Isolation and Identification of New Acid-tolerant and Starch-degradable Yeasts

Viet Anh Thi Nguyen, Atsuko Tokuda, Takashi Mishima, Yoshio Nishida¹ and Makoto Hisamatsu*

*Laboratory of Food Science and Technology, Faculty of Bioresources, Mie University
(1515, Kamihama, Tsu 514-8507, Japan)*

¹*Food Research Institute, Aichi Prefecture Government (2-1-1, Shinpukuji-cho, Nishi-ku, Nagoya 451-0083, Japan)*

Two strains of acid-tolerant and starch-degradable yeast were isolated from rivers flowing in Kusatsu and Manza, considering an effective reuse of great mass of starch-based biodegradable plastic. The isolated strains could grow in acid media of pH 1.8 and were identified as *Candida fluviatinis* and *Candida intermedia*. The yeasts could assimilate many carbon sources and ferment glucose and sucrose in acid media to alcohol.

There is a growing interest in the development of degradable plastics to enhance biodegradability of plastic product in landfills and composts.¹⁾ As a biodegradable plastic, polylactide or poly(lactic acid) (PLA) has become the focus of public attention. It is polymerized from lactic acid, which can be prepared effectively by fermentation with renewable resources such as starchy materials and cellulose.^{2,3)} But PLA is very expensive at present and a starch-based plastic blended from starch as main component and PLA as minor one is one of the most promising biodegradable polymers.⁴⁾

Research on the development of biodegradable polymers derived from renewable resources has gained considerable momentum in recent years.⁵⁾ Together with research attending to production of new degradable polymers, it is also important to study the reuse of these polymers.^{5,6)} Until now, the studies have focused on PLA degradation through enzymatic^{2,7)} and acidic⁸⁾ degradation. In the field of commercial biodegradable plastics, it seems that starch is used more than 50%, and the utilization of the starch in the plastic after use should be also investigated. Although there are some studies concerning saccharification of starch⁹⁾ and cellulose¹⁰⁾ following alcohol fermentation by

using *Saccharomyces cerevisiae*, it seems that there is room for improvement concerning economic policy.

In this paper, an isolation of acid-tolerant and starch-degradable yeasts was conducted in expectation of alcohol fermentation without sterilization.

MATERIALS AND METHODS

Isolation of acid-tolerant yeasts. The water samples (25 mL) collected from rivers flowing through areas of Kusatsu, and Manza hot springs were inoculated in 100 mL of acid medium (pH 2.5) using GPY medium containing 5% glucose, 1% polypepton and 0.5% yeast extract, and carried out at 30°C for 5 days on a rotary shaker. Appropriately diluted cultures were spread on acid agar plates (pH 2.5) containing 1% glucose or 1% soluble starch, 0.5% yeast extract, 1.8% agar and penicillin G potassium (final 150 U/mL) and incubated at 30°C for 5 days. Yeast-like colonies appearing were picked up from the plates and streaked on new plates. After cultivation, plates containing soluble starch were exposed to vapor of solid iodine by overturning the plates. Strains showing both a halo-like design around colonies on the starch plate and a comparatively large colony on the glucose plate were isolated and further

* Corresponding author.

purified three times by successive transference to acid GPY agar plates. Isolated strains growing on the acid GPY agar medium (pH 2.5) were maintained at 4°C.

Identification of yeast strains. Taxonomical characteristics of the isolated strains were investigated according to the methods described by N. J. W. Kreger-Van Rij,¹¹⁾ and J. A. Barnett, R. W. Payne and D. Yarrow.¹²⁾ Morphologies of the vegetative cells of isolated strains were tested in YM medium (pH 6.0) containing 1% glucose, 0.5% polypepton, 0.3% yeast extract and 0.3% malt extract.

Growth and fermentation conditions. The effect of pH on growth of isolated strains was studied in acid GPY medium by measuring the absorbance at 660 nm. The pH of medium was adjusted with 4.5 M and 1.0 M H₂SO₄ to pH 3.0, 2.5, 2.0, 1.8 and 1.5, respectively.¹³⁾ A fermentation test was carried out by shaking at 150 rpm at 30°C in 100 mL of acid GPY media. Effects on the carbon sources were also studied by replacing the glucose of the GPY medium with soluble starch (5%), maltose (5%), cellobiose (1%), xylose (1%), sucrose (5%), or trehalose (5%). Growth on the high concentrations of glucose and Na₂SO₄ were studied on the acid GPY media consisting of 10% and 20% glucose and 5% Na₂SO₄ respectively. Four brewing strains of *S. cerevisiae* (Kyokai No.3, Kyokai No.7, Kyokai No.9 and Shochu S2) were also tested in order to make a comparison with the isolated strains.

HPLC analysis of fermented alcohol. A culture was centrifuged at 5000 rpm for 20 min and the supernatant (20 µL) was analysed with a HPLC system (Tosoh Co., Ltd., Japan) with a refractive index detector, an analysis column (7.8 mm I.D×30 cm) of TSK gel Oapack-A (Tosoh Co., Ltd., Japan), and a guard column (6.0 mm I.D×4 cm) of TSK gel Oapack-P at room temperature using 0.75 mM H₂SO₄ as eluent at 0.6 mL/min.

RESULTS AND DISCUSSION

Characterization of the isolated strains.

From 120 acid-tolerant strains screened by using

acid agar media, two strains were selected and designated as 2C and 3A, respectively. The acid-tolerant abilities of 2C and 3A strains were examined in the media with different initial pH value. Both strains grew well in acid media until pH 2.5 and could not grow in medium of pH 1.5 (Fig. 1). Their starch-degradabilities were also checked on an acid agar-plate (pH 2.5) with 1% soluble starch (see Fig. 2). *S. cerevisiae* (Kyokai No.3, Kyokai No.7, Kyokai No.9 and Shochu S2) could grow in the acid medium but did not digest starch, while the two isolated strains could grow well and also

Fig. 1. Growth curves of 2C and 3A strains in various acid media.

GPY media (100 mL) of pH 6.0 and 3.0 were prepared by mixing with 0.07 mL and 1.1 mL of 1 M H₂SO₄, respectively, and those of pH 2.5, 2.0, 1.8 and 1.5 were also prepared by mixing with 0.45, 0.70, 0.98 and 1.1 mL of 4.5 M H₂SO₄, respectively. The growth was followed by measuring the absorbance at 660 nm. Symbols: ■, pH 6.0; ○, pH 3.0; △, pH 2.5; □, pH 2.0; ▲, pH 1.8; ×, pH 1.5.

Fig. 2. Photograph of starch-degrading abilities.

1, *S. cerevisiae* No.3; 2, *S. cerevisiae* No.7; 3, strain 2C; 4, strain 3A; 5, *S. cerevisiae* No.9; 6, *S. cerevisiae* Shochu 2S. Plate overturned was exposed to vapor of solid for appropriate time.

showed a strong ability to digest starch in low pH.

Identification of the isolated strains.

The physiological and biochemical characteristics of 2C and 3A strains were investigated according to Kreger-Van Rij⁽¹¹⁾ and are summarized in

Table 1. Two strains showed abilities to ferment glucose, sucrose, maltose and trehalose and to assimilate many carbon compounds containing galactose, xylose, lactose, starch, cellobiose and some alditols. Assimilation of nitrogen compounds, the Diazonium Blue B (DBB) color test, urease test and starch formation were negative for both strains.

There were some difference in characteristics between 2C and 3A. The growth of 2C strain in vitamin-free medium was positive, but that of 3A strain was negative. The assimilations of rhamnose and raffinose were positive in 3A strain and negative in 2C strain. Morphologies of their vegetative cells were different (see Fig. 3). Cells of 2C strain were round to short oval and those of 3A strain were oval to ellipsoidal with multilateral budding.

According to the methods described in "The yeasts a taxonomic study,"⁽¹¹⁾ and "Yeasts: Characteristics and identification,"⁽¹²⁾ it was suggested that strain 2C belong to *Candida fluvialis*, and strain 3A to *Candida intermedia*.

Table 1. Physiological and biochemical characteristics of 2C and 3A strains.

Fermentation:					
Glucose:	+	Maltose:	+	Trehalose:	+
Galactose:	—	Lactose:	—	Inulin:	—
Sucrose:	+	Raffinose:	—	Starch:	—
Melibiose:	—				
Production of ammonia from urea: —					
Starch formation: —					
Growth on 50% (w/w) glucose agar: —					
Growth at 37 °C: +					
Growth in vitamin-free medium: —/+					
Assimilation of carbon compounds:					
Glucose:	+	Salicin:	+	Ribitol:	+
Galactose:	+	Arbutin:	+	Xylitol:	—
Ribose:	—	Melibiose:	—	Glucitol:	+
Xylose:	+	Lactose:	+	Mannitol:	+
Arabinose:	—	Raffinose:	—/+	Inositol:	—
Rhamnose:	—/+	Melezitose:	+	Cellobiose:	+
Sucrose:	+	Starch:	+	Methanol:	—
Maltose:	+	Glycerol:	+	Ethanol:	+
Trehalose:	+	Erythriol:	—	Citric acid:	+
Assimilation of nitrogen compounds: Nitrate — Nitrite —					
Acid production: +					
Diazonium Blue B reaction: —					

Items indicating different contents between 2C and 3A shown by separation with slash: result of 2C is left side and that of 3A is right side.

Fig. 3. Morphologies of vegetative cells of strains of 2C (A) and 3A (B).

Vegetative cells growing in YM medium (pH 6.0) for 3 days at 30°C were observed by microscopy. Bar scale: 10 μm.

Effects of substrates on growth and alcohol fermentation.

The fermentation test shown in Table 1 was indicated by the production of carbon dioxide from carbohydrates because of taxonomical analysis. So, definite alcohol production needed to be ascertained, and *C. fluviatilis* 2C and *C. intermedia* 3A were therefore cultivated in 100 mL of acid GPY media (pH 2.5) with soluble starch, maltose, cellobiose, xylose, sucrose, glucose and trehalose, respectively. Some properties of cultures containing alcohol fermentation are shown in Table 2. It was found that only glucose and sucrose were the sub-

strates for ethanol production and their final pH values did not change, but cultures of other carbon substrates were considered as non alcohol-fermentation and their pH values rose.

Growth on high concentrations of glucose and Na₂SO₄.

For unsterilized alcohol-fermentation of acid hydrolysate derived from starch-based biodegradable plastic, it should be verified that isolated strains can grow and ferment alcohol in acid media containing high concentrations of sugar because the high concentration of substrate is normally inhibitory to fermentation.¹⁴⁾ The effect of salt resulting

Table 2. Growth and fermentation of *C. fluviatilis* 2C and *C. intermedia* 3A for various substrates in acid GPY media of pH 2.5.

Carbon source (%) ^b	OD ^a at 660 nm		Final pH of cultures		Alcohol fermentation ^c	
	2C	3A	2C	3A	2C	3A
None	0.45	0.32	7.0	7.3	—	—
Soluble starch (5)	0.88	0.74	3.9	3.5	—	—
Maltose (5)	1.12	0.69	2.6	3.2	—	—
Cellobiose (1)	0.97	0.54	5.6	6.2	—	—
Xylose (1)	0.82	0.46	2.9	3.5	—	—
Sucrose (5)	1.05	0.73	2.4	2.5	+	+
Glucose (5)	1.06	0.67	2.3	2.4	+	+
Trehalose (5)	0.93	0.56	3.4	3.3	—	—

^aAbsorbance at 660 nm of culture grown in 100 mL of acid GPY medium in 300 mL flask at 30°C for 5 days. ^bThe number in parentheses indicate the percentages of substrate. ^cAlcohol fermentation was decided by checking a peak of ethanol by HPLC.

from the neutralization of the acid hydrolysate of starch should be also considered. Our tentative hydrolysis conditions showing good yield of reducing sugar from 50% (w/v) corn starch were 0.4 M H_2SO_4 at 110°C and the concentration of sulfate after neutralization was estimated to be about 5% (w/v). So, effects of high concentrations of glucose and/or Na_2SO_4 on the growth of *C. fluvialis* 2C and *C. intermedia* 3A were tested, and some strains of *S. cerevisiae* were also tested for comparison. All strains tested could grow well in pH 2.5 media with 10 and 20% glucose. Four strains of *S. cerevisiae* could not grow in pH 2.5 medium with 10% glucose and 5% Na_2SO_4 . The growths of strains 2C and 3A measuring by optical density at A_{660} showed more than 0.5, it was indicated that they were the salt-tolerant yeasts.

Ethanol production under unsterile condition.

Ethanol fermentation of 2C and 3A strains was investigated without sterilization in GPY medium of pH 2.5 containing 10% glucose and 5% Na_2SO_4 . Although these acid-tolerant yeasts grow well in these acid conditions, their alcohol productions were very low (about 1%) and the yield was far from the value expected. However, it was expected that there is potential for developing a technology for a bio-conversion system producing fuel ethanol from starch-based biodegradable plastic.

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新規酸性耐性および澱粉分解性酵母の分離同定

ウェト アン ティ グエン, 徳田濃子
三島 隆, 西田淑男¹, 久松 眞

三重大学生物資源学部食品資源工学研究室
(514-8507 津市上浜町 1515)

¹ 愛知県食品工業センター
(451-0083 名古屋市中区新福寺町 2-1-1)

澱粉を主体とする大量な生分解プラスチックの再資源化を考慮に入れて、二株の酸性耐性および澱粉分解性酵母を草津および万座を流れる河川から単離した。単離した株は pH 1.8 の酸性培地で生育でき、*Candida fluvialis* and *Candida intermedia* と同定された。この酵母は、多くの炭素源を資化でき、さらにグルコースと砂糖からアルコールを発酵することができた。