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Substrate characterization of a NAD-dependent secondary alcohol dehydrogenase from *Rhodococcus* sp. GK1 (CIP 105335)☆

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Abstract

A NAD-dependent secondary alcohol dehydrogenase (SAD) has been extracted from cells of the sterol-degrading bacterium, *Rhodococcus* sp. GK1 (CIP 105335). The dehydrogenase was partially purified by means of ammonium sulfate fractionation (60% saturation) and filtration on a Sepharose CL-6B column. The obtained enzyme sample was active with aliphatic secondary alcohols, such as 2-hexanol, and as reductase with aliphatic monoketones and diketones, such as 2-hexanone and 2,3-hexanedione. A hydrophobic environment was required for catalysis: methyl on one side and either methyl, ethyl, propyl, butyl, pentyl or hexyl on the other side of the function being transformed. The K_m value for NAD or NADH with, respectively 2-propanol or acetone was around 1.60×10^{-4} M at pH 7.0 and 30 °C. The enzyme affinity ($1/K_m$) for the examined 2-alcohols and 2-ketones (three to eight C atoms) increased with increasing the chain length. Its activity with 2-octanone was somewhat higher than that with 3-octanone, reflecting a better enzyme affinity for a function positioned at C-2. The K_m values for the 2-alcohols (pH 7.0, 30 °C) ranged from 6.0×10^{-2} M for 2-propanol to 1.8×10^{-3} M for 2-octanol. Reciprocally, the K_m values for the 2-ketones ranged from 6.5×10^{-2} M for acetone to 2.1×10^{-3} M for 2-octanone. With 2-hexanol as the substrate, the optimal temperature was around 55 °C and the activation energy of the system was 9.49 kcal/mol. The SAD was specific for the (*S*)-(+)-stereoisomers of 2-butanol.

Keywords: *Rhodococcus*; NAD-dependent dehydrogenase; Substrate characterization; Secondary alcohols; Ketones

1. Introduction

Secondary alcohol dehydrogenases (SADs) occur in fermentation yeasts, as well as in bacteria [1–5]. These enzymes have been characterized as industrially useful catalysts for synthesis, due to their regioselectivity and enantiospecificity. Also, they are useful for quantitative metabolite determination (e.g. [6]).

A secondary alcohol dehydrogenase was unintentionally found in cells of *Rhodococcus* sp. GK1, a sterol-degrading actinomycetal bacterium, during a search for a dehydrogenase reducing the alcohol of 5-androsten-3β-ol-17-one, and a dehydrogenase reducing 3α-ol of cholic acid. The SAD was discovered, because the reaction medium for this search contained, in addition to NAD, 2-propanol used as steroid solvent. Data on substrate characterization of the enzyme, the pH effect on its activity, and its requirement for coenzyme were previously published

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[7]. Optimal pH was 7.0 or 8.5 for diacetyl reduction or 2-propanol oxidation, respectively. The SAD activity was strictly dependent on NAD(H) with no activity observed with NADP(H). However, the profile of its substrate specificity has not been completely understood. Therefore, further substrate characterization was performed for the enzyme, and the optimal temperature of its reaction was determined. The obtained data are described in the present paper.

2. Experimental

2.1. Chemicals

Except where stated otherwise, the products used were of analytical grade, obtained from commercial sources. The buffer used was a 0.05 M phosphate (a mixture of Na_2HPO_4 and KH_2PO_4) solution pH 7.05 ± 0.05.

2.2. Microorganism growth

Culture of *Rhodococcus* sp. GK1 (CIP 105335; CIP: Collection de l'Institut Pasteur, Paris) was performed in a mineral salt medium at 28–30 °C, under agitation [8]. The medium contained 3 g/l $(NH_4)_2SO_4$, as the nitrogen source, and 5 g/l of either cholic acid (NaOH-neutralized solution), phytosterols (mixture of sterols with main component β-sitosterol) or cholesterol, as the carbon source. Microbial growth was stopped at the maximum, in the first stage of the stationary phase. For enzyme extraction, cells were harvested by centrifugation, washed with cold phosphate buffer and resuspended in the buffer (0.6 g wet biomass for 10 ml). Weight of dry cells (dc) was estimated after desiccation of water-suspended cells at 100–105 °C [8]. Specific enzyme production (substrate diacetyl, pH 7.0, 30 °C) was around 80 U/g dc from cells grown on cholate, and around 90 U/g dc from cells grown on cholesterol or phytosterols.

2.3. Preparation of enzyme sample

The dehydrogenase was extracted in the phosphate buffer by disintegration of cells in the presence of glass beads [7]. Crude enzymatic extract was collected by 30 min centrifugation (27,000 × *g*, 5 °C) and fractionated with ammonium sulfate at 60% saturation. Protein precipitation was performed by stirring magnetically for 30 min at 0 °C, followed by maintenance at this temperature for 1 h. The precipitate was collected by centrifugation, dissolved in the buffer and then permeated throughout a Sepharose CL-6B column (Fig. 1), calibrated with protein markers (not shown). Elution volume of the enzyme was slightly higher than that of bovine serum albumin (65–70 kDa) yielding an apparent molecular mass of at least 70 kDa. The dehydrogenase in the final sample (specific activity with diacetyl = 0.784 U/mg, pH 7.0, 30 °C) was about six times purified with a recovery around 65%. This sample was used to characterize activity of the enzyme.

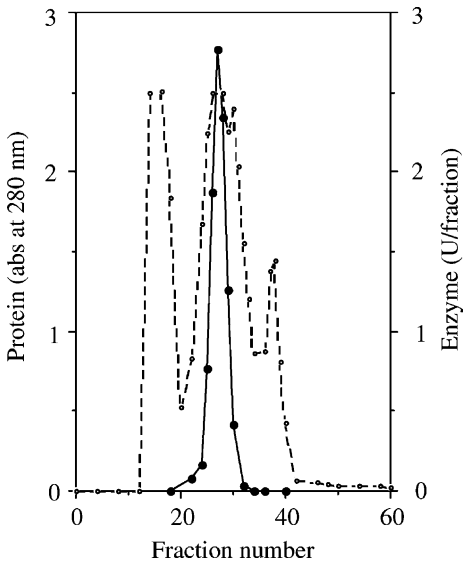

Fig. 1. Gel filtration of secondary alcohol dehydrogenase on a Sepharose CL-6B column (108 × 1.5 cm). Equilibration and elution of the column were with 0.05 M phosphate buffer pH 7.0 at 5 °C and at a flow rate of 18 ml/h. A sample of 12 ml (138 mg protein) was applied on the column and 5.4 ml fractions were collected. Protein profile (○). Enzyme activity (●) was measured during 10 min for 0.1 ml per assay (3.2 ml) with NAD and 2-propanol.

Electrophoresis of an aliquot (4.0 mg protein/2.5 ml + 0.1 ml glycerol) of the sample was carried out on a thin plate (18 cm × 16 cm) of polyacrylamide gel, under native conditions. The acrylamide concentration was 5 and 13% in the stocking and the separation gels, respectively. The electrode buffer was a solution of pH 8.3, containing 0.025 M tris(hydroxymethyl)

aminomethane and 0.19 M glycine. Protein migration was performed under 180 V tension, at 4 °C for 4 h. The dehydrogenase activity was visualized on the gel, due to formazan derivative formation (blue color) [9] using a reaction mixture that consisted of phosphate buffer (pH 7.0), 1.13 mM NAD, 31.00 mM 2-octanol, 0.61 mM nitrotetrazolium blue chloride and 0.25 mM phenazine methosulfate. One active band was observed under these conditions, reflecting the existence of only one dehydrogenase in the prepared sample. Electrophoresis of an aliquot (0.12 mg) of the sample, in the presence of SDS (denaturing conditions), resulted in the resolution of several bands (of them, six major bands), as visualized by staining with 0.25% Coomassie brilliant blue G-250 solution in acetic acid, methanol and water (1:4.5:4.5, v/v).

2.4. Enzyme assay and protein determination

SAD assay was performed at 30 °C and pH 7.0 (for either oxidation or reduction activity) in a final volume of 3.2 ml, following increase (alcohol oxidation) or decrease (ketone reduction) in absorbance of NADH at 340 nm ($\varepsilon_m = 5.78 \times 10^3$ (M cm)$^{-1}$). The reaction medium contained 150 μmol phosphate, 6 μmol NAD (or 0.6 μmol NADH) and substrate (as indicated in the Section 3) and an appropriate enzyme amount. Control assay was without substrate. A saturating NADH concentration (ca. 6 μmol per assay) could not be used, due to high absorbance of NADH at 340 nm and consequently unfeasibility of the assay. Reaction linearity was respected for all the efficient substrates.

One unit of the dehydrogenase was defined as the amount transforming 1 μmol substrate (one alcohol or ketone function) per min. Specific activity was expressed in units per mg protein. Protein amounts were determined by reading absorbance at 220 nm using an absorption coefficient of 11 ml/mg.

3. Results and discussion

3.1. Enzyme substrate specificity

3.1.1. Alcohol oxidation

The specific enzyme activities of the prepared sample with the primary alcohols ethanol, propanol, butanol or pentanol ranged from 0.010 to 0.020 U/mg. These activities were 9–18-fold lower than the specific dehydrogenase activity with 2-propanol, suggesting no specificity for primary alcohols. This is in confirmation to the previous observation concerned with ethanol and 1-propanol [7]. The dehydrogenase activities, obtained for the different secondary alcohols so far examined, are given in Table 1. No enzyme activity could be measured with glycerol or lactic acid. Meanwhile, the SAD was active with 2-propanol, 2-butanol, 2-pentanol, 2-hexanol, 2-heptanol and 2-octanol. Glycerol has two hydrophilic sides (two primary alcohols) adjacent to the secondary alcohol, the attack point of the enzyme, and lactic acid has one hydrophilic and charged side (the carboxyl group) adjacent to this point. The efficient substrates each contain two hydrophobic groups adjacent to both sides of the alcohol function being transformed: methyl on one side and either methyl, ethyl, propyl, butyl, pentyl or hexyl on the other side of the function. The structural difference between the inert and the active alcohols indicated a requirement for hydrophobic environment on both sides of the function being catalyzed. Further, the enzyme activity with cyclohexanol, relative to its activity with 2-hexanol, was remarkably weak. This suggested the enzyme is only specific for aliphatic secondary alcohols. Regarding the substrate chain length, the specific activities of the SAD with 2-alcohols of five to eight C atoms, such as 2-hexanol, were slightly higher than those with 2-alcohols of three to four C atoms, such as 2-propanol.

A weak enzyme activity was observed with 1,2-butanediol or 1,3-butanediol (Table 1), probably due to the presence of the 1-hydroxyl, conferring a hydrophilic environment on one side of the secondary alcohol.

The enzyme was examined for activity against enantiomers of 2-butanol and found to be 3.3-fold more active with the (*S*)-(+)-isomer than with the (*R*)-(−)-isomer. Considering the K_m value (around 1.0×10^{-2} M) of the enzyme for 2-butanol, its activity with the (*R*)-(−)-2-butanol can be accounted for by as little as 1% contamination of this product with (*S*)-(+)-2-butanol. These data suggested that SAD of the strain GK1 is specific for the (*S*)-(+)-isomers of 2-alcohols.

3.1.2. Ketone reduction

The reductase activities of the SAD with the examined ketones are given in Table 2. The enzyme

Table 1
Alcohols examined for the secondary alcohol dehydrogenase activity, at the given concentrations

Alcohols	Formula	Concentration (M)[a]	Specific activity (U/mg)
Glycerol	$CH_2OH–CHOH–CH_2OH$	0.371	Inactive
		0.093	Inactive
L-(+)-Lactate	$CH_3–CHOH–COO^-$	0.366	Inactive
		0.046	Inactive
2-Propanol	$CH_3–CHOH–CH_3$	0.816	0.182
		0.408	0.155
		0.204	0.140
2-Butanol	$CH_3–CH_2–CHOH–CH_3$	0.337	0.185
		0.169	0.160
2-Pentanol	$CH_3–(CH_2)_2–CHOH–CH_3$	0.229	0.220
		0.143	0.195
2-Hexanol	$CH_3–(CH_2)_3–CHOH–CH_3$	0.249	0.244
		0.124	0.274
		0.050	0.263
Cyclohexanol	$C_6H_{11}OH$	0.089	0.012
		0.059	0.022
2-Heptanol	$CH_3–(CH_2)_4–CHOH–CH_3$	0.044	0.276
		0.022	0.260
2-Octanol	$CH_3–(CH_2)_5–CHOH–CH_3$	0.038	0.275
		0.019	0.282
1,2-Butandiol	$CH_3–CH_2–CHOH–CH_2OH$	0.679	0.010
		0.340	0.010
1,3-Butanediol	$CH_3–CHOH–CH_2–CH_2OH$	0.340	0.033

The assay (3.2 ml final volume) contained 6 μmol NAD (1.88 mM). The data were from one set of assays but representative of at least two independent measurements.

[a] One saturating concentration was used at least of each efficient substrate, based on K_m value ($[C] \geq 10K_m$).

was inactive with either pyruvic acid, 2-oxobutyric acid, or 3-methyl-2-oxobutyric acid, since the activity values with these substrates were zero or close to the error limit (less than 0.01 U/mg). However, the SAD was active with acetone or 2-butanone. The inactivity of the enzyme with the keto-acids was probably due to the carboxyl group, which confers a negative and hydrophilic environment on one side of the ketone function. Similarly to the case of 2-alcohol oxidation, these observations indicated the requirement for hydrophobic environment on both sides of the ketone being reduced.

The specific activities of the SAD with the ketones of five to eight C atoms were slightly higher than those registered with the ketones containing three to four C atoms (Table 2). The enzyme affinity for these substrates increased with increasing the chain length. In that the highest affinity was for 2-octanone ($1/K_m = 476.2\,M^{-1}$) and the lowest was for acetone ($15.4\,M^{-1}$). Enzyme activity with 2-octanone was somewhat higher than with 3-octanone. Besides, the enzyme affinity for 2-octanone was higher than that for 3-octanone ($38.5\,M^{-1}$), suggesting a preference for the ketone function at the 2-position. This may be also true for the binding of secondary alcohols.

The SAD was found to be active with diketones 2,3-pentanedione, 2,3-hexanedione and 3,4-hexanedione (Table 2). With these substrates, the specific activities were higher than those with the corresponding 2-ketones. However, it has not been shown whether the enzyme reduces both ketones in the substrates or only a single ketone function. With regard to the enzyme's reduction activity with diacetyl ($CH_3–CO–CO–CH_3$) and acetoin ($CH_3–CO–COH–CH_3$), the first substrate

Table 2
Ketones examined for the reductase activity of the secondary alcohol dehydrogenase, at the given concentrations

Ketones	Formula	Concentration (M)[a]	Specific activity (U/mg)
Pyruvate	CH_3–CO–COO^-	0.053	Inactive
Acetone	CH_3–CO–CH_3	0.850 0.425 0.043	0.170 0.143 0.071
2-Butanone	CH_3–CH_2–CO–CH_3	0.348 0.035	0.191 0.091
2-Oxobutyrate	CH_3–CH_2–CO–COO^-	0.060 0.030	Inactive Inactive
3-Methyl 2-oxobutyrate	$(CH_3)_2$–CH–CO–COO^-	0.016	Inactive
2-Pentanone	CH_3–CH_2–CH_2–CO–CH_3	0.143	0.196
2-Hexanone	CH_3–$(CH_2)_2$–CH_2–CO–CH_3	0.126	0.264
2-Octanone	CH_3–$(CH_2)_4$–CH_2–CO–CH_3	0.060 0.040	0.273 0.265
3-Octanone	CH_3–$(CH_2)_4$–CO–CH_2–CH_3	0.080 0.060	0.210 0.147
2,3-Pentanedione	CH_3–CH_2–CO–CO–CH_3	0.143	0.690
2,4-Pentanedione	CH_3–CO–CH_2–CO–CH_3	0.303 0.151	Inactive Inactive
2,3-Hexanedione	CH_3–CH_2–CH_2CO–CO–CH_3	0.115	0.634
3,4-Hexanedione	CH_3–CH_2–CO–CO–CH_2–CH_3	0.122	0.727

The assay (3.2 ml final volume) contained 0.6 μmol NADH (0.19 mM). The data were from one set of assays but representative of at least two independent measurements.

[a] Except for 3-octanone, one saturating concentration was at least used of each efficient substrate, based on K_m value. Concentrations of the diketones were inferior or equal to these of the corresponding 2-ketones. This allowed enzyme activity comparison, since it was more active with the diketones than with the monoketones.

was 19-fold more efficient than the second one [7]. In addition, affinity for diacetyl was about 20-fold higher than that for acetoin. Besides, no activity was observed with acetoin as secondary alcohol substrate. It is likely that these catalytic criteria are valid for other diketones, such as 2,3-pentanedione and 2,3-hexanedione. The absence of enzyme activity with 2,4-pentanedione (Table 2), while 2,3-pentanedione and 2,3-hexanedione were efficient substrates, suggested the two ketones have to be contiguous for occurrence of catalysis.

3.1.3. Enzyme kinetics

The K_m value for NAD determined in the presence of a saturating concentration of 2-propanol (Fig. 2) was around 1.60×10^{-4} M. A similar K_m value was obtained for NADH in the presence of saturating acetone concentration.

The SAD obeyed the classical Michaelis–Menten hyperbolic saturation curve for both its oxidation and reduction activity with 2-alcohols or 2-ketones. The K_m value for 2-propanol or 2-octanol was determined to be 6.0×10^{-2} and 1.8×10^{-3} M, respectively. Those determined for acetone and 2-octanone were 6.5×10^{-2} and 2.1×10^{-3} M, respectively. The K_m values (not shown), determined for the other 2-alcohols and monoketones decreased with increasing the chain length, but kept between the above limits. It has been generally admitted that a decrease in K_m with increasing chain length demonstrates that hydrophobic interactions are important in substrate binding by alcohol dehydrogenases [4,10]. The K_m values for 2-propanol

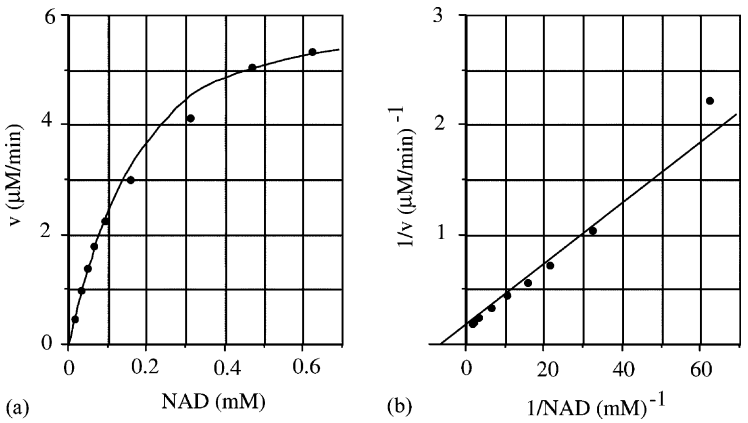

Fig. 2. Effect of NAD concentration on activity of secondary alcohol dehydrogenase: (a) Michaelis–Menten saturation curve; (b) Lineweaver–Burk plot. The enzyme was assayed at 30 °C and at pH 7.0 in a final volume of 3.2 ml containing 0.816 M 2-propanol.

and acetone, determined in the present work, were lower than those previously determined [7], which were 9.3×10^{-2} and 7.6×10^{-2} M, respectively. This discrepancy was due to the concentration of NAD(H) in the previous assay, which was 0.16 mM, lower than those used in the present work. The K_m values for diketone substrates remain to be determined.

3.1.4. Discussion

Yeasts and lactic acid bacteria produce acetoin during fermentation of sugar via diacetyl or α-acetolactic acid [1,2,11]. Acetoin is partially converted into 2,3-butanediol. Diacetyl reduction to acetoin and reduction of this last compound to 2,3-butanediol are catalyzed by specific reductases. Although the SAD of *Rhodococcus* sp. GK1 was appreciably active with diacetyl, its physiological role could not be the same as the diacetyl reductases of yeasts or lactic acid bacteria. This suggestion is probably true, because the strain GK1 is strictly aerobic and different from microorganisms that can ferment or grow in media of significantly low dissolved oxygen tension.

The SAD of *Rhodococcus* sp. GK1 seemed to resemble the secondary alcohol dehydrogenase of the alkane-degrading bacterium, *Rhodococcus erythropolis* ATCC 4277 [4] by several criteria: its dependence on NAD(H), its inactivity with glycerol and its negligible activity with primary alcohols. Optimal activity of the *R. erythropolis* enzyme was observed with linear 2-alcohols containing 6–11 C atoms. Activity of the present SAD with 2-alcohols containing five to eight C atoms was higher than with those containing three to four C atoms. However, secondary alcohols of longer chain length than 2-octanol, have to be examined with the present dehydrogenase. Regarding its stereospecificity, SAD of the strain GK1 was active with (*S*)-(+)-2-butanol, and seemed to be inactive with (*R*)-(−)-2-butanol. This stereospecificity was in accordance with that of the *R. erythropolis* dehydrogenase, as it was demonstrated with 2-octanol enantiomers. This enzyme was active as reductase with 2-ketones, such as 2-heptanone, but it was not examined with diketones. Regarding its physiological role, it has been postulated that it may be involved in oxidation of secondary alcohols, produced via an initial subterminal oxidation of alkanes. *Rhodococcus* sp. GK1, which is a potent degrador of steroids [8,12], has not yet been examined for its capability to grow on hydrocarbons. The known alcohol derivatives, which result from steroid catabolism, such as 2-hydroxy-2,4-hexadienoic acid, are structurally different from the efficient alcohols, tested with the dehydrogenase of the strain GK1. In these circumstances, it is difficult to postulate any physiological role for this enzyme.

3.2. Optimal temperature and stability of the dehydrogenase

The optimal temperature, determined for the SAD reaction with 2-hexanol, was around 55 °C (Fig. 3a). In the assay conditions, the activation energy of the reaction system was 9.49 kcal/mol (Fig. 3b). With

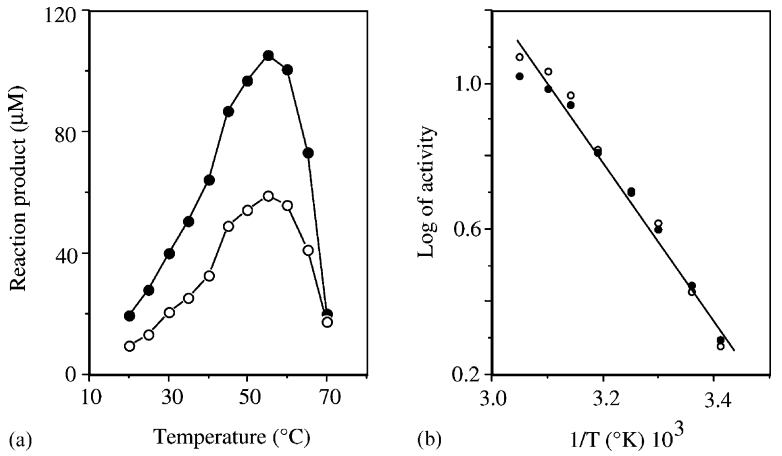

Fig. 3. Temperature effect on secondary alcohol dehydrogenase reaction. The enzyme was measured at pH 7.0, in a final volume of 3.2 ml containing 0.053 mg protein and 0.124 M 2-hexanol. (a) The reaction was followed for 5 min (○) and 10 min (●). (b) The data were derived from (a) (20–55 °C) for estimation of the activation energy (E_a) (Arrhenius equation: log activity $= -(E_a/2.3 \times R \times 1/T)$; enzyme activity was expressed in μM product/min).

regard to the SAD stability during storage, enzyme activity in the prepared sample (0.05 M, pH 7.0) was practically stable for 10 days at 4 °C and for 2 months frozen at −20 °C.

4. Conclusion

The NAD-dependent secondary alcohol dehydrogenase, characterized from cells of *Rhodococcus* sp. GK1 (CIP 105335), is active with aliphatic 2-alcohols and, as reductase, with aliphatic 2-ketones. It is probably specific for the (*S*)-(+)-enantiomers of 2-alcohols. For occurrence of catalysis, a hydrophobic environment is required on both sides of the function being transformed, e.g. methyl on one side and propyl or hexyl on the other side. The SAD is also active with diketones, such as 2,3-hexanedione. The dehydrogenase may be useful for quantitative measurement of methylketones, such as diacetyl in beer or wine, 2-heptanone and 2-octanone in cheese. The enzyme regioselectivity and stereospecificity have to be more fully studied. This and the determination of its physiological role constitute another study.

References

[1] P. Romano, G. Suzzi, Appl. Environ. Microbiol. 62 (1996) 309.
[2] W. Hummel, F. Boermann, M.-R. Kula, Biocatalysis 2 (1989) 293.
[3] A. Fauve, H. Veschambre, Biocatalysis 3 (1990) 95.
[4] B. Ludwig, A. Akundi, K. Kendall, Appl. Environ. Microbiol. 61 (1995) 3729.
[5] C. Heiss, M. Laivenieks, J.G. Zeikus, R.S. Philips, Biorg. Med. Chem. 9 (2001) 1659.
[6] S.G. Fadda, A. Lebert, R. Talon, J. Agric. Food Chem. 50 (2002) 2471.
[7] F. Krier, J. Kreit, J.-B. Millière, Lett. Appl. Microbiol. 26 (1998) 283.
[8] J. Kreit, G. Lefebvre, P. Germain, J. Biotechnol. 33 (1994) 271.
[9] D.S. Nagi, R.J. Stephens, J. Histochem. Cytochem. 25 (1977) 149.
[10] C.H. Barret, K.S. Dodgson, G.F. White, Biochem. Biophys. Acta 661 (1981) 74.
[11] M. Curic, B. Stuer-Lauridsen, P. Renault, D. Nilsson, Appl. Environ. Microbiol. 65 (1999) 1202.
[12] J. Kreit, P. Germain, in: F.J. Plou, J.L. Ibora, P.J. Halling, A. Ballesteros (Eds.), Progress in Biotechnology, vol. 15, Elsevier, Amsterdam, 1998, p. 639.