Aerobic Dissimilation of L-Rhamnose and the Production of L-Rhamnonic Acid and 1,2-Propanediol by Yeasts

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Aerobic dissimilation of L-rhamnose by various genera and species of yeasts was examined. Of 63 strains tested, 12 utilized L-rhamnose fairly well, and 7 strains, *Debaryomyces klöckeri*, *Pichia pseudopolymorpha*, *P. rhodanensis*, *P. robertsii*, *P. wickerhamii*, *Candida polymorpha* and *Torulopsis famata*, produced an appreciable amount of L-rhamnonic acid and/or 1,2-propanediol. These two main products were isolated in pure forms from the fermented broth of *C. polymorpha* and identified. *Candida polymorpha* produced L-rhamnonic acid and 1,2-propanediol from L-rhamnose in the presence of CaCO_3 at good yield of 38% of sugar consumed.

In the previous papers,^{1~3)} it was observed that the pattern of polyalcohol production in aerobic dissimilation of D-galactose, D-xylose, L-arabinose or D-ribose by various genera and species of yeasts was quite different from that with D-glucose. For instance, *Candida polymorpha* produced D-arabinitol and erythritol from D-glucose, while the yeast produced galactitol from D-galactose, xylitol from D-xylose, L-arabinitol from L-arabinose and ribitol from D-ribose at good yield of 30~40% of sugar consumed.^{1~3)} *Pichia miso* dissimilated D-xylose with the production of xylitol and two heptitols, though the yeast produced D-arabinitol, glycerol and erythritol from D-glucose.^{1,4)}

In the course of our studies on aerobic dissimilation of various sugars by yeasts, the authors observed that some yeasts utilized a methyl pentose, L-rhamnose, fairly well and gave two polyhydric products. This paper

describes the pattern of L-rhamnose dissimilation by yeasts and the isolation and identification of the two major products, L-rhamnonic acid and 1,2-propanediol.

It has so far been reported that *Bacterium rhamnosifermentans* produced propylene glycol⁵⁾ and *Pasteurella pestis* produced lactaldehyde⁶⁾ from L-rhamnose but little has been known with yeasts.

MATERIALS AND METHODS

Yeast strains. The following 63 yeast strains were examined: *Saccharomyces acidifaciens*, *Sacch. acidifaciens* var. *halomembranis*, *Sacch. rouxii*, *Sacch. rouxii* var. *halomembranis*, *Debaryomyces fabryi*, *Deb. gruetzii*, *Deb. hansenii*, *Deb. hudei*, *Deb. klöckeri*, *Deb. mandschuricus*, *Deb. matruchoti*, *Deb. membranaefaciens*, *Deb. membranaefaciens* var. *Zingiberi*, *Deb. miso* var. 1, *Deb. sake*, *Deb. sake* Forma 1, *Deb. tyrocola*, *Pichia alcoholophila*, *P. belgica*, *P. farinosa*, *P. hyalosporea*, *P. mandschurica*, *P. membranaefaciens*, *P. miso*, *P. polymorpha*, *P. pseudopolymorpha*, *P. quercibus*, *P. rhodanensis*, *P. robertsii*, *P. scoolyti*, *P. wickerhamii*, *Hansenula anomala*, *H. anomala* var. *productiva*, *H. saturnus*, *H. schneeggii*, *H. suaveolens*, *H. subpelliculosa*, *Candida albicans*, *C. arborea*, *C. guilliermondii*, *C. krusei*, *C. parapsilosis*, *C. polymorpha*, *C.*

1) H. Onishi, *Bull. Agr. Chem. Soc. Japan*, **24**, 131 (1960).

2) H. Onishi and T. Suzuki, *J. Bacteriol.*, **95**, 1745 (1968).

3) H. Onishi and T. Suzuki, *Agr. Biol. Chem.*, **30**, 1139 (1966).

4) H. Onishi and M. B. Perry, *Can. J. Microbiol.*, **11**, 929 (1965).

5) A. J. Kluyver and C. H. Schnellen, *Enzymologia*, **4**, 7 (1937).

6) E. Englesberg, *J. Bacteriol.*, **74**, 8 (1957).

tropicalis, *C. utilis*, *Torulopsis famata*, *T. halophila*, *T. nodaensis*, *T. sake*, *T. versatilis*, *Torula rubra*, *Kloeckera africana*, *Kl. apiculata*, *Trichosporon behrendii*, *Trich. cutaneum*, *Trigonopsis variabilis*, *Cryptococcus albidus*, *Cry. laurentii*, *Cry. neoformans*, *Rhodotorula rosa*, *Monilia formosa*, *M. sitophila* and *M. vini*. Among them, typical L-rhamnose-assimilating yeasts,^{7,8)} *Pichia pseudopolymorpha*, *P. rhodanensis*, *P. robertsii*, *P. scolyti* and *P. wickerhamii* were obtained through the courtesy of Dr J. Boidin Professor of Lyon University, France.

Medium and fermentation condition. Aliquots (0.5 ml) of freshly cultured yeast suspension were inoculated into large test tubes each containing 8 ml of a sterilized medium of the following composition: L-rhamnose monohydrate (Nutritional Biochemicals Corp.) 10%, D-glucose 0.1%, KH_2PO_4 0.1%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05%, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.01%, NaCl 0.01%, casamino acids (Difco) 0.4%, yeast extract (Difco) 0.1%, with or without the addition of CaCO_3 (0.3 g/tube). The tubes were shaken on a reciprocal

FIG. 1. Isolation and Purification Procedure of the Fermented Products from the Fermented Broth.

7) J. Boidin, M. C. Pignal, Y. Lehodey, A. Vey and F. Abadie, *Bull. de la Soc. Mycol. de France*, **80**, 396 (1964).

8) J. Boidin, M. C. Pignal and M. Besson, *ibid.*, **81**, 566 (1965).

shaker operating at 300 rpm with an amplitude of 2 cm at 30°C for 3~7 days.

Analytical methods. After removal of the yeast cells by filtration, clear fermentation liquid was analyzed according to the method of Neish⁹⁾ for reducing sugar and total polyalcohol. 1,2-Propanediol and glycerol were separately determined after fractionating by the celite column chromatography of Neish.¹⁰⁾ Aldonic acid was assayed by the method of Hestrin¹¹⁾ as the lactone after heating the fermented broth in the presence of 1 N HCl in a boiling water

bath for 15 min. In this case, pure L-rhamnonic (1,4)-lactone was used as a standard and the results were corrected for L-rhamnose interference. Paper-chromatography was performed by the ascending method on Whatman No. 1 filterpaper using *n*-propanol-ethylacetate-water (7:1:2 v/v)¹²⁾ as the mobile phase. The reducing sugar was detected with aniline hydrogen phthalate reagent¹³⁾ and polyhydric compound with KIO₄-tetra base by the method of Yoda.¹⁴⁾ The lactone was detected with NH₂OH-FeCl₃ by the method of Abdel-Akher and Smith.¹⁵⁾

TABLE I. AEROBIC DISSIMILATION OF L-RHAMNOSE AND PRODUCTION OF POLYHYDRIC COMPOUNDS BY VARIOUS YEASTS

Strains	Presence of CaCO ₃	Fermentation period (days)	% of L-rhamnose consumed	% of total polyalcohol produced (as 1,2-propanediol)	Yield of total polyalcohol on L-rhamnose consumed (as 1,2-propanediol)	Polyhydric compounds of fermented broth*			
						<i>Rf</i> = 0.78	<i>Rf</i> = 0.54	<i>Rf</i> = 0.41	<i>Rf</i> = 0.31
<i>Debaryomyces huederoi</i>	{ -	7	3.90	0.29	7.4%	±	≡ (≡)†	—	—
	{ +	7	6.02	0.65	10.8	+	≡ (≡)	—	—
<i>Deb. klöckeri</i>	{ -	7	4.48	0.92	20.5	±	≡ (≡)	—	+
	{ +	7	8.33	1.63	19.6	≡	≡ (≡)	—	≡
<i>Pichia pseudopolymorpha</i>	{ -	7	10.23	0.92	9.0	+	+(+)	—	—
	{ +	3	8.59	1.60	18.6	≡	≡ (≡)	—	±
<i>P. rhodanensis</i>	{ -	7	6.28	0.05	0.8	—	≡ (≡)	—	+
	{ +	7	10.84	0.52	4.8	+	— (—)	—	≡
<i>P. robertsii</i>	{ -	7	7.69	0.17	2.2	—	≡ (≡)	+	+
	{ +	7	9.94	0.45	4.5	+	+(+)	—	≡
<i>P. scolyti</i>	{ -	7	7.61	0.07	0.9	—	≡ (≡)	—	—
	{ +	7	6.10	0.05	0.8	—	≡ (≡)	—	—
<i>P. wickerhamii</i>	{ -	7	9.15	0.82	9.0	+	≡ (≡)	—	—
	{ +	3	9.65	2.56	26.5	≡	— (—)	—	±
<i>Hansenula saturnas</i>	{ -	7	8.11	0.11	1.4	—	≡ (≡)	+	+
	{ +	7	6.23	0.10	1.6	—	≡ (≡)	—	≡
<i>Candida polymorpha</i>	{ -	7	5.77	1.82	31.5	≡	≡ (≡)	—	±
	{ +	4	9.35	1.83	19.6	≡	≡ (≡)	—	≡
<i>Torulopsis famata</i>	{ -	7	4.00	0.29	7.3	±	≡ (≡)	—	+
	{ +	7	9.41	0.25	2.7	±	≡ (≡)	—	≡
<i>Cryptococcus laurentii</i>	{ -	7	6.03	0.10	1.7	—	≡ (≡)	—	—
	{ +	7	9.04	0.09	1.0	—	≡ (≡)	—	+
<i>Cry. neoformans</i>	{ -	7	7.95	0.06	0.8	—	≡ (≡)	—	—
	{ +	7	8.16	0.06	0.7	—	≡ (≡)	—	+

* Polyhydric compounds with *Rf* of 0.78, 0.54 and 0.31 are assumed to be 1,2-propanediol, L-rhamnitol-like compound and L-rhamnose, and L-rhamnoic acid, respectively. — = no detectable spot, ≡ = intense spot.

† Parenthesis shows the spot of residual L-rhamnose.

9) A. C. Neish, "Analytical Methods for Bacterial Fermentations," 2nd Rev. National Research Council of Canada, N. R. C. No. 2952 (1952).

10) A. C. Neish, *Can. J. Res.*, **B28**, 535 (1950).

11) S. Hestrin, *J. Biol. Chem.*, **180**, 249 (1949).

12) J. Cerbulis, *Anal. Chem.*, **27**, 1400 (1955).

13) S. M. Partridge, *Nature*, **164**, 443 (1949).

14) A. Yoda, *J. Chem. Soc. Japan*, **73**, 18 (1952).

15) M. Abdel-Akher and F. Smith, *J. Am. Chem. Soc.*, **73**, 5859 (1951).

Isolation of fermentation products. Isolation and purification procedures of L-rhamnonic acid and 1,2-propanediol from the fermented broth are illustrated in Fig. 1.

Reagent. L-Rhamnonic (1,4)-lactone was prepared from L-rhamnose by the method of Jackson and Hudson.¹⁶⁾

RESULTS

I) Aerobic dissimilation of L-rhamnose and production of polyhydric compounds by various genera and species of yeasts

Of 63 yeast strains tested, 12 could utilize L-rhamnose fairly well and the analytical data of the fermented broth were shown in Table I. The paperchromatogram showed two main polyhydric products of *Rf* 0.78 and 0.31. The spot of *Rf* 0.54 corresponds to those of L-rhamnose and unknown product. *Candida polymorpha*, *Debaryomyces klöckeri*, *Pichia pseudopolymorpha* and *P. wickerhamii* produced a large amount of polyhydric compound of *Rf* value of 0.78, and *Candida polymorpha*, *Debaryomyces klöckeri*, *Hansenula saturnas*, *Pichia rhodanensis*, *P. robertsii* and *Torulopsis famata* produced much polyhydric compound of *Rf* value of 0.31. It was generally observed that the production of polyhydric compounds was enhanced by adding CaCO_3 to the medium. On the other hand, *Cryptococcus laurentii*, *Cry. neoformans* and *Pichia scolyti* produced only a trace amount of polyhydric compound in spite of their good utilization of L-rhamnose.

As the fermented broth in the presence of CaCO_3 of *Hansenula saturnas* containing appreciable amount of the only one product of *Rf* 0.31 gave negligible value in polyalcohol determination, the product did not seem to be polyalcohol and was presumed to be aldonic acid by the characteristic mauve color reaction after treating water extract of the spot with 1N HCl. Therefore, the other product of *Rf* 0.78 was supposed to be poly-

TABLE II. ANALYTICAL RESULTS OF THE FERMENTED BROTH OF *Candida polymorpha* AND *Pichia robertsii*

	<i>Candida polymorpha</i>		<i>Pichia robertsii</i>	
Presence of CaCO_3	—	+	—	+
Fermentation period (days)	7	6	7	6
% of L-rhamnose consumed	5.44	10.84	7.62	8.82
% of total polyalcohol produced (as 1,2-propanediol)	1.80	1.56	0.16	0.33
Yield of total polyalcohol on L-rhamnose consumed (as 1,2-propanediol)	33.1%	14.4%	2.1%	3.7%
% of pure 1,2-propanediol produced	1.55	1.19	0.09	0.33
% of total adonic acid produced (as L-rhamnonic acid)	0.25	2.96	0.70	2.37
Yield of total aldonic acid on L-rhamnose consumed (as L-rhamnonic acid)	4.6%	27.3%	9.2%	26.9%

alcohol, and the *Rf* value agreed with that of 1,2-propanediol and was different from that of glycerol.

Next, more detailed analyses of two major products were carried out using the fermented broth of *Candida polymorpha* and *Pichia robertsii*, two excellent producers of polyhydric compounds, and the results were shown in Table II. As 76~86% of the total polyalcohol in the fermented broth of *Candida polymorpha* were recovered as pure 1,2-propanediol by celite column chromatography, 1,2-propanediol would be a major polyalcohol produced. Any polyalcohol could not be detected in the glycerol fraction after celite chromatography. The result shows that *Candida polymorpha* produced L-rhamnonic acid and 1,2-propanediol from L-rhamnose in the presence of CaCO_3 at good yield of 38% of sugar consumed.

II) Isolation and identification of L-rhamnonic acid and 1,2-propanediol

The isolation of two major polyhydric products from the fermented broth of *Candida*

16) E. L. Jackson and C. S. Hudson, *ibid.*, **52**, 1270 (1930).

polymorpha was carried out according to the procedure shown in Fig. 1.

a) **Identification of L-rhammonic acid.** In aqueous solution, 4.86 g of crude calcium salt, obtained from 224 ml of the fermented broth containing 2.70% of aldonic acid (as L-rhammonic acid), were treated with Amberlite CG-120 type II (H^+) and the resulted free acid was lactonized under the acidic condition with HCl. A yield of 0.69 g of crystalline lactone was obtained by three times recrystallization.

FIG. 2. Infrared Spectra of Isolated and Synthetic L-Rhammonic (1,4)-lactone. (Nujol mull)

-----: Isolated crystal.

—: Synthetic L-rhammonic (1,4)-lactone.

stallization of the lactone from acetone. The crystals showed mp 150~151°C, which was undepressed to admix with synthetic L-rhammonic (1,4)-lactone, and $[\alpha]_D^{20} -37.9^\circ$ ($c=4.33$, water, 20 min after solution). *Anal.* Found: C, 44.51; H, 6.22%. Calcd. for $C_6H_{10}O_5$: C, 44.45; H, 6.22%. The infrared spectrum of the crystals coincided with that of synthetic L-rhammonic (1,4)-lactone (Fig. 2).

b) **Identification of 1,2-propanediol.** Poly-alcohol fraction of R_f 0.78 was purified by paperchromatography on Whatman No. 3 MM filterpaper and the cut section was extracted with distilled water and the extract was evaporated *in vacuo* to dryness. The phenylurethan derivative was prepared from completely dried sample with phenylisocyanate. The crystals, obtained by crystallization from benzene and petroleum benzine, were found to be mp 149~150°C, undepressed on admixture with the phenylurethan of authentic 1,2-propanediol by the same preparation pro-

FIG. 3. Infrared Spectra of Isolated and Synthetic Phenylurethan of 1,2-Propanediol. (Nujol mull)

-----: Crystalline derivative of the sample.

—: Synthetic phenylurethan of 1,2-propanediol.

cedure. *Anal.* Found: C, 65.01; H, 5.56; N, 9.25%. Calcd. for $C_{17}H_{18}O_4N_2$: C, 64.96; H, 5.77; N, 8.91%. The infrared spectrum of the crystals was superimposable with that of synthetic 1,2-propanediol phenylurethan (Fig. 3).

DISCUSSION

The fermentation product of L-rhamnose by yeasts has not so far been reported. In bacteria, Kluyver and Schnellen reported that *Bacterium rhamnosifermentans* produced propylene glycol from L-rhamnose,⁵¹ and Englesberg found that *Pasteurella pestis* accumulated a small amount of lactaldehyde from L-rhamnose.⁶¹ It was also suggested that the L-rhamnose molecule was split into glyceraldehyde and lactaldehyde by *Bacterium rhamnosifermentans*, the latter being then reduced to propylene glycol.⁶¹ The metabolic pathway which leads to form dihydroxyacetone phosphate and L-lactaldehyde from L-rhamnose has been well established with the enzyme system of *Escherichia coli*.¹⁷⁻²¹ The present studies showed that yeasts produced 1,2-propanediol as the main metabolic product of

17) G. Tecce and M. DiGirolamo, *Giorn. Microbiol.*, **1**, 286 (1956).

18) D.M. Wilson and S. Aji, *J. Bacteriol.*, **73**, 410, 415 (1957).

19) Y. Takagi and H. Sawada, *Biochim. Biophys. Acta*, **92**, 10, 18 (1964).

20) H. Sawada and Y. Takagi, *ibid.*, **92**, 26 (1964).

21) T. H. Chiu and D. S. Feingold, *ibid.*, **92**, 489 (1964).

L-rhamnose.

As for the biological formation of L-rhamnonic acid from L-rhamnose, only the formation by buckwheat seedlings and other seedlings has been found.²²⁾ This paper would be the first demonstration of microbial formation of L-rhamnonic acid.

Candida polymorpha and *Pichia robertsii* produced a large amount of L-rhamnonic acid, an oxidation product of L-rhamnose, in the presence of CaCO_3 , but L-rhamnitrol corresponding to a reduction product of L-rhamnose was not found, instead 1,2-propanediol being produced. The authors have found that *Pichia quercibus* produced both xylitol, a reduction product, and D-xylonic acid, an oxidation product of D-xylose, by aerobic dissimilation of D-xylose at rather good yield.²³⁾

However, the small spot of R_f 0.54 was still detected on the paperchromatogram after removing residual L-rhamnose from the fermented broth of *Candida polymorpha*, and careful examination is now going on to identify this L-rhamnitrol-like product.

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22) G. A. Barber and W. Z. Hassid, *Bull. Res. Council Israel* Sect. A. **11**, 249 (1963).

23) T. Suzuki and H. Onishi, *Agr. Biol. Chem.*, **31**, 1233 (1967).