STUDIES ON THE MICROORGANISM PRODUC-
ING ISOPROPANOL FROM ACETONE
PART 2. ENZYME INVESTIGATION ON THE OXIDA-
TION-REDUCTION OF LACTOBACILLUS BREVIS
VAR. HOFUENSIS

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INTRODUCTION

In a previous communication (1) it was demonstrated that the newly isolated organism, *Lactobacillus brevis* var. *hofuensis* has the specific activity for the oxidation-reduction between acetone and isopropanol, and that secondary alcohols and polyols are easily dehydrogenated by this microorganism. This paper deals with the enzymatic investigation on isopropanol oxidation and acetone reduction.

The dehydrogenation of isopropanol has been considered as the side reaction of alcohol dehydrogenase. BARRON (2) indicated that the isopropanol specificity of yeast alcohol dehydrogenase is 6.6% compared with ethanol, while THEORELL (3) proved no activity of liver alcohol dehydrogenase for isopropanol. The specificity for secondary alcohols of this microorganism is supposed to be the interaction of the enzyme which is different from alcohol dehydrogenase. In order to distinguish the difference of the enzyme system from alcohol dehydrogenase the oxidation-reduction between ethanol and acetaldehyde is subjected to the experiment as the reference reaction.

MATERIALS AND METHODS

L. brevis var. *hofuensis* was cultured under stationary condition on the media described as follows. Intact cell for the purpose of enzymatical experiments are obtained from the broth by centrifugation and washing. Dried cell was prepared by GUNSALUS's method (4). Crude cell-free extract was by sonic oscillation (Raytheon magnetic constriction oscillator 9 KC) and SiO₂-grinding.

Unit for oxidation of isopropanol and ethanol was measured by 2,6-dichlorophenol-indophenolas described in the previous paper (1). Oxidation-reduction potential was determined by comparing the intensity of discoloring with various dyes such as tetrazolium, janus green and neutral red.

O₂-uptake: by Warburg manometer measurement. Catalase was obtained by the courtesy of Dr. NAKAMURA of Tokyo Institute of Technology and used for O₂-uptake experiments.

Enzymatical estimations about oxidation-reduction of pyridine nucleotide were carried out spectrophotometrically by the method of RACKER (5) and VENNESLAND (6).

Cytochrome c reductase was proved by SLATER'S (7) method of TPNH oxidation. Oxidation-reduction of cytochrome c and flavoprotein were examined by modification of the method by THEORELL (8). Mammalian cytochrome c was prepared by the method of KEILIN and HARTREE (9).

DPN and TPN were prepared from pig liver by the method of LEPAGE (10) and HORECKER (11). TPNH was prepared enzymatically by SLATER'S (7) method. Concentration of cytochrome and TPNH were calculated by use of extinction coefficient of SLATER (7) and HORECKER (12).

RESULTS

MEDIUM. Concerning the composition of medium for propagation experiments were given in the previous report (1). In order to obtain the cells which are highly active in catalyzing oxidation-reduction between isopropanol and acetone ingredients were examined. After these experiments the following two media were selected.

Medium 1: Glucose 1%, Polypeptone 1%, NaCl 0.1%, K₂HPO₄ 0.05%
KH₂PO₄ 0.05%, MgSO₄·7H₂O 0.02%, MnSO₄·H₂O 0.001%.

Medium 2: Sucrose 1%, Polypeptone 1%, Meat extract 1%, NaCl 0.1%
K₂HPO₄ 0.05%, KH₂PO₄ 0.05%, MgSO₄·7H₂O 0.02%,
MnSO₄·H₂O 0.001%, pH 6.5, adjusted by NaOH.

Fig. 1. Development of enzyme activity in two different media.

○—○ unit for isopropanol.
△---△ unit for ethanol.
×---× wet cell.

Developments of enzyme and cell were illustrated in Fig. 1. Medium 1 is used as inoculum and medium 2 as accumulation medium.

Reduction of Acetone.

As previously described acetone reduction by the intact cell of this microorganism was promoted by addition of glucose. The test was applied to dried cell, crude cell-free extract and residue of cell-free extract and the similar results have been obtained as indicated in Fig. 2; however, the loss of activity by sonic oscillation was observed. As seen in intact cell glucose also plays

Fig. 2. Reduction of acetone by cell and cell-free extract.

Intact cell: 0.052 mg wet per ml.

Dried cell: 0.01 mg dried matter per ml.

Crude cell-free extract: 6.2 mg protein per ml.

Sonic residual: 12.5 mg per ml.

Acetone: 0.02 M.

Temp. 37C.

a role in the reduction of acetone.

Oxidation-Reduction Potential.

It has been known that the dehydrogenation system exhibits the oxidation-reduction potential in response to the tendency of electron transport. WURMSER (13) measured the potentials of yeast concerning isopropanol/acetone and ethanol/acetaldehyde systems. As previously mentioned *L. brevis* var.

Table 1. Reaction of oxidation-reduction dyes about various dehydrogenation systems of *L. brevis* var. *hofuensis*.

dye E_0' (dye)	2.6 Dichloro phenol- indo- phenol	Methylene blue	Neo tetra- zolum	Janus green	Neutral red	Methyl viologen
Substrate	+0.217	+0.01	-0.07	-0.08	-0.33	-0.441
Ethanol	+	+	+	+	-	-
Propanol	++	++	+	++	++	-
Isopropanol	++	++	+++	++	+++	-
Butanol	+	+	-	+	-	-
Isobutanol	++	+	+	+	-	-
Secondary butanol	++++	+++	++++	+++	++	-
Glycerol	+	+	++	+	++	-
1.4 Butylene glycol	+	++	++	+	-	-
1.3 Butylene glycol	++	+++	++	++	+++	-
2.3 Butylene glycol	++++	++	++++	+++	-	-

hofuensis has many dehydrogenation systems and they are supposed to show the potentials respectively.

Oxidation-reduction dyes which change their colors by reduction were employed in this experiment. The results are summarized in Table 1. Symbols indicate the relative intensity of discoloring. From the result the potential of isopropanol/acetone system of this organism is considered below -0.3 volt, while ethanol/acetaldehyde is more positive, since neutral red is not reduced by ethanol. These results were also confirmed by crude cell-free extract.

O₂-Uptake.

The intact cell and the crude cell-free extract of *L. brevis* var. *hofuensis* absorb molecular oxygen in the presence of substrate such as isopropanol and ethanol. These were measured by means of Warburg's manometer. As illustrated in Fig. 3 the endogenous respiration by cell and cell-free extract

Fig. 3. O_2 -Uptake by cell and cell-free extract.

Isopropanol 0.1 M.	0.1 ml.
Ethanol 0.5 M.	0.1 ml.
Cell-free extract 2.5 mg protein per ml.	0.2 ml.
Cell 200 mg (wet) per ml.	0.2 ml.
Phosphate buffer 0.2 M. (pH 7.3)	1.0 ml.
Total volume	2.5 ml.
Catalase	10^{-8} M.

which are used in this experiment is negligible and that CO_2 formation is not recognized. These oxygen uptakes were inhibited by KCN partially; furthermore, the product in the absence of methylene blue is supposed water and not peroxide from the results by catalase experiment. This indicates the presence of oxidation system such as cytochromes. Table 2 shows the

Table 2. Effect of buffer for O₂-uptake of isopropanol by *L. brevis* var. *hofuensis*.

Buffer (pH)	O ₂ -uptake (μ l/min/dried cell mg)
M/ 15 phosphate (pH 6.2)	49
M/ 15 phosphate (pH 6.8)	50
M/ 15 phosphate (pH 8.0)	44
M/ 30 phosphate (pH 6.8)	44
M/ 60 phosphate (pH 6.8)	45
M/120 phosphate (pH 6.8)	45
Citrate (pH 6.2)	45
Acetate (pH 5.6)	28
M/ 25 glycylglycine (pH 8.7)	36
Veronal (pH 8.6)	42

Isopropanol 0.02 M.
Cell (dried) 7 mg in ml.

effect of buffers and phosphate buffer (pH 7.0) which appears suitable for the O₂-uptake experiment.

As it has been considered that the dye reduction and oxygen uptake are similar reactions except the potential of dyes, the units were compared as to the same cell preparation by way of dye reduction of 2.6 dichlorophenol-indophenol and O₂-uptake with methylene blue. These results are summarised in Table 3 and the units by 2.6 dichlorophenol-indophenol are found to exceed O₂-uptake; furthermore, it is concluded that on the whole the velocity for isopropanol is higher than in the case of ethanol.

Table 3. Comparison of unit by dye-reduction and O₂-uptake

pH	Substrate	2.6 Dichlorophenol-indophenol		O ₂ -uptake	
		Isopropanol	Ethanol	Isopropanol	Ethanol
6.8		670	255	498	283
7.0		580	237	467	206
7.5		414	180	460	192
8.5		292	163	340	136

Phosphate buffer M/30.

Protein (crude cell-free extract by SiO₂ grinding) 45 mg/ml.

Cofactor.

Reduction of cofactor by electron transport were verified by many workers and used for the stoichiometric treatment. RACKER (14) reported the reduction of DPN by yeast alcohol dehydrogenase. DEMOSS (15), on the other hand,

confirmed the bacterial alcohol dehydrogenase linked to DPN and TPN. Furthermore, BONNICHSEN (16) investigated the DPN linked alcohol dehydrogenase of liver, while STAFFORD (6) reported the germ alcohol dehydrogenase which reduced DPN and TPN. The pathway of electron from isopropanol and ethanol in this microorganism is also supposed to link with cofactor considering their oxidation-reduction potentials. The reductions of DPN and TPN were measured spectrophotometrically by RACKER's (17) test system with crude cell-free extract. These results are illustrated in Fig. 4 and the

Fig. 4. Oxidation of substrate and reduction of pyridine nucleotide by crude cell-free extract.

Substrate	0.1 M	0.1 ml.
TPN	2.5×10^{-3} M	0.1 ml.
DPN	3.5×10^{-3} M	0.1 ml.
Protein (2.18 mg per ml)	0.05 ml (Ethanol, TPN and Ethanol, DPN, TPN)	0.5 ml. (others)
Tris (hydroxymethyl)-aminomethane buffer	0.2 M (pH 8.6)	1.0 ml.
Total volume		3.0 ml.

linkage with DPN and TPN are proved. The TPN-linkage is more specific than DPN in this organism.

The substrate specificity seems important for the enzymatic clues of this microorganism. Reduced pyridine nucleotide is formed by isopropanol in high degree, while the rate of formation is slow compared with the case of ethanol. These were witnessed in both cases of DPN and TPN. Effect of cofactor concentration in two substrates is also investigated and the different situation observed as shown in Fig. 5. It has been supposed that in case of isopropanol a side reaction such as secondary oxidation of reduced pyridine nucleotide should be considered. Such information

Glycolysis and Acetone Reduction.

As previously mentioned glucose plays a role in the reduction of acetone in *L. brevis* var. *hofuensis*. Concerning the glycolysis two pathways, namely Embden-Meyerhof-Parnas and monophosphate shunt, are considered. It seems significant that the aldolase reaction of this organism is weak compared with that of yeast. Another experiments indicated the inhibition of glycolysis and acetone reduction by monoiodoacetic acid, while fluoride had the stimulating effect as shown in Table 4. The inhibition by monoiodoacetic acid described above is supposed to be the blockage of triosephosphate dehydrogenation and decarboxylation via gluconic acid. DAVIES (18) reported the inhibition of monoiodoacetic acid against decarboxylation of acetoacetate. Moreover, addition

Fig. 5. Effect of TPN concentration on the reduction.

Substrate	0.1 M	0.1 ml.
TPN	1150 μ M	0.2 ml. (1); 0.1 ml. (1/2); 0.04 ml. (1/5) 0.02 ml. (1/10)
Protein (2.18 mg. per ml.)		0.15 ml.
Tris (hydroxymethyl) aminomethane buffer 0.2 M (pH 8.2)		1.0 ml.
Total volume		3.0 ml.

Table 4. Effect of the inhibitor about glycolysis and reduction of acetone.

Ingredient (M)				Acetone reduction (%)			Sugar consume (%)		
Glucose	Acetone	CH ₂ ICOOH	NaF	2 hrs	4 hrs	6 hrs	2 hrs	4 hrs	6 hrs
	0.004			0	0	0			
0.0025	0.004			0	5.3	40.4	11.2		29.7
0.0025							3.6	13.6	17.6
0.0025	0.004		0.02	10.9	71.6	98.8	9.9	34.2	45.2
0.0025	0.004		0.01	0	11.2	45.8	0.2	11.2	21.2
0.0025	0.004	0.01		0	0	0	0	0	0
0.0025	0.004	0.005		0	0	2.3	0	0.7	6.9
0.0025	0.004	0.0025		0	0	3.9	0		7.7

Cell (dried): 0.01 mg. per ml.

of fluoride seemed to inhibit enolase and finally result in the increase of decarboxylation of gluconic acid. Thus Embden-Meyerhof-Parnas and monophosphate shunt are considered. Besides, it is possible to suppose that the reduction of acetone and acetaldehyde is incorporated with the latter glycolysis. These speculations are consistent with TPN-specificity of this microorganism. From this point of view oxidation-reduction between glucose-6-phosphate or 6-phosphogluconate and acetone or acetaldehyde has been experimented. TPN was used as the cofactor for these reactions. The results were illustrated in Fig. 6 and the linkage of oxidation of glucose-6-phosphate and 6-phos-

Fig. 6. Oxidation-reduction of TPN by various substrates.

Acetone, Methylethylketone	1.0 M	0.03 ml.
Acetaldehyde	0.5 M	0.03 ml.
Glucose-6-phosphate, 6-Phosphogluconate	0.02 M	0.05 ml.
TPN	0.002 M	0.5 ml.
Mg Cl ₂	0.1 M	0.2 ml.
Protein (25mg per ml.)		0.05 ml.
Glycylglycine buffer 0.04 M (pH 7.5)		1.0 ml.
Total volume		2.8 ml.

phogluconate; reduction of acetaldehyde, acetone and methylethylketone was proved. They are incorporated through the oxidation-reduction of TPN. Comparing these two reducing systems, i.e. isopropanol/aceton and ethanol/acetaldehyde, the velocity of the reduction of acetaldehyde is much higher than acetone reduction. It has been found that the velocities in reduction and oxidation (O₂-uptake) bear opposite relationship concerning these two systems. The discrepancy will be discussed again in the following paragraph.

Effect of inhibitors such as iodoacetic acid, p-chloromercuribenzoate and others was examined as to the oxidation of TPNH by acetone and acetaldehyde. Iodoacetic acid had no inhibition, while p-chloromercuribenzoate showed the marked inhibition in oxidation of TPNH by acetaldehyde. Nevertheless the oxidation by acetone has not been inhibited by p-chloromercuri-

benzoate. From these experiments these two systems are considered to depend upon different enzyme systems (Fig. 7).

Comparison of Velocity

About two Systems.

As previously described, the activity of acetone reduction is damaged considerably by sonic extraction and the rate of TPNH formation by isopropanol is less than that of ethanol. Extraction of the enzyme is not to be increased by prolongation of vibration. In this experiment SiO_2 -grinding was attempted in order to raise the yield of the enzyme. After washing the wet cell was ground with sterilized SiO_2 and extracted in phosphate buffer. The yield was promoted about two fold as compared with sonicated extract. Furthermore, what is remarkable is that the rate of TPNH formation by isopropanol was increased by means of grinding. Velocity of three reactions

(O_2 -uptake, formation of TPNH and oxidation of TPNH) were compared by use of sonicated and ground extracts. These experiments were undertaken with the extracts of same cell preparation. The results are illustrated in Fig. 8. O_2 -uptake by isopropanol is higher than ethanol, while the velocity of acetaldehyde reduction by TPNH exceeds acetone reduction regardless of whatever extracting method employed. The TPNH formation is supposed to be an intermediary reaction and by selecting extracting method the extract of whichever high specificity can be obtained. From these experiments it has been concluded that the isopropanol/acetone system is more oxidative than ethanol/acetaldehyde.

Respiratory System.

L. brevis var. *hofuensis* belongs to facultative anaerobe and is supposed

Fig. 7. Inhibition of p-Chloromercuribenzoate against TPNH oxidation by acetaldehyde.

Acetaldehyde	0.5 M	0.03 ml.
6-Phosphogluconate	0.02 M	0.05 ml.
TPN	0.002 M	0.5 ml.
MgCl_2	0.1 M	0.2 ml.
p-Chloromercuribenzoate	0.05 M	0.1 ml.
Protein (26.8 mg per ml.)		0.05 ml.
Glycylglycine buffer 0.04 M (pH 7.5)		1.0 ml.
Total volume		2.8 ml.

Fig. 8. Oxidation-reduction by two substrate systems about sonicated and ground extracts.

- Oxidation-reduction between isopropanol and acetone.
- - - Oxidation-reduction between ethanol and acetaldehyde.
- · · · TPNH-formation by 6-phosphogluconate.

to have the oxidase system such as cytochrome from the O_2 -uptake experiment. SLATER has proved the cytochrome c reductase by the oxidation of DPNH prepared in the laboratory. In the present experiment the oxidation of TPNH was examined as shown in Fig. 9. From these results flavoprotein, like cytochrome reductase, is recognized as a member of the respiratory chain. Since HOGNESS (19) demonstrated the flavoprotein which does not link

Fig. 9 Oxidation of TPNH by crude cell-free extract.

TPNH 2.1×10^{-5} M
 Protein 1.5 mg per ml
 Phosphate buffer 0.125 M (pH 7.3)

with cytochrome c, a proper cytochrome except c is considered as the respiratory enzyme. In this experiment it was examined whether or not it links with mammalian cytochrome c by use of mammalian c preparation. As the result of this experiment the reduction of mammalian cytochrome c has not been observed, viz. the negative linkage was recognized. However, it is considered that *L. brevis* var. *hofuensis* has a proper terminal oxidase by the experiment of KCN inhibition and negative effect by catalase as shown in the previous paragraph.

Flavoprotein.

L. brevis var. *hofuensis* has the flavoprotein and seems to play a role in the oxidation-reduction between isopropanol and acetone. BRODIE, in the investigation of glycerol dehydrogenase of *E. coli*, described that the linkage of dehydrogenase and flavoprotein was tight, that the wave of reduced pyridine nucleotide (340 m μ) was not observed and that as the result of heat treatment of cell-free extract at 65 C 90 minutes the formation of reduced pyridine nucleotide was recognized. Heat treatment of crude cell-free extract has been attempted with this organism where inactivation was occurred at 42 C 10 minutes treatment. Nevertheless heat resistance of the enzyme is observed in the presence of substrate, i.e. after 52 C 10 minutes treatment with 1% isopropanol the yellow supernatant was obtained. After 0.9 saturation with ammonium sulfate and resuspend in the buffer the yellow solution exhibited a specific difference spectrum as shown Fig. 10. The peaks of spectrum are found at 380 m μ and 445 m μ . Furthermore the shift of substrate specificities was demonstrated comparing crude cell-free extract with flavoprotein solution as indicated in Table 5, the time required for the reduction of 2.6 dichlorophenol-indophenol is recorded therein.

The specificity of isopropanol, 1,2 propylene glycol and secondary butanol show increase incorporated with flavoprotein accumulation. These substances are included in secondary alcohol group. It has been supposed that the flavoprotein links with the enzyme which relates to dehydrogenation of secondary alcohols. The remarkable activity for secondary alcohols in this microorganism has been reported in the previous communication (1).

Fig. 10. Spectra of purified solution.

DISCUSSION

It has been demonstrated that the oxidation of isopropanol in *Lactobacillus brevis* var. *hofuensis* incorporated the reduction of TPN as cofactor. From the results of inhibitor experiments in glycolysis and acetone reduction,

Table 5. Shift of substrate specificity after the purification of isopropanol dehydrogenase.

Substrate	Enzyme solution	1	2
Ethanol		60'	120'
Propanol		4'50''	12'
Isopropanol		5'25''	5'
Hutanol		60'	120'
Isobutanol		8'10''	120'
Secondary butanol		4'30''	6'30''
1.2 propylene glycol		9'45''	7'6''
1.3 butylene glycol		11'	120'
1.4 butylene glycol		60'	120'
2.3 butylene glycol		4'20''	10'40''
Glycerol		60'	120'

Enzyme solution 1: crude cell-free extract (1/1).

Enzyme solution 2: purified solution (1/4).

dehydrogenation of glucose-6-phosphate or 6-phosphogluconate and reduction of acetone or acetaldehyde are considered to link by way of oxidation-reduction of TPN. Thus the biochemical aspect of oxidation-reduction between isopropanol and acetone peculiar to this microorganism has been revealed.

The activities of oxidation-reduction about these reaction are measured by several reactions, however, the activity by O_2 -uptake is more weak than other reactions, especially in ethanol/acetaldehyde system. It is supposed that these discrepancies depend upon the oxidative inclinations about these two systems.

The dehydrogenation of isopropanol is considered as a side reaction of yeast alcohol dehydrogenase by many workers. However, as previously mentioned, isopropanol is more oxidative than ethanol in this microorganism.

This property is also illustrated by the fact that the potential of isopropanol dehydrogenation of this organism is -0.3 volt, whereas in yeast -0.261 volt. Thus the potentials of isopropanol dehydrogenation in *L. brevis* var. *hofuensis* is more negative than yeast. Accordingly, isopropanol is easily oxidized in this microorganism, while ethanol is oxidized in yeast. Recently BARRON proved that the specificity for dehydrogenation of isopropanol in yeast is 6.6% as compared with ethanol specificity.

It has also been demonstrated that these two systems were established as having different reactions toward specific inhibitory effect by p-chloromercuribenzoate. The author has proposed the name of isopropanol dehydrogenase for the new enzyme of this microorganism. It is suggested that this enzyme also relates to the oxidation of secondary butanol and 1.2 propylene glycol.

From the enzymatical point of view the linkage of isopropanol dehydrogenase with flavoprotein is considered due to the shift of potential. KAPLAN (20) indicated the importance of cofactors linkage involving potential drop of systems. BONNICHSEN reported 0.06 volt of potential drop as the result of the linkage of liver alcohol dehydrogenase and DPN. Furthermore, GREEN (21) demonstrated the 0.25 volt drop by coupling xanthine oxidase and flavoprotein. Isopropanol specificity and yellow coloring of the organism were increased in parallel with purification. The peaks of difference spectrum of this flavoprotein were found at 380 $m\mu$ and 445 $m\mu$. The peaks of flavoprotein which were investigated by STRAUB (22) were 370 $m\mu$ and 450 $m\mu$, while VERNON reported that his reductase had peaks at 350 $m\mu$ and 440 $m\mu$. These fluctuations are considered to depend upon their enzyme sources. From these results in experiments of oxidation-reduction by isopropanol dehydrogenase and their relationship to the flavoprotein, the linkage of isopropanol dehydrogenase with flavoprotein is supposed so tight that the potential of isopropanol/acetone system are reduced and results in the oxidation of isopropanol.

SUMMARY

(1) The composition of medium for the enzymatical experiments of *Lactobacillus brevis* var. *hofuensis* was investigated and two media were determined as inoculum and accumulation media.

(2) Acetone was reduced by intact cell, cell-free extract and residue of extract and it was found that glucose played a role for the reduction of acetone by these preparations.

(3) Oxidation-reduction potentials of the dehydrogenation systems by alcohols and polyols were measured and the potential of isopropanol dehydrogenation was proved at -0.3 volt.

(4) Oxygen-uptake by isopropanol and ethanol was examined under various conditions and it was demonstrated that isopropanol exceeded ethanol.

(5) Cofactors about dehydrogenation of isopropanol and ethanol are DPN and TPN. DPN-specificity is less than 1/10 of TPN.

(6) The incorporation between glycolysis and reduction of substrate was elucidated. Oxidation of glucose-6-phosphate or 6-phosphogluconate and reduction of acetone, acetaldehyde or other substances were coupled by way of oxidation-reduction of TPN.

(7) Inhibitors experiments clarified the reductions of acetone and acetaldehyde as the different reactions.

(8) Comparing the oxidation-reduction of isopropanol/acetone with ethanol/acetaldehyde it was suggested that the former reaction is oxidative.

(9) This microorganism has the flavoprotein analogous to cytochrome c reductase and the specific terminal oxidase.

(10) The flavoprotein exhibits the peaks of different spectrum at 380

m μ and 445 m μ and links to the dehydrogenase of isopropanol and results in its oxidative activity.

(11) The name of isopropanol dehydrogenase was designated to the enzyme concerning oxidation-reduction between isopropanol and acetone.

(12) This enzyme relates to the dehydrogenation of secondary butanol and 1,2 propylene glycol.

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