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Immunological Relationships of Alcohol Dehydrogenases in the Genus *Rhizopus*

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Summary

The production of ethanol and acids were compared as well as intracellular activities of alcohol dehydrogenase and their antigenicities of four strains of *Rhizopus* species. The amounts of ethanol produced were in the following order: *R. javanicus* ATCC 44037 > *R. delemar* IFO 4746 > *R. javanicus* IFO 5441 > *R. oryzae* IFO 5384, while the amounts of acids produced were in the following order: *R. oryzae* IFO 5384 > *R. javanicus* ATCC 44037 > *R. javanicus* IFO 5441 > *R. delemar* IFO 4746.

The specific activity of alcohol dehydrogenase was proportional to the amount of ethanol produced by these strains and also the alcohol dehydrogenases of tested strains were antigenically similar. Therefore we assume that the variable amounts of ethanol produced by these strains are the results of the regulation by the degree of alcohol dehydrogenase activity in cells rather than the influence of alcohol dehydrogenase which has altered molecular properties.

Introduction

Amount of ethanol and organic acids production by various species of *Rhizopus* vary with the type of strain and/or species [3]. The formation of these products might be controlled by intracellular levels of enzymes which are responsible for the formation of these products. No information is available whether these regulation of fermented products was resulted from the qualitative difference or from the quantitative one of related enzymes of the fermented products.

GASSER and GASSER [2] investigated the immunological relationships among lactic dehydrogenases in the genena *Lactobacillus* and *Leuconostoc* and showed that the enzymes of some strains had a different antigenicity even though they belong to the same genus.

Alcohol dehydrogenases (alcohol: NAD oxidoreductase, EC 1.1.1.1) (ADH), which play an essential role in alcohol fermentation, show the differences with respect to their numbers in cells, location, molecular weight, subunit number, and substrate specificity depending on the organism that produces the enzyme [1,5,6,8].

We previously described two ADHs of *R. javanicus* ATCC 44037 and assumed that they possess different properties in living cells, although they showed the same antigenicity [10].

The present study compares the alcohol and acid production of four strains of *Rhizopus* species and the immunological properties of their ADHs.

Materials and Methods

1. *Source of cultures and their growth conditions*

The strain of *R. javanicus* ATCC 44037 used in this investigation was the same as previously described [10] and the strains of *R. delemar* IFO 4746, *R. javanicus* IFO 5441, and *R. oryzae* IFO 5384 were obtained from the Institute for Fermentation (Osaka, Japan).

Growth medium containing glucose, 10% ; asparagine, 0.25% ; $MgSO_4.7H_2O$, 0.3% ; and

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KH_2PO_4, 0.1% (pH 6.5) was used. Cells were grown at 30°C for 1 week on a potato-dextrose agar (Difco Laboratories) slant and washed off with 4 ml of sterile water. A spore suspension was inoculated at 1% (V/V) level into 150 ml of medium in 500 ml shaken flasks (amplitude 7 cm, 120 revolutions/min) and incubated at 30°C for appropriate periods.

2. *Preparation of cell-free extract*

Mycelia were collected by centrifugation at 10,000 g for 25 min, and washed twice with 0.05 M phosphate buffer (pH 6.2). Washed cells were suspended in 0.05 M Tris-HCl buffer (pH 7.8) containing 10 mM mercaptoethanol, and the cell suspension was homogenized with a Braun homogenizer (glass beads, 0.45 to 0.50 mm in diameter). The homogenized material was filtered through sterilized gauge, the filtrate was centrifuged at 10,000 g for 25 min, and the supernatant fluid was used as the enzyme source. All procedures were carried out at the temperature of 0—5°C.

3. *Analysis*

Measurements of ethanol content, ADH activity and acidity were performed previously described[9]. Protein concentration was determined by the method of LOWRY et al.[4], using bovine serum albumin as a standard.

4. *Immunodiffusion*

A rabbit antiserum prepared against the ADH of *R. javanicus* ATCC 44037 was the same as previously described[10]. Two-dimensional immunodiffusion[7] was carried out on petri plates at 4°C for at least 7 days, using 1% agar containing 0.9% NaCl. The ADHs of yeast and horse liver used were obtained from Sigma Chemical Co. (St. Louis, Mo.).

Results and Discussion

1. *Comparison of the amounts of ethanol and acids formation and ADH activities*

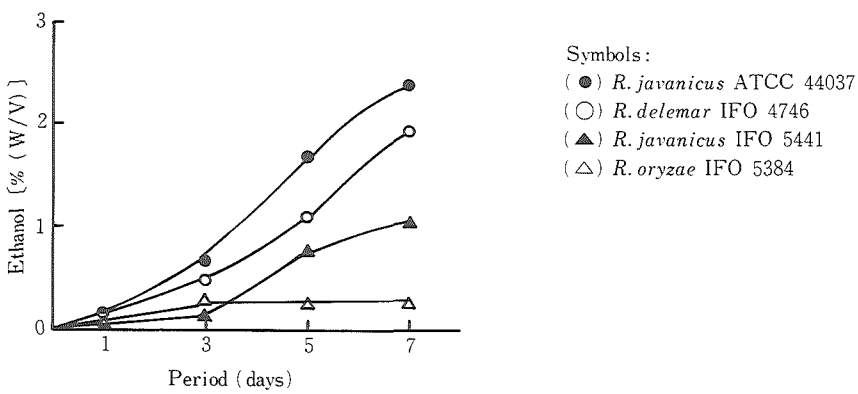

Fig. 1 Time course of ethanol production by *Rhizopus* species.

Fig. 1 illustrates the time course of ethanol production of each strain. *R. javanicus* ATCC 44037 produced the highest amount of ethanol among the tested strains, whereas *R. oryzae* IFO 5384 produced the lowest amount of ethanol, yield of ethanol was approximately ten times lesser than that of *R. javanicus* ATCC 44037. On the other hand, acid production of R. *oryzae* IFO 5384 culture (incubated for 96 h) was higher than that of other strains (Table 1). According to INUI et al.[3], *R. javanicus* and *R. delemar* produce higher quantities of ethanol and the produced organic acids are mainly fumaric acid, whereas *R. oryzae* produces smaller amounts of ethanol and mainly produces lactic acid. Therefore the former two species are used for ethanol production using Amylo method from sweet potatoes.

Table. 1 Specific activities of NAD-dependent ADH and titratable acidities of the cultures of tested strains grown for 96 hours

Strain	Sp. act*	TA**
R. javanicus ATCC 44037	0.56	5.46
R. belemar IFO 4746	0.54	4.25
R. javanicus IFO 5441	0.09	4.82
R. oryzae IFO 5384	0.04	8.51

* Specific activity. Given in μmoles of NADH formed per min per mg protein in the reaction mixture.

** Titratable acidity. Given in mls of 0.1N NaOH/10 g medium.

The ADH activities in the cell-free extract of each strain are shown in Table 1. The highest specific activity of ADH was observed in the cell-free extract of *R. javanicus* ATCC 44037, which produced the highest amount of ethanol as compared to other tested strains. *R. oryzae* IFO 5384, which produced ethanol little, showed the lowest specific activity of the enzyme. The amounts of ethanol produced by the tested strains were in proportional to the degree of specific activities of ADH.

2. *Immunodiffusion*

Since the differences in the amounts of ethanol and acid formation as well as ADH activity were observed among the tested strains, we compared the immunological properties of the ADHs in order to clarify the interspecific structural relationships among them. A rabbit antiserum prepared against the ADH of *R. javanicus* ATCC 44037 was the same as previously described[10]. Fig. 2 shows the immunodiffusion which was carried out according to the method of OUCHTERLONY[7].

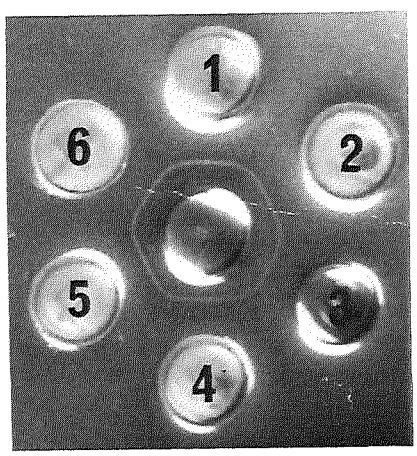

Fig. 2 immumodifiusion of ADH of *Rhizopus* species with rabbit antiserum prepared against purified ADH of *R. javanicus* ATCC 44037. Center well contains antiserum. The sample (outer) wells contain extracss of the following strains:
(1) *R. delemar* IFO 4746
(2) *R. javanicus* IFO 5441
(3) *R. oryzae* IFO 5384
(4)—(6) *R. javanicus* ATCC 44037
The protein content of each extract was prepared approximately 2 mg/ml.

Cell-free extract of all strains reacted with the antiserum and formed a single-fused precipitin band. Therefore, the ADHs of four strains were assumed to possess the same antigenic sites. The same reaction was carried out using ADHs of yeast and horse liver (Sigma Chemical Co.), but no precipitin line was observed (Table 2).

Table. 2 Results of cross-reaction experiments with serum prepared against ADH of *R. javanicus* ATCC 44037

Organism	Cross-reactivity
R. javanicus ATCC 44037	+
R. javanicus IFO 5441	+
R. delemar IFO 4746	+
R. oryzae IFO 5384	+
Yeast	—
Horse liver	—

The present results suggest that the difference in the ethanol production of these strains is due to the degree of ADH activity in cells rather than the difference in the molecular properties of the enzymes.

We previously reported the multiple forms of ADHs of *R. javanicus* ATCC 44037 and found the existence of two main components which are separable by electrophoresis or ion exchange chromatography[10]. Although the antigenic sites of these two enzymes were the same, these are thought to have two different roles in living cells; that is, one of them is a fermentative enzyme which is responsible for ethanol formation and the other one is an oxidative enzyme involved in ethanol metabolism. Since the relationship of the occurrence of ADH multiple forms and ethanol and organic acids productions are observed in *R. javanicus* ATCC 44037, it may be interesting to study the multiple forms of ADHs and production profiles of ethanol and organic acids in various *Rhizopus* species.

Acknowledgement

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References

1) B-HEICK N. and H.M.C.HECIH: Biochim. Biophys. Acta **212**, 13−20 (1970)
2) GASSER F. and C.GASSER: J. Bacteriol. **106**, 113−125 (1971)
3) INUI T., Y.TAKEDA and H.IIZUKA: J. Gen. Appl. Microbiol. **11**, Suppl. 81−91 (1965)
4) LOWRY O.H., N. J. ROSEBROUGH, A. L. FARR and R. J.RANDALL: J. Biol. Chem. **193**, 265−275 (1951)
5) SCHIMPFESSEL L.: Biochim. Biophys. Acta **151**, 317−329 (1968)
6) SCHWARZ M., J.O'DONNELL and W.SOFER: Arch. Biochem. Biophys. **194**, 365−378 (1979)
7) STOLLAR D. and L.LEVINE: Methods Enzymol. **6**, 848−854 (1963)
8) WILLS C. and J.PHELPS: Arch. Biochem. Biophys. **167**, 627−637 (1975)
9) YONEYA T. and Y.SATO: Appl. Environ. Microbiol. **37**, 1073−1078 (1979)
10) YONEYA T. and Y.SATO: Appl. Environ. Microbiol. **40**, 967−969 (1980)

Rhizopus 属菌株間のアルコール脱水素酵素の活性および抗原性の比較

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Rhizopus 属の3菌種4株を用いてそのエタノール，酸生成の比較を行ない，更にそれらの株の菌体内アルコール脱水素酵素の活性，抗原特異性を検討した．エタノール生産量では，*R. javanicus* ATCC 44037＞*R. delemar* IFO 4746＞*R. javanicus* IFO 5441＞*R. oryzae* IFO 5384の順であった．一方，生酸量では，*R. oryzae* IFO 5384＞*R. javanicus* ATCC 44037＞*R. javanicus* IFO 5441＞*R. delemar* IFO 4746の順であった．エタノール生成に必須であるアルコール脱水素酵素の菌体内における比活性の大小はエタノール生産量と同様の関係が認められた．以上のことと供試した4株のアルコール脱水素酵素の抗原性に差異が見られないことから，Rhizopus 属のエタノール生産量の菌株，菌種間の差異はアルコール脱水素酵素本体の差異によるものではなく細胞内での活性の大小により調節されるものと推論した．

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正　誤　表 (Errata)

頁 (Page)	行 (Line)	誤 (Erratum)	正 (Correct)
4	Fig. 2	Berry size (mm)	Berry size (mm)
21	2	胸深(r =0.484), 尻長(r =0.528),	胸深(r =0.484**), 尻長(r =0.528**),
40	17	プロ・ピオン酸	プロピオン酸
43	6	Tabl 5	Table 5
46	29—30	抑制遅延	抑制・遅延
51	Fig. 2	extracss	extracts