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SHORT COMMUNICATION

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Role of the pectinolytic enzyme in the lactic acid fermentation of potato pulp by *Rhizopus oryzae*

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Abstract *Rhizopus oryzae* strain NBRC 4707 produced lactic acid and ethanol more efficiently than strain NRRL 395 in potato pulp, an agricultural by-product of the starch industry. The two strains developed comparable activities of xylanase, cellulase, α-amylase, and glucoamylase, while the polygalacturonase activity of strain NBRC 4707 was double that of strain NRRL 395. The addition of commercial pectinase enhanced the formation of metabolites, suggesting that the degradation of pectic substances determines the fermentation of potato pulp by *R. oryzae*. Orange and apple peel were more effective in the induction of polygalacturonase activity than potato pulp, sugarbeet pulp, or wheat bran when used as a principal carbon source for fungal growth in a solid-state culture. The fungal cells in both types of fruit peel stimulated the fermentation of potato pulp and increased the quantity of lactic acid and ethanol to higher levels than those in other agricultural by-products.

Keywords Pectic substances · Polygalacturonase · Potato pulp · Lactic acid

Introduction

Silage, which is produced to preserve forage with a high moisture content by controlled fermentation, is an important winter feed for cattle [23]. During the fermentation process, lactic acid bacteria convert water-soluble carbohydrates predominantly to lactic acid under anaerobic conditions. A low pH, in combination with the toxicity of the undissociated acids, can suppress the activities of the microorganisms responsible for spoilage. The raw materials used for silage include grass, maize, legumes, grains, potatoes, cabbage, and the residues resulting from processing these crops [18]. Potato pulp (composed of starch, cellulose, hemicellulose, and pectic substances) is an agricultural by-product in the starch industry [11], but it is not rapidly fermented by an amylolytic lactic acid bacterium [15]. *Rhizopus oryzae* is used to convert complex carbohydrates to lactic acid under aerobic conditions, as an alternative inoculant for ensiling potato pulp [15]. Species of *Rhizopus* produce organic acids, such as lactic, fumaric, and malic acids, in the presence of excess carbon and limited sources of nitrogen [5]. So far, strain NRRL 395 has been selected [9], studied [19, 20], and widely used to optimize and enhance lactic acid production from starch [24], corn [6], and steam-exploded wood hydrolysate [22]. However, strain NBRC 4707 is unexpectedly more suitable for the lactic acid fermentation of potato pulp than strain NRRL 395 and 37 other strains tested [15]. The differences between NBRC 4707 and NRRL 395 may be governed by enzymes hydrolyzing the polysaccharides of potato pulp to fermentable sugars, because similar levels of lactic acid are formed in a medium containing glucose as the sole carbon source [16]. In the present experiments, the key enzyme that determines the production rate of lactic acid in potato pulp is assessed to develop a practical method for rapid ensiling.

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Materials and methods

Organisms

R. oryzae NBRC 4707 (formerly known as IFO 4707) and NRRL 395 were obtained from the Institute for Fermentation, Osaka, Japan and the Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, United States Department of Agriculture, Peoria, Ill., respectively.

Culture and fermentation

Potato pulp (dry matter 20.8%) was donated from a local industrial plant that was manufacturing starch from potato tubers. After the pulp was autoclaved at 121 °C for 15 min and cooled to room temperature, it was bagged in 30-g amounts in polyethylene. Fungal spores that formed on potato dextrose agar for 5 days were suspended in 0.01% Tween 80. The sterilized pulp was inoculated with a final concentration of 10^5 spores g^{-1} and sealed by hand to keep air out. A lump of the pulp was incubated at 25 °C for 7 days and crumpled daily.

Fungal cells were optionally grown as starter cultures on 10 g of potato pulp, sugarbeet pulp, orange peel, apple peel, or wheat bran in a Petri dish for 3 days and successively fermented for 7 days after mixing with 90 g of the sterilized pulp under air-tight conditions, as described above.

Analytical procedures

The fermented pulp (10 g) was mixed with 30 ml of distilled water and centrifuged to obtain the supernatant. Lactic acid and ethanol were analyzed using a high-performance liquid chromatograph (model SCL-10A; Shimadzu Co., Kyoto, Japan) equipped with a Shodex RS pack KC-811 column (Showa Denko Co., Tokyo, Japan) and a refraction index detector (model RID-10A; Shimadzu Co.). Phosphoric acid (0.1%) was used in the mobile phase at a flow rate of 1.0 ml min^{-1}. Soluble sugars were analyzed by thin-layer chromatography using a solvent system of *n*-butanol:*i*-propanol:water:acetate (7:5:4:2). Spots were visualized on the plate by spraying with anisaldehyde–sulfuric acid reagent [17].

Enzyme assays

The crude enzyme was extracted from the fermented pulp by an extraction buffer (50 mM malic acid, 50 mM NaCl, 100 mM NaOH, 2 mM $CaCl_2$, pH 5.8) with efficiency over 95% and used for the assays of five enzymes (Table 1).

The reaction mixture for the polygalacturonase assay contained 2.0 mg of sodium polygalacturonic acid (Sigma) in 0.2 ml of a 50 mM acetate buffer (pH 5.5) and 0.05 ml of the crude enzyme. After incubation of the mixture at 37 °C for 30 min, the reaction was stopped by the addition of a Nelson reagent [13] and the reducing sugars produced were determined as galacturonic acid.

Xylanase, cellulase, α-amylase, and glucoamylase were assayed using tablets in the commercial kits supplied by Megazyme International Ireland Co., (Wicklow, Republic of Ireland). The principles of the assays are based on the determination of the colored product released from each chromogenic substrate (Table 1). The activities of xylanase and cellulase were measured by adding one tablet to 0.5 ml of the crude enzyme. After incubation for 30 min at 37 °C, 10.0 ml of 2% Tris buffer were added in order to stop the reaction. The amount of released azurine was determined at 590 nm after obtaining the supernatants by centrifugation. α-Amylase and glucoamylase were assayed by the methods described by Oda et al. [14]. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of product under the above conditions.

A commercial pectinase was derived from *Rhizopus* sp. (Sigma Chemical Co.).

Reproducibility

All data are shown as the average values and standard deviations from three independent experiments, unless otherwise stated.

Results and discussion

Strains NBRC 4707 and NRRL 395 were grown in potato pulp to compare the metabolite concentrations and enzyme activities (Fig. 1). Strain NBRC 4707 produced both lactic acid and ethanol more efficiently than strain NRRL 395, while the activities of most enzymes depicted similar curves. Glucoamylase activity showed a slight increase after 3 days; and xylanase activity was not detected. Cellulase and α-amylase activities were maintained at a low level through the culture period tested. A significant difference was found in polygalacturonase among the enzymes assayed. The activity of strain NBRC 4707 was twice that of strain NRRL 395.

Figure 2 shows the formation of soluble saccharides in the potato pulp fermented by strain NBRC 4707. Poly- and oligosaccharides were rapidly produced during fermentation, as judged from blots near the origin of the chromatogram. Glucose from starch degradation was not detected, probably because of its rapid consumption by the growing cells. Galacturonic acid appeared as a major monosaccharide, in accordance with the gradual increase of polygalacturonase activity (the lowermost graph in Fig. 1). Polygalacturonase seemed to be related to the degradation of the structural components of potato pulp.

When a commercial preparation of pectinase was added to potato pulp inoculated with fungal spores and incubated for 7 days, both strains effectively produced larger amounts of lactic acid and ethanol (Table 2). These data indicate that the fermentation of potato pulp depends on the degradation of pectic substances in NRRL 395 and NBRC 4707.

Starch-manufacturing plants in Japan produce potato pulp for about 3 months in a year. The major

Table 1 List of enzymes assayed. For details of substartes, see Materials and methods

Enzyme	Substrates in the reaction mixture	Product determined
Polygalacturonase	Polygalacturonic acid	Galacturonic acid as reducing sugar
Xylanase	Xylazyme AXT tablet (azurine-cross-linked wheat arabinoxylan)	Azurine
Cellulase	Cellazyme C tablet (azurine-cross-linked HE-cellulose)	Azurine
α-Amylase	Blocked *p*-nitrophenyl maltopeptaoside, glucoamylase, α-glucosidase	*p*-Nitrophenol
Glucoamylase	*p*-Ntrophenyl-*β*-maltoside, *β*-glucosidase	*p*-Nitrophenol

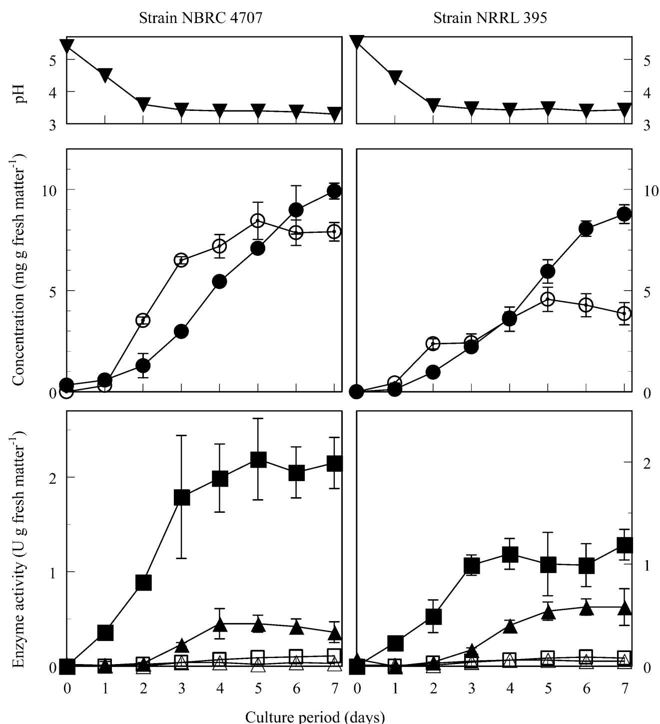

Fig. 1 Production of metabolites and enzymes in potato pulp fermented by *Rhizopus oryzae* strains NBRC 4707 and NRRL 395. Each fermented pulp was extracted and used for metabolite analysis and enzyme assays as described in the Materials and methods. *U* Units of enzyme activity, ▼ pH, ○ lactic acid, ● ethanol, ■ polygalacturonase, □ cellulase, △ α-amylase, ▲ glucoamylase

components of fresh pulp are starch, cellulose, hemicellulose, and pectic substances [11]. However, the content of each substance is not precisely equal when compared with that in other samples. Among them, the pectic substances, which are complex heteropolysaccharides with a backbone of galacturonic acid, are located in the middle lamella and primary cell walls of higher plants, including the potato tuber [21]. A crucial and physiological role of pectic substances is adhesion between the cells, allowing the transfer of water among them [21]. Heating plant tissues separates the internal cells by melting the pectic substances; and individual cells adhere to each other again as they cool. From the present experiments, the pectinolytic enzyme secreted from *R. oryzae* may cause intracellular starch to become susceptible to amylases when the cell surface is uncovered.

Pectic substances are chemically classified into protopectins, pectinic acids, pectins, and pectic acids and degraded by three types of enzymes: de-esterifiying enzymes, depolymerizing enzymes, and protopectinases [1, 8]. In the present experiments, a qualitative assay indicated that pectic substances are degraded by both exo- and endo-depolymerizing activities (Fig. 2). It is still unknown whether cleavage of the α-1,4-linkage in the polygalaturonic acid backbone is catalyzed by simple hydrolysis (hydrolase) or by a *trans*-eliminative hydrolysis (lyase).

Efforts were successively made to improve the fermentation rate of potato pulp by using a mutant overproducing polygalacturonase. After UV irradiation, fungal spores of NBRC 4707 were spread on agar plates containing pectin as a carbon source and incubated for 3 days. However, none of the colonies showed a larger stained zone (using ruthenium red [12]) than the parental strain.

A method to enhance polygalacturonase activity was then developed for a starter culture. The cells were grown aerobically on agricultural by-products, such as sugarbeet pulp, orange peel, apple peel, and wheat bran, in addition to potato pulp as a control culture (Table 3). The enzyme activities varied in response to the materials used for the solid-state culture. As compared with potato pulp, polygalacturonase was elevated 3-fold by apple peel and orange peel, both of which abound in pectic substances. These activities exceeded the value (1.2 units g^{-1} fresh matter) that was effective to increase

Table 2 Effects of commercial pectinase on the lactic fermentation by *Rhizopus oryzae*. Sterilized potato pulp was fermented by each strain at 25 °C for 7 days with and without the addition of commercial pectinase at the final activity of 1.2 units g^{-1} fresh matter

Strain	Addition of pectinase	Concentrations (mg g^{-1}fresh matter)	
		Lactic acid	Ethanol
NBRC 4707	–	7.2 ± 0.9	5.4 ± 0.2
	+	12.9 ± 1.0	10.2 ± 0.8
NRRL 395	–	3.6 ± 0.6	3.6 ± 0.6
	+	6.4 ± 0.3	8.5 ± 0.7

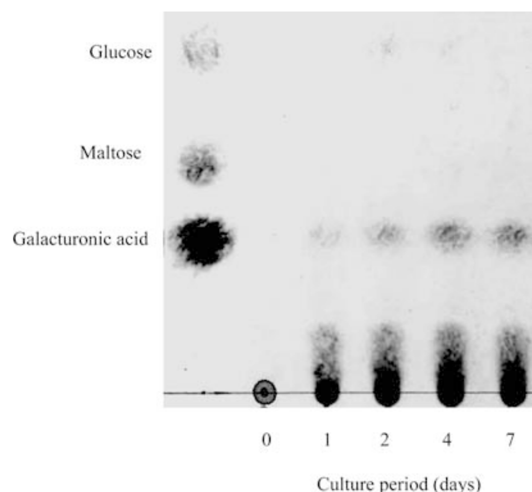

Fig. 2 Formation of soluble saccharides in potato pulp fermented by *R. oryzae* NBRC 4707. One microliter of the extract used for metabolite analysis in Fig. 1 was applied to thin-layer chromatography and developed as described in the Materials and methods. There were 10 μg of each of the authentic sugar references (*shown at the left*)

Table 3 Enzyme activities in the starter culture of *R. oryzae* NBRC 4707. Each starter culture was grown at 25 °C for 3 days after inoculation of fungal spores and used for the preparation of crude enzyme

Starter material	Activity (units g^{-1} fresh matter)			
	Polygalacturonase	Cellulase	α-Amylase	Glucoamylase
Potato pulp	2.1 ± 0.5	0.4 ± 0.1	< 0.1	< 0.1
Sugarbeet pulp	0.2 ± 0.2	3.1 ± 0.3	0.2	0.3
Orange peel	5.8 ± 1.9	0.7 ± 0.1	0.1	0.1
Apple peel	5.7 ± 2.3	0.3	< 0.1	< 0.1
Wheat bran	< 0.1	1.8 ± 0.7	0.2	1.8 ± 0.4

Table 4 Concentrations of metabolites in potato pulp fermented by starter cultures of *R. oryzae* NBRC 4707. Each starter culture (10 g) grown for 3 days was mixed with 90 g of sterilized potato pulp and fermented for a further 7 days at 25 °C

Starter material	Concentrations (mg g^{-1}fresh matter)	
	Lactic acid	Ethanol
Potato pulp	8.0 ± 0.3	7.2 ± 2.0
Sugarbeet pulp	7.7 ± 0.9	9.3 ± 1.3
Orange peel	13.7 ± 3.7	7.7 ± 2.5
Apple peel	14.6 ± 3.1	10.0 ± 2.4
Wheat bran	5.2 ± 1.8	7.9 ± 1.6

metabolite formation (Table 2). Table 4 summarizes the results of fermentation conducted using these starter cultures. The starter cultures composed of apple orange peel and apple peel stimulated the fermentation of potato pulp and increased the quantity of lactic acid and ethanol to higher levels, as we expected.

R. nigricans consumed soluble sugars liberated from lemon peel by the action of pectinolytic enzymes [7]. Blandino et al. [2] reported that whole wheat flour acted as a good nutrient source for *R. stolonifier* to produce extracellular polygalacturonases. Pectinase production by an *Aspergillus* species has been found to be higher in a solid-state culture than in a submerged process [3, 4] and *R. oryzae* NBRC 5318 was grown on wheat bran supplemented with pectin for the production of polygalacturonase [10]. The present study confirms previous observations that a solid-state culture using fruit peel can induce fungal production of the pectinolytic enzyme and further highlights its practical application for improving the lactic acid fermentation of potato pulp by *R. oryzae*.

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