth yield and prolonged lag time. The specific growth rate of the yeast was changed, depending upon the initial concentrations of ethanol and acetaldehyde during the exponential period, but was constant on acetic acid without regard to the initial substrate concentrations, giving a value of $0.107h^{-1}$. The highest μ value was obtained on ethanol and acetadehyde substrates and the respective values were 0.270 at 20 g/L and $0.041h^{-1}$ at 0.2g/L. The maximum overall growth yields were appeared to be 32.6% for ethanol of 10g/L, 25.6% for acetic acid of 20g/L, and 45 % for acetaldehyde of 0.2g/L. The respective cellular contents of crude protein and nucleic acids were determined to be 41.5 and 4.9wt% on ethanol and 40.2 and 4.7wt% at the concentration revealing maximal growth yield.

INTRODUCTION

The researches on the ethanol-, acetic acid-, or acetaldehyde-assimilating yeasts among compounds of two carbon atoms by batch culture were largely upon the cultural conditions, or cell yield. There are reports concerning the application of substrate consumption and respiration by Saccharomyces cerevisiae (1), or the activities of several key enzyme from the genus *Candida* (2). Some experiments were also discussed on the cell yield and optimal condition of *Candida brassicae* nov. sp. (3, 4) and *Pichia guilliermondii* Wickerham (5). The batch cultures of *Candida*

† Corresponding Author

methanolica and *Torulopsis methanolovescens* (6) as acetic acid-assimilating yeasts and those of *Candida utilis* MG-1 (7) as an acetaldehyde-assimilating yeast were also performed. Besides these reports, the cell yields of *Hansenula miso* IFO 0176 (8), *Saccharomyces cerevisiae* FRI 14 (9), and *Candida tropicalis* (10) as acetic acid-assimilating yeasts were also examined. Moreover, no informations are available regarding production of single-cell protein of *Rhodotorula* species from ethanol, acetic acid or acetaldehyde so far. The present study was concentrated on the growth properties of an isolated strain, *Rhodotorula* sp. Y-55 including cell yield, and specific growth rate in ethanol, acetic acid and acetaldehyde among compounds of two carbon atoms by the batch culture and the results were compared with those of the previously studied yeast strains.

MATERIALS AND METHODS

Microorganism

Rhodotorula sp. Y-55 showing the maximum growth rates in ethanol, acetic acid and acetaldehyde substrates was kept on the stock culture medium(glucose, 1.0g; ammonium sulfate, 0.3g; sodium phosphate monobasic, 0.1g; magnesium sulfate, 0.05g; agar, 1.6g; distilled water, 100mL; pH 5.5). The strain was used through all the experiments.

Cultivation method.

The utilization of alcohols or organic acids was examined in 500mL Erlenmeyer flasks with 90 mL of medium(pH 7.0) containing basal salts as presented in Table 1. 1% each alcohols or acidic salt was prepared and cultivated at 30℃ on the reciprocal shaker(110rev. at 6cm stroke). Batch culture was conducted in jar fermentor(Bioflow model-30, New Brunswick Scientific Co., Inc.). Prior to jar cultivation, the yeast cells were grown overnight at 30℃ on agar slants containing ethanol, acetic acid, or acetaldehyde. A loopful of inoculum was transferred to test tubes with the medium composition of the basal salts

Table 1. Composition of medium for the isolation of ethanol-, acetic acid-, and acetaldehyde-assimilating yeast(pH 5.5)

Chemical	Amount
Ethanol	1.0g
Acetic acid	0.2g
Acetaldehyde	0.05g
Chloramphenicol	0.03g
Basal salts	
Ammonium sulfate	0.3g
Potassium phosphate monobasic	0.15g
Magnesium sulfate	0.05g
Ferrous sulfate	0.30mg
Calcium chloride	0.30mg
Manganese sulfate	0.50mg
Cupric sulfate	0.05mg
Distilled water	100mL

with ethanol 0.3mL, acetic acid 0.2mL, or acetaldehyde 0.01mL in a 100mL distilled water(pH 7.0). After the tubes were cultivated on the reciprocal shaker at 30℃ for 18h. 2mL of subcultures were withdrawn and inoculated to the jar fermentor(working volume, 375mL) with 300mL of the medium(pH 7.0) composed of ethanol 1.5 ~18mL, acetic acid, 1.5~18mL, or acetaldehyde, 0.03~0.12mL. The aeration rate was controlled as 0.5L/m and the agitator speed was maintained at 400r.p.m. Silicone oil was incorporated as an antifoaming agent. The pH of medium for optimal growth was automatically monitored and controlled with 1*N* NaOH, or 1*N* HCl solution. 10mL of routine samples were always withdrawn in duplicate from the culture.

Analytical method.

Cell desity was determined by the measurement of the turbidity. The culture fluid was centrifuged at 12,000r.p.m.(Hitachi 20, PR-5) for 10min. The pellet was washed twice, suspended with physiological saline solution and the absorbance was read at 610nm(Shimadzu recording spectrophotometer UV 240, Japan). Cell dry matter(mg dry wt. per l) was determined by centrifugation of 10mL of outflowing culture medium, washing

with the saline and drying overnight at 105℃. Apparent overall growth yield was expressed as the ratio of maximum dry cell quantity formed to the substrate quantity incorporated initially. To determine the μ value during the exponential growth, the data of absorbance were plotted on semilogarithmic paper and a straight line was graphically fitted and then the μ value was calculated. The contents of crude cellular proteins(wt %, total nitrogen content×6.24) were determined by Kjeldahl method (11). The measurement of nucleic acids(wt%) was made by Schneider's method (7) and monitored by high-performance liquid chromatography(Waters liquid chromatograph model 244, 440 absorbance-detector, column; μ-Bondapak c/18, solvent; Na_2SO_5, 6% +$(NH_4)_2HPO_4$(0.1M), pH 4.6).

RESULTS

Utilization of alcohols and organic acids.

Of the various alcoholic compounds tested, ethyl alcohol, glycerol and mannitol were found to support marked growth(Table 2). Additional substrates that observed not to support growth included amyl alcohol, methyl alcohol, 2-mercaptoethanol, benzyl alcohol and inositol. Of the acidic compounds tested, acetic acid, pyruvic acid and propionic acid revealed marked growth(Table 3). Our yeast could also grow on compounds present in the tricarboxylic acid cycle(succinic acid, fumaric acid and citric acid), whereas formic acid, *n*-butyric acid, glycollic acid and β-hydroxybutyric acid did not reveal growth. At a final concentration of 1g(or 1mL of liquid substrates) per 100mL of medium, the cell yields were found to be 2,600mg/L on ethanol and 1,200mg/L on glycerol, while a cell yield of 3,100mg/L was obtained on glucose. The value obtained on acetic acid was thought to be 16% lower than that on ethanol with our yeast.

Growth on ethanol substrate by batch culture

For various initial concentrations of ethanol substrate, the growth curves of the strain were

Table 2. Utilization[a] of various alcohols as sole sources of carbon

Alcohol	Yield of cells(mg dry wt./l)
Amyl alcohol	0
2-Amyl alcohol	30
2-Butyl alcohol	63
3-Butyl alcohol	108
Ethyl alcohol(ethanol)	2610
Methyl alcohol	0
Glycerol	1216
n-Propyl alcohol	18
2-propyl alcohol	570
2-Mercaptoethanol	0
Polyvinyl alcohol	46
Ethylene glycol	73
Diethylene glycol	65
Propylene glycol	203
Benzyl alcohol	0
Mannitol	1032
Inositol	0
Glucose	3100

[a]The shaking flask with 90mL of medium(pH 7.0) containing 1% each alcohol and basal salts(refer to Table 1) was prepared and cultivated by the shaking method for 3 days at 30℃.

Table 3. Utilization[a] of various organic acids as sole sources of carbon

Organic acid	Yield of cells(mg dry wt./l)
Acetic acid	2010
n-Butyric acid	0
Citric acid	120
Fumaric acid	752
Formic acid	0
Glycollic acid	0
β-Hydroxybutyric acid	0
Lactic acid	690
Malonic acid	151
Propionic acid	915
Pyruvic acid	1026
Succinic acid	730
Tartaric acid	668

[a]The method was the same as described in Table 2, except 1% each organic acid, and acidic salts were used as organic acids.

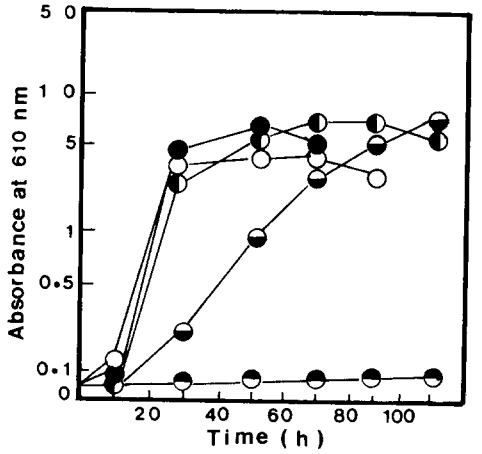

Fig. 1. Growth curves of *Rhodotorula* sp. Y-55 on various concentrations of ethanol. Concentrations of ethanol(g/L): ○, 5: ●, 10: ◐, 20: ◒, 40: ◓, 60.

Table 4. Growth characteristics[a] of *Rhodotorula* sp. Y-55 on ethanol, acetic acid and acetaldehyde substrates

Substrate	Initial conc. (g/L)	Maximum dry cell weight (g/L)	Apparent overall growth yield(%)	$\mu(h^{-1})$[b]
Ethanol	5.0	1.55	31.0	0.213
	10.0	3.26	32.6	0.249
	20.0	3.76	18.8	0.270
	40.0	7.34	18.3	0.077
	60.0	0.10	0.01	0.016
Acetic acid	5.0	0.95	19.0	0.107
	10.0	2.00	20.0	0.107
	20.0	5.12	25.6	0.107
	40.0	8.45	21.1	0.107
	60.0	1.50	0.3	0.107
Acetalde-hyde	0.2	0.09	45.0	0.041
	0.5	0.19	38.0	0.035
	1.0	0.27	27.0	0.022
	5.0	0	0	0

[a]The cultivation was carried out in the culture vessel of fermentor with 300mL of medium containing various concentrations of substrates and basal salts(refer to Table 1) at pH 7.0 at 30℃.

[b]Specific growth rate.

presented in Fig. 1 Y-55 could grow at the initial concentrations below 50g/L and the maximum

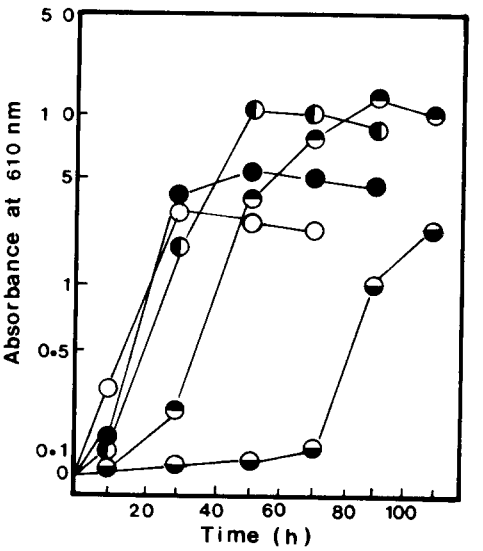

Fig. 2 Growth curves of *Rhodotorula* sp. Y-55 on various concentrations of acetic acid. Concentrations of acetic acid(g/L): ○, 5: ●, 10: ◐, 20: ◒, 40:, ◓, 60.

absorbance increased generally with the initial ethanol concentration, while no growth was observed at concentrations above 80g/L. Furthermore, the active growth took place commonly after lag period. The growth characeristics of our yeast on ethanol were summarized in Table 4. The μ value during the exponential growth period at various concentrations of ethanol decreased generally in proportion to the increase in the initial concentration of ethanol. In addition, as the initial ethanol concentration increased, the lag time was proportionally prolonged and the apparent overall growth yield(growth yield) decreased. The highest yield of 32.6% was observed at the initial ethanol concentration of 10g/L, giving a value of 3.26g/L for dry cell weight.

Growth on acetic acid substrate by batch culture

Fig. 2 showed the growth curves of Y-55 cells at various initial concentrations of acetic acid. It was likely that the strain could grow at the initial concentrations higher than 60g/L, but not at 80g /L. The absorbance of cells generally diminished in proportion to the initial acetic acid concentra-

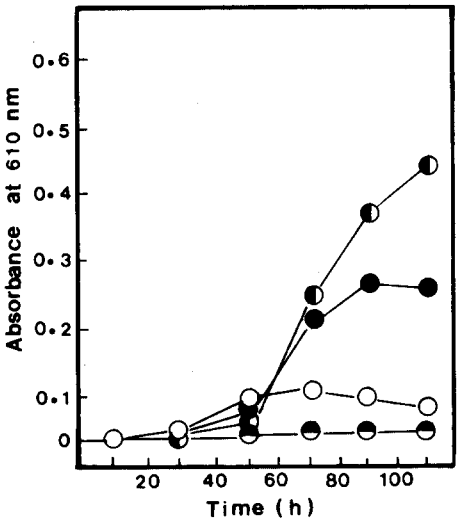

Fig. 3. Growth curves of *Rhodotorula* sp. Y-55 on various concentracetaldehyde. Concentrations of acetaldehyde(g/L): ○, 0.1: ●, 0.5: ◐, 1.0: ⊖, 5.0.

tions. The growth characteristics on acetic acid summarized in Table 1 indicated that the increased dry cell weight was accompanied by the increase of initial concentration of acetic acid. The μ values were observed during the exponential growth. As long as the growth proceeded, the μ values remained constant regardless of the initial concentrations of acetic acid. The μ value with our yeast was calculated to be $0.107h^{-1}$. The highest growth yield of 25.6% was achieved at an initial concentration of 20g/L, giving a value of 5.12g/L for the dry cell weight. The growth yields decreased in general and the lag time was prolonged in proportion to the initial concentrations of acetic acid.

Growth on acetaldehyde substrate by batch culture

The growth curves of Y-55 cells on acetaldehyde at various initial concentrations were shown as in Fig 3. A significant growth inhibition occurred on acetaldehyde substrate. Our results obtained meant typical patterns of inhibition on cell growth. By increasing the initial concentrations the lag time was prolonged and the maximum absorbance generally increased, while *Rhodotorula* was unable to grow at the initial acetaldehyde concentrations higher than 5.0 g/L. The growth characteristics on acetaldehyde substrate at various initial concentrations were also summarized (Table 1). The μ values were changed with the initial concentrations of acetaldehyde. The growth yields also decreased in proportion to the initial concentrations of acetaldehyde as observed on ethanol substrate. The maximum growth yield of 45.0% was obtained at 0.2g/L, giving a value of 0.09g/L for dry cell weight.

Table 5. Content of crude protein and nucleic acids on ethanol, acetic acid and acetaldehyde

Substrate	Crude protein(wt%)	Nucleic acids(wt%)
Ethanol(10g/L)	41.5	4.9
Acetic acid(20g/L)	40.2	4.5
Acetaldehyde(0.2g/L)	35.4	3.9

Content of crude protein and nucleic acids

Crude protein and nucleic acid contents were determined from the dried cells at concentration presenting maximal growth yield. As shown in Table 5, the content of crude protein was found to be 40.2wt% at 20g/L acetic acid and 35.4wt% at 0.2g/L acetaldehyde, respectively.

DISCUSSION

With *Rhodotorula* sp. Y-55 strain, substrate utilization tests were made for alcoholic compounds and for organic acids. Our yeast could utilize ethyl alcohol, whereas no growth was observed in amyl alcohol and methyl alcohol, giving a marked cell yield on acetic acid. From these results, batch culture experiments were exerted to establish the effects of various concentrations of ethanol, acetic acid or acetaldehyde as a sole carbon source on cell growth. It was reported that *Torulopsis methanolovescens* and *Candida methanolica* could grow at the initial ethanol concentrations below about 30g/L, whereas no growth occurred at the initial ethanol concentrations

above about 50g/L (6). As compared with these, our strain was considered to have higher affinity, or tolerance to ethanol. The cell dry weight was found to increase with the initial concentration of ethanol, whereas overall growth yield decreased with prolonged lag time for all substrates used. This corresponded with that from cells of *Torulopsis methanolovescens* and *Candida methanolica*. In addition, Goto *et al.* (6) noted that the prolonged lag time was found to be increased with the increase of the initial concentration of the substrate during the cultivations of *Candida* and *Torulopsis* yeasts. The same was true for our yeast. Furthermore, reductions in the μ values were revealed for our yeast with the increase in the initial concentrations of ethanol, similar to those from above yeasts. It is well documented that growth yields are shown to be 75.8% for *Torulopsis methanolovescens*, 58.5% for *Candida methanolica* (6), 68% for *Candida utilis* (12) and 77.4% for *Candida brassicae* sp. nov. E-17 (3) on ethanol, containing malt extract, yeast extract and peptone. Besides these, there are other reports that the growth yields appeared to be values of 27% and 61.2% for *Pichia guilliermondii* Wickerham (5) and for *Candida* sp. JY-5 (13), respectively on minimal medium. Our value was lower than that for JY-5, but higher than that for *Pichia guilliermondii* Wickerham. As regards value, It is reported that differences in its value is accompanied by the initial ethanol concentrations (3, 6). Similar results were obtained with our yeast. Goto *et al.* (6) demonstrated that the μ values were observed to be $0.318h^{-1}$ for *Candida methanolica* and $0.299h^{-1}$ for *Torulopsis methanolovescens* at the initial concentration of 0.53% and 4.02% ethanol, respectively. A μ value of $0.72h^{-1}$ was shown at 0.5% ethanol with *Candida brassicae* sp. nov. E-17 (3). They obtained the values from yeasts on complete medium containing malt extract, yeast extract and peptone. Our yeast showed a μ value of $0.270h^{-1}$, a relatively high value at 20% ethanol on minimal medium and is comparable to those descibed above, because both cell yields and μ values increase on complete medium (14).

Few informations are available for the production of single-cell protein from acetic acid, especially from acetaldehyde so far. Goto *et al.* (6) reported that two yeasts could grow at the initial concentrations of acetic acid lower than 20g/L, whereas no growth occurred at those higher than about 50g/L. As compared with this, our yeast could grow at the concentrations below 70g/L and not at those above 80g/L. Therefore, 70g/L of acetic acid was thought to be the threshold concentration for batch culture of the yeast. This means that our strain possesses a relatively high resistance to acetic acid and has ability to overcome the substrate inhibition. It is well known that maximum growth yields are shown to be 44.7% for *Torulopsis methanolovescens*, 42.8% for *Candida methanolica* (6) and 34~36% for *Candida utilis* (12, 14) on complete medium with malt extract, yeast extract and peptone. Our value was lower than that of *Candida* sp. JY-5 (13) on minimal medium, but similar to that of *Candida utilis*.

Goto *et al.* (6) reported that the μ values of two yeasts remained constant without regard to the initial concentrations of acetic acid. Similar results were obtained with our yeast, but it differed markedly from those of Cama and Edwards (14) in respect of stimulatory effect. We have no explanation for the constant or different values on acetic acid. According to Goto *et al.* (6), it was thought that our yeast could adapt to acetic acid in the early period of batch culture. Our value was lower than those from others (7, 14) in spite of the fact that Y-55 cells had ability to grow at a relatively high concentration of ethanol, but slightly higher than that with *Candida* sp. JY-5.

At 0.125% of acetadehyde, the growth of *Candida utilis* MG-1 (7) is inhibited and the optimum concentration of acetaldehyde is observed at 0.03%, whereas our yeast could grow at 0.5% acetaldehyde with a value of $0.042h^{-1}$ comparable with that of JY-5 (15). There was, however, an inclination to rapid reduction in the maximum dry

cell weight at concentrations above 1.5g/L, probably due to the toxicity of the compound. Our yeast seemed not to utilize 2.0g/L of acetaldehyde and showed 4.1% of growth yield, giving a value of about 45% for growth yield at 0.2g/L. Therefore, we considered that it was essential to screen the powerful strains tolerant to the compound. Of three substrates, the crude protein and nucleic acid content of cells grown on ethanol was highest, giving a value of 41.5wt% at 10g/L ethanol and that of 4.9wt% for nucleic acids. From the above results, we considered *Rhodotorula* sp. Y-55 to be a useful and possible yeast for single-cell protein.

요 약

Rhodotorula sp. Y-55 효모의 증식특성을 ethanol, acetic acid 또는 acetaldehyde 상에서 batch culture로 조사하였다. 기질농도가 증가함에 따라 증식수율이 감소하고 유도기가 연장되었다. 비성장속도(μ)는 지수기에서 ethanol과 acetaldehyde의 초기농도에 따라 변화하였으나 acetic acid는 이와 상관이 없었으며 그 값은 0.107h^{-1}로 나타났다. 최대 비성장속도는 20g/L의 ethanol에서 0.270h^{-1}, 0.2g/L의 acetaldehyde에서 0.041h^{-1}이었다. 또 최대 증식수율은 10g/L의 ethanol에서 32.6%, 20g/L의 acetic acid에서 25.6%와 0.2g/L의 acetaldehyde에서 45%였다. 세포내 조단백질과 핵산 함량은 ethanol상에서 각각 41.5wt%와 4.9wt%였고 acetic acid상에서는 각각 40.2wt%와 4.7wt%로 나타났다.

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