Production of D-malate by maleate hydratase from *Pseudomonas pseudoalcaligenes*

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The research described in this thesis was performed in collaboration with and financed by DSM Research, Geleen. Most of the investigations were carried out at the Division of Industrial Microbiology, Wageningen Agricultural University, Wageningen. From January 1993 until July 1993 the research was performed at the Bio-organic Chemistry Section, DSM Research, Geleen.

STELLINGEN

1. Bij afwezigheid van gegevens omtrent de evenwichtsligging van enzym-gekatalyseerde reacties is optimalisatie van de reactiecondities niet erg zinvol.

   Dit proefschrift.

2. De door Visscher en Taylor beschreven kwantitatieve omzetting van mercaptoappelzuur in fumaarzuur door celextracten van *Rhodopseudomonas* sp. is in tegenspraak met de ligging van het evenwicht van deze reactie en het niet ophopen van L-appelzuur door het aanwezige fumarase.

   Visscher, P.T. and B.F. Taylor. 1993. Organic thiols as organolithotrophic substrates for growth of phototrophic bacteria. *Appl. Environ. Microbiol.* **59**, 93-96.
   Vairavamurthy, A. and Mopper, K. 1987. Geochemical formation of organosulphur compounds (thiols) by addition of H_2S to sedimentary organic matter. *Nature* **329**, 623-625.

3. De door D'cunha *et al.* beschreven omzetting van de methylester van kaneelzuur door phenylalanine ammonia-lyase uit hele cellen van *Rhodotorula glutinis* is in tegenspraak met het reactiemechanisme van dit enzym en is waarschijnlijk het gevolg van de aanwezigheid van esterase-activiteit in dit micro-organisme.

   D'cunha, G.B., Satyanarayan, V. and P.M. Nair. 1994. Novel direct synthesis of L-phenylalanine methyl ester by using *Rhodotorula glutinis* phenylalanine ammonia-lyase in an organic-aqueous biphasic system. *Enzyme Microb. Technol.* **16**, 318-322.
   Hermes, J.D., Weiss, P.M. and Cleland, W.W. 1985. Use of Nitrogen-15 and deuterium isotope effects to determine the chemical mechanism of phenylalanine ammonia-lyase. *Biochemistry* **24**, 2959-2967.

4. De activatie-energie voor L-serine dehydratase wordt door Farais *et al.* ten onrechte gelijkgesteld aan de Gibbs-vrije-energie van activering ($\Delta G^{\neq}$).

   Farais, M.E., Strasser de Saad, A.M., Pesce de Ruiz Holgado, A.A. and G. Oliver. 1988. Characterization of a L-serine dehydratase activity from *Streptococcus faecalis*. *Le Lait* **68**, 177-188.

5. Om verzekerd te zijn van een continue stroom van publikaties dient het aanbeveling om de afbraak van, telkens een andere, gesubstitueerd polycyclische aromatische verbinding door, telkens een andere, wit-rot schimmel te bestuderen.

6. Een grote tijdsspanne tussen publikatie en acceptatie van wetenschappelijke artikelen remt de voortgang van de wetenschap.

7. Sportlieden die zich voor meer dan 100% kunnen inzetten, bezitten bovenmenselijke gaven.

8. De "kracht" van Omo Power ligt niet alleen in de marketing.

9. De media besteden vaak onevenredig veel aandacht aan hun interne aangelegenheden.

10. Geboortenbeperking is op de lange termijn de meest effectieve milieumaatregel.

11. Als je werk je hobby niet is, kun je beter geen A.I.O. worden.

Stellingen behorende bij het proefschrift "Production of D-malate by maleate hydratase from *Pseudomonas pseudoalcaligenes*".

Mariët J. van der Werf
Wageningen, 19 oktober 1994

VOORWOORD

Aan het eind van mijn AIO bestaan wil ik graag een aantal personen bedanken die bijgedragen hebben aan het tot stand komen van dit proefschrift.

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CONTENTS

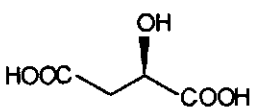
CHAPTER 1

GENERAL INTRODUCTION

D-MALATE: APPLICATIONS AND SYNTHESIS

D-Malate is an optically active α-hydroxy acid that only sporadically occurs in nature where it has been detected recently for the first time in the plant *Fumaria officinalis* (17). The opposite enantiomer of D-malate, L-malate, is considered the naturally occurring isomer (59). However, in the past the enantiomeric composition of the malate present in natural sources was usually not established. Apples, pears and grapes, however, only contain the L enantiomer of malate (14). The detection of D-malate in juices of these fruits is therefore a convenient method to determine if any DL-malic acid has been added to adulterate the juice (14). Since babies are not able to convert D-malic acid, which may lead to a watery motion, it is not allowed to add DL-malic acid to food for infants (23). Several properties of D-malate are listed in Table 1.

Table 1. Properties of D-malic acid

Other names		(*R*)-(+)-Malic acid, (*R*)-hydroxysuccinic acid, (*R*)-2-hydroxybutanedioic acid
Structural formula		
Molecular formula		$C_4H_6O_5$
Molecular weight		134.09 g·mol^{-1}
Melting point		98-102°C
Density		1.601 kg·m^{-3}
Dissociation constant	pKa_1	3.40
	pKa_2	5.11
$[\alpha]_D^{20}$		+27° (c=3, H_2O)
Safety		Harmful by inhalation and if swallowed
Taste (sodium salt)		Tasteless (24) (L enantiomer tastes salt)

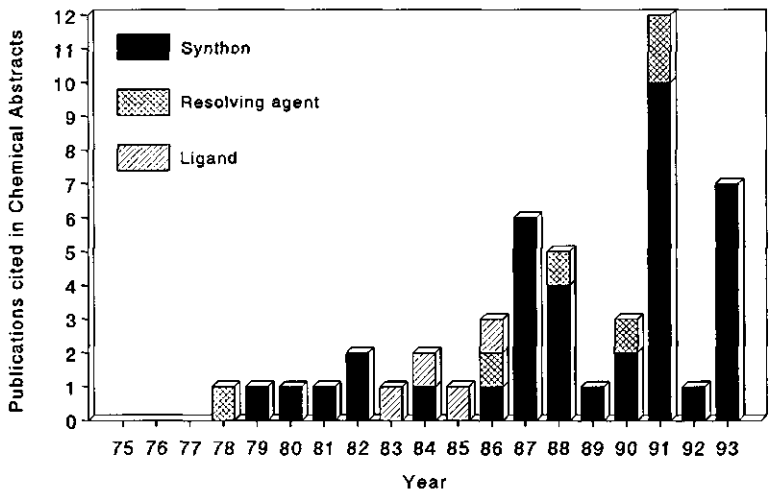


Figure 1. Number of publications (from Chemical Abstracts) during the last 20 years using D-malic acid in organic synthesis. The different application areas in which the D-malic acid was used are indicated.

APPLICATIONS OF D-MALATE IN ORGANIC CHEMISTRY

D-Malate is becoming a valuable source of chirality for various applications, and in recent years an increase in the number of publications on its application in organic chemistry has been observed (Fig. 1). Many of these publications are in the patent literature, indicating the industrial importance of the compound. The three major areas for the use of D-malate in organic chemistry are discussed below.

Application of D-malic acid as a chiral synthon

Optically active malic acid is a versatile chiral synthon (building block for organic syntheses) in the synthesis of various important compounds (13,41). In this area, this hydroxy acid is especially useful as it is available in both enantiomeric forms. D-Malic acid is, however, not as readily available as the natural occurring L enantiomer (51) and, consequently, is much more expensive (55). Therefore, many more syntheses starting with L-malic acid rather than with D-malic acid have been described. However, the procedures as described for L-malic acid in the synthesis of enantiomerically pure compounds are also applicable to D-malic acid and will result in the opposite enantiomer of the product (41).

Malic acid is not the most optimal chiral synthon as it lacks highly electrophilic centres (41). However, malic acid can easily be converted into several other chiral compounds which subsequently can be used readily as synthons (41). The strategies which have most frequently

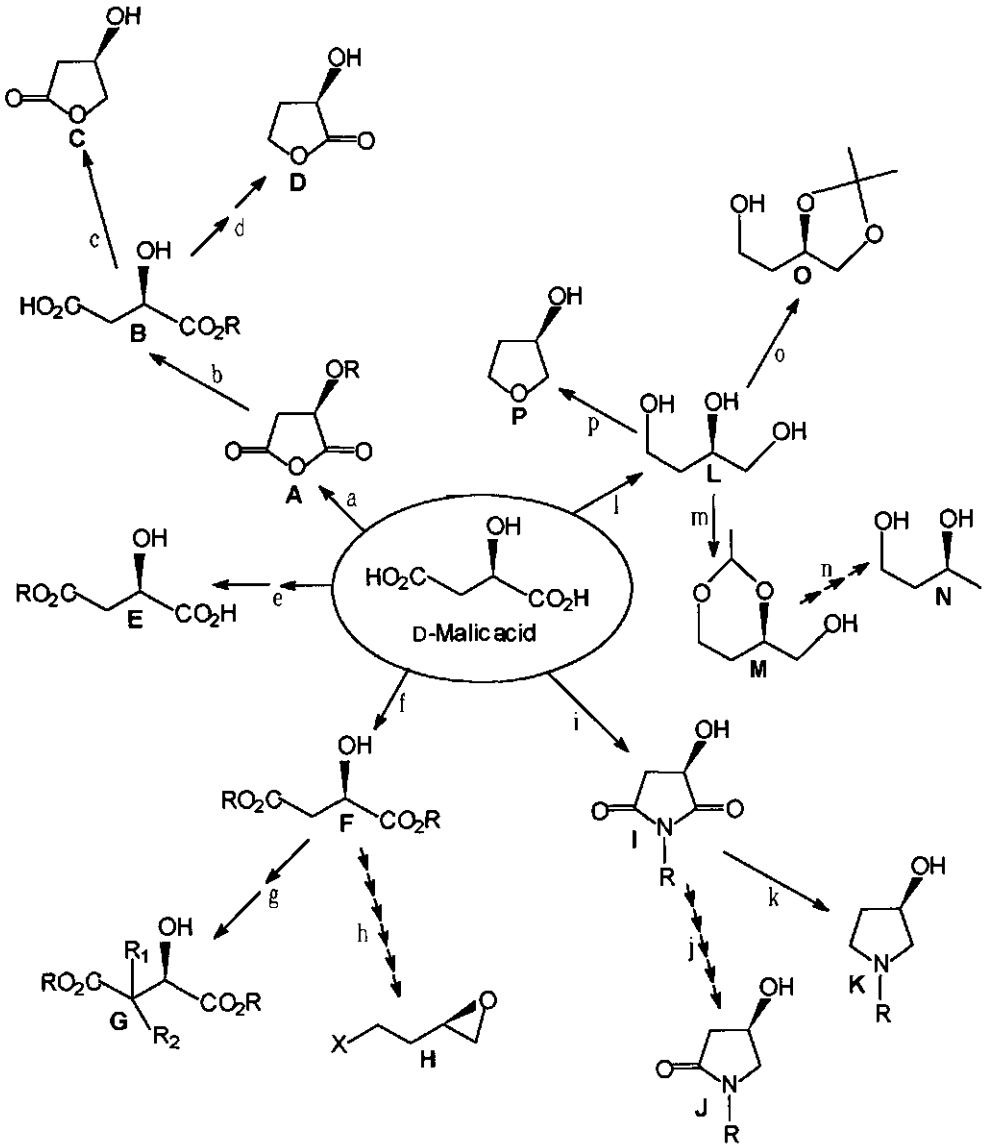


Figure 2. Most frequently used initial transformation steps of D-malic acid in organic synthesis. A, D-Malic anhydride; B, D-malic acid 1-mono ester; C, (*R*)-3-hydroxy-4-butyrolactone; D, (*R*)-2-hydroxy-4-butyrolactone; E, D-malic acid 4-mono ester; F, D-malate diester; G, D-3,3-dialkyl-malate diester; H, (*R*)-1,2-epoxy-4-halobutane; I, (*R*)-1-alkyl-3-hydroxy-2,5-pyrrolidinedione; J, (*R*)-1-alkyl-4-hydroxy-2-pyrrolidinone; K, (*R*)-1-alkyl-3-pyrrolidinol; L, (*R*)-1,2,4-butanetriol; M, (*R*)-2-alkyl-4-hydroxymethyl-1,3-dioxane; N, (*S*)-1,3-butanediol; O, (*R*)-2,2-dialkyl-1,3-dioxolane-4-ethanol; P, (*R*)-3-hydroxy-tetrahydrofuran. a: 1 step (19,28,38,47); b: 1 step (19,28,47); c: 1 step (19); d: 2 steps (47); e: 2 steps (9); f: 1 step; g: 2 steps (mono alkylering) or 4 steps (dialkylering)(58); h: 6 steps (21,42); i: 1 step (48); j: 5 steps (2); k: 1 step (48); l: 1 step (20,7); m: 1 step (20); n: 3 steps (22); o: 1 step (7,31); p: 1 step (53).

been adopted for the further conversion of D-malic acid are illustrated in Fig. 2. The C_4-synthons formed in this way can be used for the synthesis of various chiral pharmaceuticals (2,6,7,47,48), antibiotics (28,54), D- and L-carnitine (9,38), the pheromone (*S*)-ipsdienol (31) and the vitamin (*R*)-pantothenic acid (58). D-Malic acid has also been used in the synthesis of many natural compounds (1,37,43,44,49,57) to establish the stereochemistry of these compounds.

Application of D-malic acid as a resolving agent

D-Malic acid is also used as a resolving agent for the resolution of racemic bases by diastereomeric salt formation. Diastereomeric salt formation is based on the interaction of a racemic product with an optically active compound (resolving agent), to give two diastereomeric salts. These diastereomeric salts have different physical properties and, consequently, can be separated in a number of ways, e.g. by crystallization (8). Resolutions in which D-malic acid is used as a resolving agent are the resolution of a neuroleptic (11) and of a muscle relaxant (45).

Application of D-malic acid as a ligand in asymmetric synthesis

The last application area for D-malic acid in organic synthesis is to use it as a ligand (chiral catalyst) in asymmetric synthesis to enhance the stereospecificity of the reaction. Due to the interaction of D-malic acid with the catalyst(s) and/or the substrate(s), the enantiomeric purity of the product can be enhanced. Illustrative examples of the use of D-malic acid as a ligand are found in asymmetric hydrogenation (18,52). However, the products formed in these studies had only moderate optical purities (18,52).

PRODUCTION OF D-MALATE

In contrast to L-malic acid, which is made biocatalytically from fumarate with the enzyme fumarase (51), D-malic acid is not easily prepared. Several ways to produce D-malate have been described. In this paragraph an overview is given on chemical, physical and bio(techno)logical ways to produce D-malate.

Chemical D-malate production

In literature, several ways to chemically produce D-malate have been described (Table 2). The classical route is from (2*R*,3*R*)-tartaric acid (41), a compound of the chiral pool (i.e. relatively inexpensive, readily available optically active natural products). (2*R*,3*R*)-Tartaric acid is the 'natural' stereoisomer of tartaric acid and is a waste product of wineries (41). Six different ways to chemically transform (2*R*,3*R*)-tartaric acid into D-malate of high

Table 2. Chemical and physical methods to produce D-malate

Starting compound(s)	Asymmetric catalyst/ Resolving agent	Steps	Molar yield (%)	Enantiomeric purity (%)	Product[a]	Refs
Chemical D-malate production						
Chiral-pool approach:						
(2*R*,3*R*)-Tartaric acid		3	48	>98	E	15
(2*R*,3*R*)-Tartaric acid		4	42	>95	E	22,41
(2*R*,3*R*)-Tartaric acid		4	47	>95	E	22,41
(2*R*,3*R*)-Tartaric acid		3	56	>98	E	12
(2*R*,3*R*)-Tartaric acid		2	67	pure	E	3
(2*R*,3*R*)-Tartaric acid		4	55	pure	E	16
(+)-Phyllodulcin		1		>98	A	4
D-Aspartic acid		1	85	>96	A	19,25
Asymmetric synthesis:						
Ketene and chloral	Quinine	3	79	89	A	60
Epoxysuccinate	Rhodium catalysts	1	62	<81	A	10
Physical D-malate production						
Optical resolution:						
Malonic acid and chloral	Quinine	3	<22	>98	A	27
DL-Malic acid	(*S*)-(-)-1-Phenylethylamine	1	13	>98	A	43
DL-Malic acid	(-)-1-(Isopropylphenyl)-ethylamine	1		97	A	39
DL-Malic acid	Propylammonium-(+)-malate	1	16	95	A	46

[a] A, free acid; E, diester of D-malate.

enantiomeric purity have been described (Table 2). D-Malate can also be synthesized from the more expensive optically active compounds D-aspartic acid and the natural product (+)-phyllodulcin (Table 2).

Another approach to produce D-malic acid chemically is by asymmetric synthesis. In this case a prochiral compound is converted into optically active D-malate. Two different methods to produce D-malate using the asymmetric synthesis approach have been described in literature (Table 2). The asymmetric synthesis of D-malate from ketene and chloral using quinine as the asymmetric catalyst (60) has been commercialized by Lonza (Basel, Switzerland)(50,55).

D-Malate production by physical resolution

D-Malate can be produced by enantioselective crystallization of the diastereomeric salts, formed in the presence of a chiral resolving agent, by making use of the different physical

properties of opposite diastereomeric salts. Four different optically active amines have been described in literature for the separation of D-malic acid from DL-malic acid (Table 2).

Bio(techno)logical D-malate production

The bio(techno)logical production of D-malate has only recently been investigated: all publications have appeared after 1990 (Table 3). D-Malate can be produced either microbiologically or enzymatically.

Microbiologically, D-malate can be produced by using a microorganism which only metabolizes L-malate. When such a microorganism is grown on DL-malate, the L enantiomer is consumed leaving optically pure D-malate in the culture broth. Several microorganisms used for the production of D-malate in this way have been described (26,29,30)(Table 3). Disadvantages of this microbiological method are the fact that high substrate concentrations

Table 3. Bio(techno)logical methods to produce D-malate

Biocatalyst	Product concentration (g/l)	Molar yield (%)	Enantiomeric purity (%)	Product[a]	Refs
Microbiological D-malate production					
Stereoselective assimilation					
Pseudomonas putida	5	49	99.8	A	26
Acinetobacter lwoffi	35	35	100	A	29,30,35
Enzymatic D-malate production					
Enzymatic resolution					
Pig liver esterase	1.7	42	96.5	E	40
Rhizopus lipase	4	20	>98	E	56
Asymmetric synthesis					
a. Stereospecific reduction					
Lactobacillus fermentum	1.2	58	90	E	40
b. Stereospecific addition					
Arthrobacter globiformis	5	50	90.5	A	33
Pseudomonas fluorescens	6.9	69	99.5	A	36
Providencia stuarti	9.1	78.8	100	A	34
Ustilago sphaerogena	41	49.6	99.6	A	32
Arthrobacter sp.	87	72	100	A	5,61
Pseudomonas pseudoalcaligenes	215	99.4	99.96	A	This thesis

[a] A, free acid; E, diester of D-malate.

severely hamper the growth of the microorganism and the fact that besides D-malate also huge amounts of biomass are produced.

Enzymatic methods to produce D-malate comprise both enantiomeric resolution and asymmetric synthesis approaches (Table 3). Enzymatic resolution of the diester of malate can be achieved using lipases or esterases, resulting in the transformation of one of the isomers to the mono-ester-mono-acid (40,56). The enantiospecific reduction of prochiral diethyl oxalacetate can be used to produce the diethyl ester of D-malate (40). The most recently described biotechnological approach for the production of D-malate involves the stereospecific addition of water to maleate thus forming D-malate (Table 3).

Comparison of the different D-malate synthesis methods

Chemical, physical and bio(techno)logical methods can be used to produce D-malate (Table 2 and 3). The chiral pool approach and physical resolution generally result in D-malate of high (>95%) optical purity. However, with chemical asymmetric synthesis, D-malate with an enantiomeric purity of only 80-90% was produced (Table 2). The yields obtained with both chemical and physical methods are, however, quite low (~50%). This is generally due to the fact that more than one reaction step is required to produce D-malate.

The bio(techno)logical routes also result in D-malate of high optical purity, but the yield depends very much on the method used (Table 3). While the bio(techno)logical production of D-malate by means of resolution and stereoselective assimilation have maximal yields of only 50%, the production of D-malate by means of enantiospecific reduction or enantiospecific addition can, theoretically, result in a yield of 100%. However, when D–malate is produced by means of enantiospecific reduction, cofactor recycling is required, making the process very complex.

Therefore, the production of D-malate using maleate hydratase seems to be the most promising method. The substrate, maleate, is a cheap bulk chemical which is presently used in the production of polyester resins. It is converted into D-malate in a simple one step bioconversion. This process does not require cofactor regeneration and the theoretical yield of the reaction is 100% while the enantiomeric purity of hydroxy acids produced by hydratases is generally 100%.

OUTLINE OF THIS THESIS

In this thesis, several aspects of the conversion of maleate into D-malate catalyzed by maleate hydratase (malease; EC 4.2.1.31) are described. Malease belongs to the enzyme class of the lyases (EC 4). Lyases are enzymes catalyzing addition and, reversibly, elimination reactions. These enzymes are potentially very interesting for the production of

optically pure compounds as they do not require cofactor recycling, show an absolute stereochemistry, and have a theoretical yield of 100%. Until now, however, not many of these enzymes have been studied for their capability to produce optically active compounds. Chapter 2 gives an overview of lyase-catalyzed reactions.

In Chapter 3 the screening of over 300 microorganisms for the presence of malease is described. *Pseudomonas pseudoalcaligenes* NCIMB 9867 was selected as the best biocatalyst. The optimal growth substrate for the induction of malease activity in this microorganism has been determined (Chapter 4). Subsequently, malease from *P. pseudoalcaligenes* was purified and characterized, especially in relation to cofactor dependency and substrate specificity (Chapter 5). To be able to determine the effect of pH and temperature on the maximal yield of this hydration reaction, the thermodynamic constants of the maleate hydration reaction were determined (Chapter 6). As *P. pseudoalcaligenes* probably lacks a transport mechanism for maleate (Chapter 3) cells need to be permeabilized to be able to use them for the conversion of maleate into D-malate (Chapter 7). The effect of the maleate counter-ion on the malease activity and substrate and product inhibition were studied (Chapter 8) in relation to the conversion of high concentrations of maleate. Ca^{2+} was the best counter-ion resulting in the formation of a crystal-liquid two-phase system. This crystal-liquid two-phase system was used to convert 1 kilogram of maleate into D-malate (Chapter 9). Finally, in Chapter 10, the production of D-malate with malease from *P. pseudoalcaligenes* is compared with other malease containing biocatalysts.

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CHAPTER 2

THE POTENTIAL OF LYASES FOR THE INDUSTRIAL PRODUCTION OF OPTICALLY ACTIVE COMPOUNDS

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SUMMARY

Lyases catalyze the cleavage of C-C, C-N, C-O and other bonds by elimination to produce double bonds or, conversely, catalyze the addition of groups to double bonds. These enzymes do not require cofactor recycling, show an absolute stereospecificity and can give a theoretical yield of 100% compared with only 50% for enantiomeric resolutions. Lyases are therefore attracting considerable interest as biocatalysts for the production of optically active compounds, and have already found application in several large commercial processes.

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INTRODUCTION

The dramatic variation in biological activity that enantiomers of a chiral compound can display is of key importance to the pharmaceutical, agrochemical and food industries (13). For example, the presence of the 'wrong' stereoisomer in a preparation of a chiral pharmaceutical compound can have severe adverse effects on patients or, in the case of a chiral agrochemical, may cause an additional environmental load (8). To obtain approval for new chiral drugs, pharmaceutical companies are required to determine the effects of all stereoisomers present, and any stereoisomers without the requisite function are considered as impurities (8,53). Therefore, a (cheap) source of optically pure compounds is of prime importance, especially for the pharmaceutical industry.

Most optically active compounds can be produced by chemical synthesis, biocatalytically, by a combination of both these approaches, or by extraction from plant material. However, the biocatalytic route has several advantages. Biocatalysts operate under mild reaction conditions, and they are also reactionspecific, regiospecific and stereospecific, thus yielding fewer side-products than more conventional routes - an important environmental consideration.

Almost all classes of enzymes have been investigated for their potential to synthesize optically active compounds, and enzymes such as transferases, hydrolases and lyases which, fortuitously, do not require (expensive) cofactors, have been found to be particularly suitable for the commercial production of optically active compounds.

Lyases catalyze the cleavage of C-C, C-N, C-O and other bonds to yield double bonds, and also catalyze the addition of groups to double bonds (see Box 1). However, the use of lyases to produce optically active compounds has attracted relatively little attention from the scientific community, in comparison with the extensive work carried out on hydrolytic enzymes (54), despite the fact that some of the oldest, large-scale commercialized biocatalytic processes exploit lyases (Table 1).

In recent years, several lyases (aldolases, oxynitrilases, acyloin condensing enzymes) have attracted renewed attention because of their (bio)synthetic and stereoselective versatility. This review considers the reactions catalyzed by lyases and discusses the properties of these enzymes, placing emphasis on their stereospecificity.

Lyase catalyzed reactions

Over 300 lyase enzymes, catalyzing a wide variety of reactions, have been described (45). However, the majority of lyases may be classified according to the broad types of reactions catalyzed (see below).

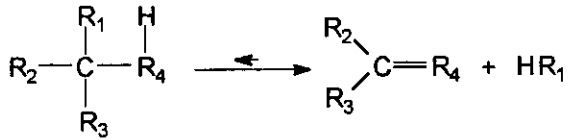
Addition to -C=C- bonds

The addition of water or ammonia to a carbon-carbon double bond is catalyzed by

Box 1. Reactions catalyzed by lyases

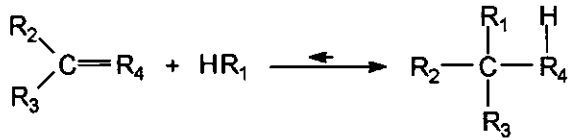
Lyases catalyze the reversible cleavage of C-O, C-C, C-N or other bonds by elimination to produce double bonds or, conversely, catalyze the addition of groups to double bonds. The direction of the reaction will depend primarily on the equilibrium constant for the reaction concerned, but may also be driven in a particular direction by the reaction conditions used. The elimination and addition reactions catalyzed by lyases are described below.

Elimination reactions



Reaction	Lyase
Decarboxylation	Decarboxylase
Dehydration	Dehydratase
Deamination	Ammonia-lyase
Cyanohydrin cleavage	Oxynitrilase
Aldol cleavage	Aldolase
α,β-Elimination	PLP-dependent lyase
α,γ-Elimination	PLP-dependent lyase

Addition reactions



Reaction	Lyase
Hydration	Hydratase
Ammonia addition	Ammonia-lyase
Acyloin condensation	TPP-dependent α-keto acid decarboxylase, synthase
Cyanohydrin formation	Oxynitrilase
Aldol condensation	Aldolase
Claisen-type condensation	Synthase, lyase
Reverse α,β-elimination	PLP-dependent lyase
Reverse α,γ-elimination	PLP-dependent lyase

Table 1. The use of lyases in commercialized processes for the production of (optically active) fine-chemicals

	Product	Substrate	Lyase	Origin of biocatalyst
Lyase-catalyzed fine-chemical production by addition				
1.	L-Aspartate	Fumarate	Aspartase	*Escherichia coli*
2.	L-Phenylalanine	*trans*-Cinnamic acid	Phenylalanine ammonia-lyase	*Rhodotorula rubra*
3.	L-DOPA	Pyruvate, catechol, NH_4^+	Tyrosine-phenol lyase	*Erwinia herbicola*
4.	L-Malate	Fumarate	Fumarase	*Brevibacterium flavum*
5.	D-β-Hydroxy isobutyric acid	Isobutyric acid	An enoyl-CoA hydratase	*Candida rugosa*
6.	L-Carnitine	γ-Butyrobetaine	An enoyl-CoA hydratase	*Rhizobiaceae*
7.	(*R*)-Phenylacetylcarbinol	Benzaldehyde, acetaldehyde	Pyruvate decarboxylase	*Saccharomyces* sp.
8.	(*R*)-Cyanohydrins	Aldehydes, HCN	(*R*)-Oxynitrilase	*Prunus amygdalus* (bitter almonds)
9.	Acrylamide	Acrylonitrile	Nitrile hydratase	*Rhodococcus rhodochrous*
Lyase-catalyzed fine-chemical production by elimination				
10.	L-Alanine	L-Aspartate	L-Aspartate-β-decarboxylase	*Pseudomonas dacunhae*
11.	L-Alanine + D-Aspartate	DL-Aspartate	L-Aspartate-β-decarboxylase	*Pseudomonas dacunhae*
12.	Urocanic acid	L-Histidine	L-Histidine ammonia-lyase	*Achromobacter liquidum*

[a] Several other companies now also produce L-aspartic acid on a commercial scale using aspartase (58,73).
[b] Process is no longer running.
[c] First production runs have recently been performed.

Table 1. (Continued)

	Method	Concentration of product in broth ($g \cdot l^{-1}$)	Molar yield (%)	Annual production	Company	Refs
Lyase-catalyzed fine-chemical production by addition						
1.	1960-1973, fed-batch culture, growing whole cells	56	95	6000 tons	Tanabe (Osaka, Japan)[a]	11,32
	Since 1973, continuous process, immobilized cells	133	100			
2.	1984-1985, fed-batch process, with cell recycling	50	90	Hundreds of tons[b]	Genex (Gaithersburg, USA)	30
3.	Since 1992, fed-batch process, whole cells	53	-	200 tons	Ajinomoto (Tokyo, Japan)	71
4.	1966-1974, batch process, permeabilized cells in presence of Ca^{2+}	113	98	500 tons	Tanabe (Osaka, Japan)	12,63
	Since 1974, continuous process, immobilized cells	94	70			
5.	Since 1980s, fed-batch culture, growing whole cells	200	>90	Dozens of tons	Kaneka (Osaka, Japan)	46
6.	Since 1993, fed-batch culture, growing cells with cell recycling	100	99	c	Lonza (Basel, Switzerland)	38
7.	Since 1930s, fed-batch process, fermenting cells	~15	70	Hundreds of tons	Malladi (Madras, India) Knoll (Ludwigshafen, Germany)	
8.	Since 1991, batch process, pure enzyme solution, organic-two phase system, recycling of the aqueous phase	130	>90	Multi kg scale	Solvay Duphar (Weesp, The Netherlands)	37
9.	Since 1985, fed-batch process, immobilized cells	500	100	30000 tons	Nitto (Tokyo, Japan)	2,43
Lyase-catalyzed fine-chemical production by elimination						
10.	1965-1982, batch process, permeabilized cells	267	100	~100 tons	Tanabe (Osaka, Japan)	10,11
	Since 1982, continuous process, immobilized cells	178	100			
11.	Since 1988, continuous process, immobilized cells	166 (D-asp)	50	<1 ton	Tanabe (Osaka, Japan)	51
		111 (L-ala)	50			
12.	Since 1970s, batch process, heat-treated cells	110	100	?	Tanabe (Osaka, Japan)	52

hydratases and ammonia-lyases, respectively. Over 100 different hydratases have been identified; however, fewer than ten ammonia-lyases have been described. These enzymes can be used to transform steroids (24), fatty acids (72), terpenoids (7), hydroxy acids (12,64) and amino acids (11), and several hydratase- and ammonia-lyase-catalyzed conversions have been commercialized (Table 1).

Carbon-carbon bond-forming reactions

It is rather difficult to control the stereochemistry in the synthesis of carbon-carbon bonds using a chemical-synthesis approach. However, some lyases catalyze this reaction very efficiently and, therefore, are attracting quite a lot of attention from organic chemists.

Acyloin condensation

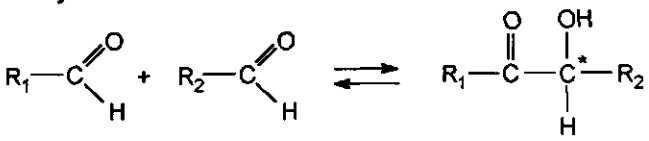
The acyloin-condensation reaction involves the condensation of two aldehydes to yield α-hydroxy ketones (acyloins) (Fig. 1a), which are versatile synthons (starting compounds for synthesis) for the fine-chemicals industry (15). Several lyases dependent on thiamine pyrophosphate (TPP) and divalent metal ions; for example, propioin synthase (42) and acetohydroxy synthase (25), catalyze acyloin-condensation reactions. Two other lyases, pyruvate decarboxylase (5) and benzoylformate decarboxylase (67), which are TPP-dependent 2-oxo acid decarboxylases, are able to catalyze the acyloin condensation reaction as a side-reaction.

The industrial synthesis of L-ephedrine has used yeast pyruvate decarboxylase to catalyze the condensation of benzaldehyde and acetaldehyde to form (*R*)-phenylacetylcarbinol, the first step in the formation of L-ephedrine, since the 1930s. This industrial process was one of the first to combine microbiological and chemical syntheses to produce optically active compounds. Yeast pyruvate decarboxylase can also catalyze the formation of several other aromatic and aliphatic aldehydes with 'active' (i.e. bound to cofactor) formaldehyde, acetaldehyde, propionaldehyde or butyraldehyde (23,36).

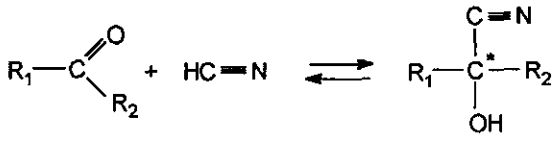
Cyanohydrin formation

Oxynitrilases (α-hydroxynitrile lyases) catalyze the addition of hydrogen cyanide to an aldehyde or a ketone to form a cyanohydrin (Fig. 1b). Both (*R*)- and (*S*)-oxynitrilases have been described (55), and can be used to produce a large variety of optically pure cyanohydrins (37,55). However, there are two problems associated with the enzymic formation of cyanohydrins: (i) the competing, nonspecific chemical addition of cyanide to aldehydes; and (ii) racemization of the product in aqueous buffer. Recently, workers at Solvay Duphar (Weesp, The Netherlands) have overcome these problems by using an organic two-phase system, and have introduced the first optically pure cyanohydrins produced with oxynitrilases onto the market (37).

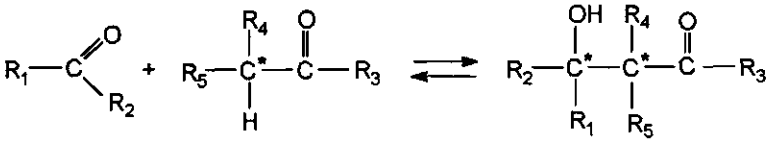
a. Acyloin condensation



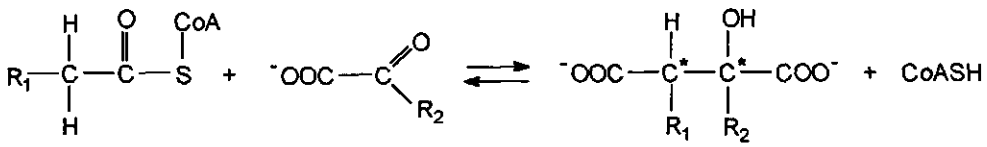
b. Cyanohydrin formation



c. Aldol condensation



d. Claisen-type condensation



e. α,β-Elimination

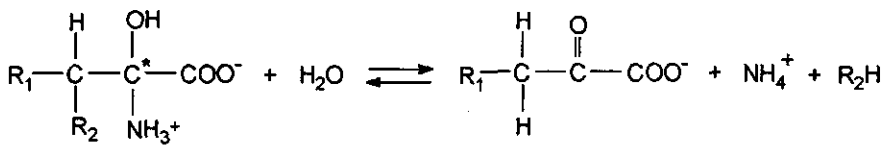


Figure 1. Reactions catalyzed by lyases that form carbon-carbon bonds.

Aldol condensation

The reversible aldol condensation reaction involves attack by the deprotonated α-carbon atom of an aldehyde or ketone on the carbonyl carbon of another aldehyde or ketone, resulting in a β-hydroxyaldehyde or a β-hydroxyketone (Fig 1c). This reaction is catalyzed by aldolases, which are involved in the degradation or transformation of sugars, amino acids and aromatic compounds. Over the past decade, these enzymes have attracted attention from organic chemists, particularly for the production of unusual sugars (69), which are of interest for the pharmaceutical and food industries (e.g. as non-nutrient substituents, sweeteners), and certain amino acids.

To date, more than 30 aldolases have been described; however, the substrate specificity

of only a few of these enzymes has been studied extensively. In general, aldolases have a restricted substrate specificity for the nucleophile, but show a broad acceptance of the electrophilic aldehyde (59). Currently, aldolase-catalyzed production of sugars and amino acids is performed on a small (gram to kilogram) scale.

Claisen-type condensation

Enzymes catalyzing condensation reactions in which the nucleophile is the α-carbanion of a thioester and the electrophilic component can be a variety of α-keto acids are called 'Claisen enzymes' (62) (Fig. 1d). Although these reactions are not formally Claisen condensations, they are distinct from aldolases in terms of their reaction mechanism (50). Claisen enzymes have not yet been exploited for their synthetic abilities because acyl-CoA compounds and α-keto acids are rather expensive.

Pyridoxal-phosphate-dependent amino acid lyases

Pyridoxal phosphate (PLP) is a cofactor required by enzymes catalyzing the synthesis, degradation or interconversion of amino acids (48). A group of $\sim$25 different PLP-dependent lyases are able to catalyze α,β-elimination (Fig. 1e), β-replacement, α,γ-elimination and/or γ-replacement reactions (56). Many of these enzymes are multifunctional, i.e. one enzyme is able to catalyze more than one of these reaction types (71).

A process using tyrosine-phenol lyase to catalyze the synthesis of L-3,4-dihydroxy-phenylalanine (L-DOPA), a compound used in the treatment of Parkinson's disease, has recently been commercialized. This process is unique in that the enzyme catalyzes the condensation of three different substrates (see Table 1).

Decarboxylation

Decarboxylases constitute a major class of lyases. These enzymes are particularly suitable for resolving mixtures of enantiomers as they exhibit absolute stereospecificity and the reactions that they catalyze are not reversible. In 1988, Tanabe (Osaka, Japan) commercialized a process in which aspartate-β-decarboxylase is used to produce both D-aspartate and L-alanine from racemic aspartate (51) (Table 1). Another potentially interesting application of decarboxylases is the decarboxylation of prochiral compounds. Recently, Miyamoto and Ohta (41) described the use of whole cells of *Alcaligenes bronchisepticus* to decarboxylate disubstituted malonic acids to chiral carboxylic acids with high enantiomeric excess.

Stereochemistry of lyase catalysis

Most lyases show absolute stereochemical control with respect to the carbon atoms at the reaction site - only the adenosylcobalamin-dependent lyases transform both stereoisomers (35).

Several strategies can be followed to synthesize optically active compounds (Table 2).

Table 2. Strategies for the biocatalytic production of optically active compounds

	Class	Starting material	Product	Maximal theoretical yields (%)	Reactions catalyzed by lyases
I.	Optical resolution	Racemic substrate	Optically active	50%	Elimination reactions, decarboxylations
II.	Chiral pool approach	Optically active substrate	Optically active	100%	Decarboxylases, β- and γ-replacement reactions
III.	Asymmetric synthesis	Prochiral substrate	Optically active	100%	Addition reactions, carbon-carbon bond forming reactions, decarboxylases
IV.	Deracemization	Racemic substrate	Optically active	100%	Acetolactate decarboxylase (14), tyrosine-phenol lyase (19), deoxyribose-5-phosphate aldolase (9)
V.	Stereoinversion	Racemic product	Optically active	100%	Not (yet) described

From an economic, and also from an environmental, point of view, strategies that can, in principle, convert 100% of the substrate into product are preferable. Despite this, a number of enzyme-catalyzed optical-resolution processes, with a maximum theoretical yield of only 50%, are actually applied on a commercial scale (e.g. lipases, acylases). In most instances, an additional product cycle has to be included to racemize the unwanted enantiomer (54). The decarboxylation of DL-aspartate is the only commercialized application of a lyase-catalyzed enantiomeric resolution (Table 1). However, in this case, both the unconverted substrate and the product are valuable products (51).

Elimination reactions with a 100% yield can be achieved if an optically active compound is used as the substrate. The commercial application of lyases catalyzing this type of reaction is limited to enzymes transforming compounds belonging to the chiral pool (i.e. relatively inexpensive, readily available, optically active natural products). An example is the commercialized process for the production of L-alanine (Table 1). L-Aspartate, which is produced from fumarate by the same company (Table 1), is decarboxylated by L-aspartate-β-decarboxylase.

The asymmetric-synthesis approach, by which relatively cheap prochiral substrates are converted into optically active compounds, also has a theoretical yield of 100%, and enzymes catalyzing this type of reaction have attracted widespread attention from organic chemists. Several reactions of this type [for example, the addition of either water or ammonia to fumarate, catalyzed by fumarase and aspartase, respectively, and the acyloin condensation of acetaldehyde to benzaldehyde, yielding (*R*)-phenylacetylcarbinol] have been in commercial use for several decades (Table 1).

Strategies IV and V (presented in Table 2) have been identified only recently and, to date, have been limited to a few specific uses. For example, acetolactate decarboxylase catalyzes the decarboxylation of both (*R*)- and (*S*)-α-acetolactate to (*R*)-acetoin in 100% yield and

>98% enantiomeric excess (14). This enzyme is thought to be able to catalyze the rearrangement of the, in principle, unreactive enantiomer, thereby making it accessible for decarboxylation (14). Another type of reaction in which a racemic substrate is converted into an optically active product is catalyzed both by tyrosine-phenol lyase (19) and by deoxyribose-5-phosphate aldolase (9). These enzymes are able to accept both enantiomers of a racemic substrate in a condensation reaction, but yield an optically pure compound.

Recently, the production of an optically active compound from the corresponding racemate has been described (28). This type of interconversion is catalyzed by more than one enzyme. For example, *Candida parapsilosis* is able to produce several (*S*)-diols in high yields and high enantiomeric excess from the corresponding racemic diols (28). This interconversion is kinetically controlled and is catalyzed by two dehydrogenases. However, as yet, their are no reports of any lyases that are able to catalyze this type of (kinetically controlled) stereoinversion.

One disadvantage of using biocatalysts to produce optically active compounds is that, frequently, an enzyme is available for the preparation of only one of the enantiomers. However, lyases can sometimes be used to produce both enantiomers by exploiting the reversible nature of some of the lyase-catalyzed reactions. Using different reaction conditions, one enzyme can catalyze the production of one of the enantiomers by asymmetric synthesis, and the other enantiomer by optical resolution (21) (Fig. 2). There are also a few examples of stereochemically complementary lyases. For instance, four, stereochemically

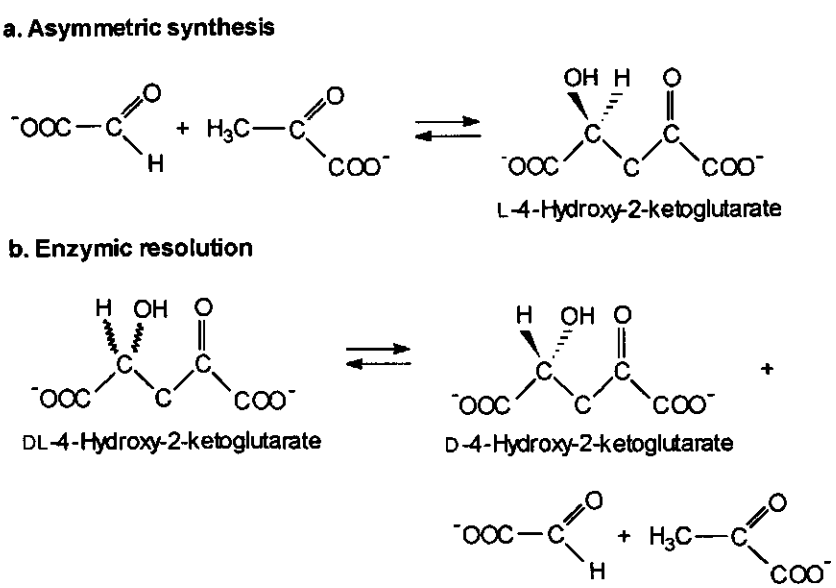


Figure 2. Strategy for synthesizing both enantiomers of 4-hydroxy-2-ketoglutarate using a single lyase. a, Synthesis of the L isomer by asymmetric synthesis; b, synthesis of the D isomer by enzymic resolution of the racemate.

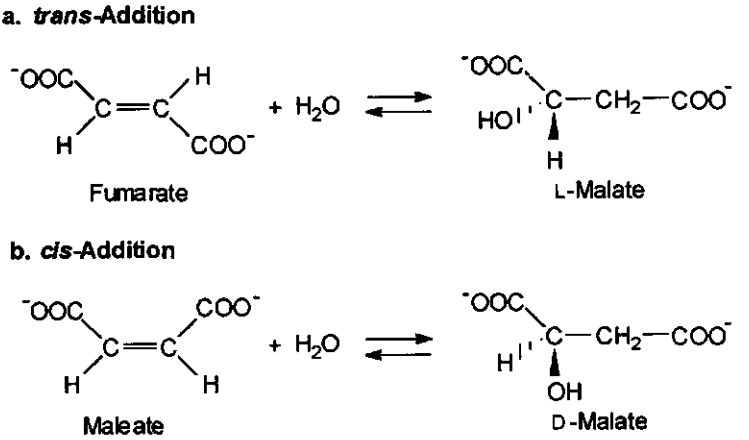


Figure 3. Addition to (a) *trans*- and (b) *cis*- compounds, leading to the formation of opposite enantiomers.

distinct aldolases exist, which are able to produce each of the four diastereoisomers (20). Stereochemically complementary oxynitrilases have also been described (55).

Lyases catalyzing addition reactions with *cis* or *trans* carbon-carbon double bonds have similar reaction mechanisms (50). Thus, addition to *cis* compounds results in the generation of the opposite enantiomer to that produced on addition to the *trans* isomer (12,64) (Fig. 3).

Lyases also often show some stereoselectivity towards both the α- and β-substituents of a substrate. For example, D-fructose-1,6-diphosphate aldolase also shows some stereoselectivity towards α-substituents of the electrophile aldehyde; it catalyzes the condensation of dihydroxyacetone phosphate with (*R*)-3-azido-2-hyroxypropanal faster than with the (*S*) enantiomer, although both isomers are accepted as electrophile and a mixture of stereoisomers is formed (47). This stereoselectivity towards α-substituents is highly dependent on the electrophilic aldehyde used (39).

Production of non-chiral compounds

Lyases may also be used to synthesize non-chiral compounds, as some lyases are able to catalyze reactions which, if performed by the more conventional chemical route, would result in the generation of large numbers of by-products, or have low yields, or require (expensive) catalyst regeneration etc. In such cases, synthesis via the enzymatic process is advantageous, since it is simpler and more economical. For example, nitrile hydratase catalyzes the hydration of a cyanide group to an amide group and is used commercially in the production of the non-chiral commodity chemical acrylamide (1,43) (Table 1). Nitrile hydratase might also be able to catalyze the synthesis of several other amides from nitriles on a commercial scale (40). Another example of the use of lyases to produce non-chiral compounds is the commercialized process for the production of urocanic acid, a compound with UV-blocking properties. In this case, an optically active compound, L-histidine, is converted into a non-

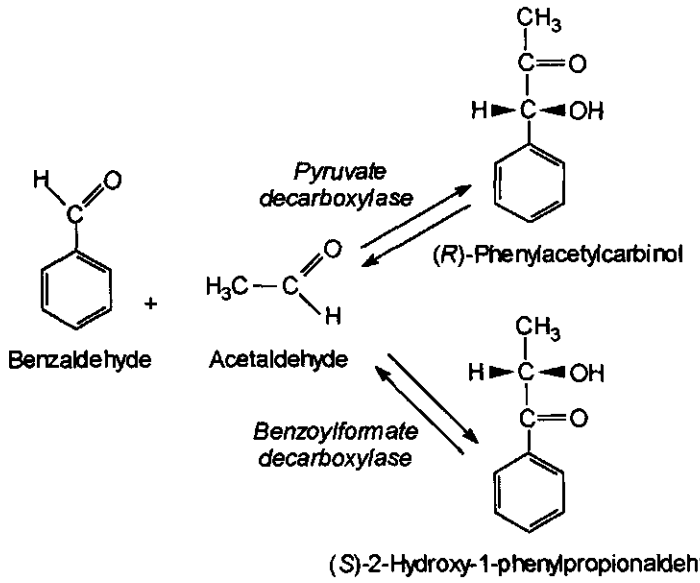


Figure 4. The condensation of acetaldehyde to benzaldehyde catalyzed by pyruvate decarboxylase results in the formation of (*R*)-phenylacetylcarbinol, while the same reaction catalyzed by benzoylformate decarboxylase results in the formation of (*S*)-2-hydroxy-1-phenylpropionaldehyde.

chiral compound (52) (Table 1).

Regiospecificity

One characteristic of enzymes (and of lyases in particular) is that they show an absolute regiospecificity in the reactions that they catalyze. Thus, in contrast to conventional chemical approaches, lyases are able to catalyze both Markovnikov and anti-Markovnikov addition reactions. For example, the chemical addition of water to acrylate results exclusively in the formation of β-hydroxypropionate (3), whereas hydratases have been described that hydrate acrylyl-CoA to either β-hydroxypropionyl-CoA (61) or to (*R*)-lactoyl-CoA (6).

The value of regiospecificity is enhanced by the fact that enzymes catalyze reactions under mild reaction conditions. For example, pyruvate decarboxylase catalyzes the condensation of benzaldehyde to acetaldehyde, forming (*R*)-phenylacetylcarbinol (1-hydroxy-1-phenylpropanone)(36), and benzoylformate decarboxylase catalyzes the condensation of acetaldehyde to benzaldehyde to form the tautomeric compound (*S*)-2-hydroxy-1-phenylpropionaldehyde (67)(Fig. 4). Under physiological conditions, acyloins do not tautomerize and, thus, both tautomers can be isolated separately.

Substrate specificity

A narrow substrate specificity is usually regarded as a drawback for the commercial

exploitation of an enzyme, since it limits the flexibility of such an enzyme as a tool for the production of related compounds. Lyases are generally, but not always, associated with a narrow substrate specificity. The substrate specificity of most hydratases and ammonia-lyases is indeed quite narrow, but the substrate specificity of aldolases, decarboxylases and oxynitrilases is much broader. Moreover, the substrate specificity of a specific lyase may vary according to its source. For instance, malease from rabbit kidney (18) only hydrates maleate, while malease from *Pseudomonas pseudoalcaligenes* (65) also hydrates several α-substituted maleates, such as citraconate.

A relaxed substrate specificity is not an absolute prerequisite for commercialization: indeed, several of the lyases in commercial use (Table 1) have a rather narrow substrate specificity.

Cofactor requirements of lyases

The requirement for (expensive) cofactors can severely limit the commercial potential of enzymes. Since lyase-catalyzed addition or elimination does not involve a net oxidation or reduction, the requirement for cofactors would not be expected. However, most of the lyases identified to date do require cofactors (Table 3). These cofactors are required for substrate binding, polarization of the substrate, temporary binding of the nucleophile, stabilization of reaction intermediates, etc. However, the majority of these cofactors are not very expensive, or are covalently bound to the enzyme and, therefore, do not constitute a barrier to the commercial use of lyases.

The use of cofactor-requiring enzymes can sometimes be avoided. Lyases isolated from different sources can vary in their requirement for cofactors. For instance, both iron-sulphur-containing hydratases and their iron-sulphur-free counterparts have been described (26), and

Table 3. Cofactor requirements of lyase enzymes

Cofactor	Lyase(s)	Refs
Divalent metal ion	Aldolases, (de)hydratases	47,68
Trivalent metal ion	Nitrile hydratase	31
Flavin adenine dinucleotide (FAD)	Oxynitrilase, hydratases	6,55
Thiamine pyrophosphate (TPP)	α-Keto acid decarboxylases	33
Pyridoxal phosphate (PLP)	Amino acid lyases	48
Coenzyme A (CoA);		
Substrate bound	Hydratases, ammonia-lyases	6
Enzyme bound	Citrate and citramalate lyase	17
Enzyme bound NAD^+	Urocanase, dehydroquinate synthase	22
Adenosine triphosphate (ATP)	Hydratases	6
Iron-sulfur cluster	Hydratases	26,57
Pyrroloquinoline quinone (PQQ)	Nitrile hydratase, amino acid decarboxylase(?)	16,44
Covalent bound pyruvate	Amino acid decarboxylases	49
Covalent bound dehydroalanine	Phenylalanine ammonia-lyase	29
Adenosylcobalamin (Coenzyme B_{12})	Diol dehydratases, ethanolamine ammonia-lyase	35
Glutathione	Dichloromethane dehalogenase	34

while aldolases obtained from plants and animals are cofactor-independent, aldolases obtained from lower microorganisms usually require a divalent metal ion for activity (47).

Even if relatively expensive cofactors are required, this need not preclude the commercial application of such enzymes. The production of L-alanine and D-aspartate is catalyzed by the PLP-dependent enzyme aspartate-β-decarboxylase (Table 1). In this case, the commercial process for the production of these amino acids is performed in the presence of 0.1 mM PLP (51).

Thermodynamics

Lyase-catalyzed reactions are equilibrium reactions. In some instances, for example the production of L-phenylalanine, catalyzed by phenylalanine ammonia-lyase, the equilibrium is very unfavourable and a large excess of ammonia has to be added to obtain reasonable yields (27) whereas, in other cases, such as the production of D-malate from maleate (66), the equilibrium strongly favours product formation.

The need for high yields (>99%) assumes particular importance when expensive downstream processing is required (38). The yield of a reaction with an unfavourable equilibrium constant can be influenced in several ways: the easiest being the addition of an excess of substrate. Reaction yields can also be improved by coupling the transformation to a more thermodynamically favoured process, or by using an enzyme that catalyzes an analogous reaction, but which has coupled this reaction to the hydrolysis of an activated bond (e.g. CoA or phosphate), thereby attaining a more favourable equilibrium (4,60). The use of two-phase systems can also result in higher yields. For instance, by performing the fumarate hydration reaction in the presence of Ca^{2+}, resulting in the precipitation of both substrate and product, the yield of this reaction increased from 80% to 98% (63).

Prospects for the application of lyases on a commercial scale

Lyases catalyze numerous commercially interesting addition reactions, resulting in the formation of optically pure compounds, and several of these enzymes have been exploited commercially for several decades (Table 1). These commercial processes combine the high stability of the enzymes [for example, immobilized fumarase has a half-life of 310 days (11)], with high productivity, high product concentrations and high yields (Table 1), although the limited substrate specificity and cofactor dependence of these enzymes can inhibit their commercial application. However, lyases are not highly conserved in nature, which is illustrated by the fact that more than one lyase catalyzing the same reaction may be present in a particular microorganism [e.g. *E. coli* contains three different fumarases (70)]. Thus, screening might not only result in enzymes with novel catalytic activities, but also in the selection of a biocatalyst with more favourable reaction characteristics.

Lyases have attracted considerable attention from organic chemists and biochemists/microbiologists. Organic chemists have focused on the application of these enzymes as catalysts for asymmetric synthesis and, in general, have concentrated on the

easily obtainable (i.e. commercially available) lyases, with respect to their stereochemical and catalytic (substrate specificity) properties. On the other hand, biochemists/microbiologists have concentrated on lyases from a physiological point of view (i.e. optimal reaction conditions, cofactor dependency). A closer collaboration between organic chemists and biochemists/microbiologists might result in the development of new and more efficient methods for the synthesis of optically active compounds by lyase-catalyzed reactions.

In conclusion: lyases already play, and will, in the future, play an even larger role in the biocatalytic production of fine-chemicals.

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CHAPTER 3

SCREENING FOR MICROORGANISMS PRODUCING D-MALATE FROM MALEATE

Mariët J. van der Werf, Will J.J. van den Tweel and Sybe Hartmans

SUMMARY

More than 300 microorganisms were screened for their ability to convert maleate into D-malate as a result of the action of maleate hydratase. Accumulation of fumarate during incubation of permeabilized cells with maleate was shown to be indicative of one of the two enzymes known to transform maleate. The ratio in which fumarate and malate accumulated could be used to estimate the enantiomeric composition of the malate formed. Many strains (n = 128) were found to be capable of converting maleate to D-malate with an enantiomeric purity of more than 97%. *Pseudomonas pseudoalcaligenes* NCIMB 9867 was selected for more detailed studies. Although this strain was not able to grow on maleate, permeabilized cells were able to degrade maleate to undetectable levels, with a concomitant formation of D-malate. The D-malate was formed with an enantiomeric purity of more than 99.97%.

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INTRODUCTION

Biological formation of optically active synthons (starting compounds for chemical synthesis) for the fine-chemicals industry is one of the major application areas in biotechnology (12). Lyases (e.g. hydratases, ammonia-lyases) are very interesting enzymes in this respect. They are generally cofactor independent, quite stable, and have a theoretical yield of 100% compared to only 50% for enantiomeric resolutions.

Our goal was to screen microorganisms for novel lyase activities to ultimately produce optically active amino acids or hydroxy acids on a commercial scale.

Lyases have been used on a commercial scale for some decades. L-Malate has been produced since 1974 by the Japanese firm Tanabe Seiyaku in a continuous process that uses immobilized *Brevibacterium flavum* cells containing high fumarase activity (36). An example of an ammonia-lyase which is used on a commercial scale is aspartase (6). This enzyme stereospecifically adds ammonia to fumarate, forming L-aspartate. Since 1958, L-aspartate has been produced on an industrial scale by the same Japanese firm by using *Escherichia coli* containing high aspartase activity. Since 1973, this process has been operated continuously by using immobilized cells (6).

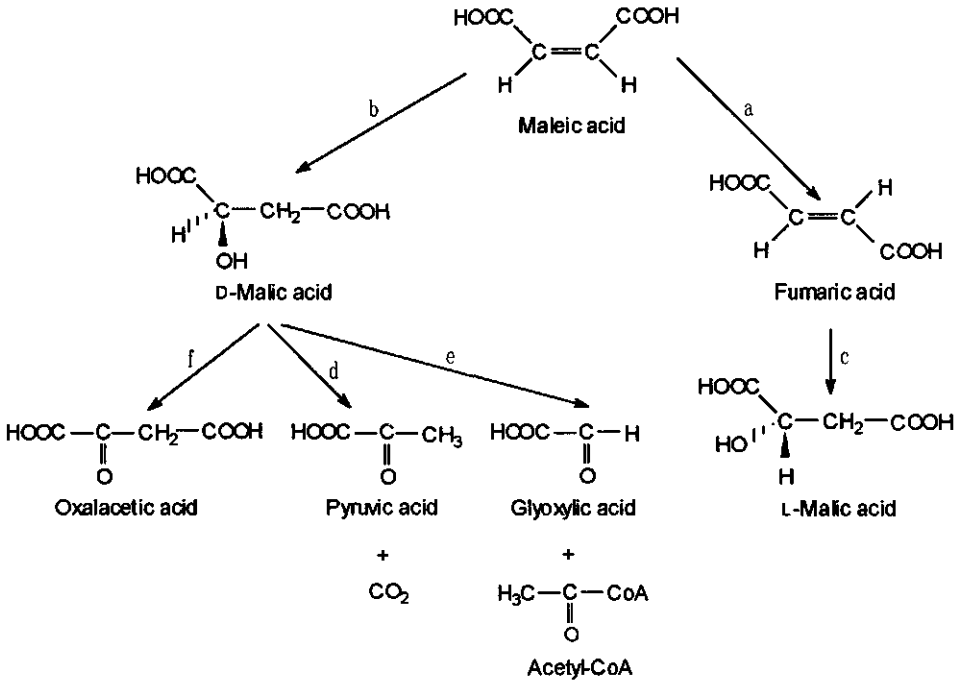

Figure 1. Microbial degradation pathways of maleate. Enzymes involved in pathways: a, maleate *cis-trans*-isomerase; b, maleate hydratase; c, fumarase; d, D-malic enzyme; e, D-malate synthase; f, D-malate dehydrogenase.

A chiral α-hydroxy acid with potential commercial applications is D-(*R*)-malate. D-Malate can be used as a chiral synthon (37) or as a resolving agent in racemic resolutions (3,29). D-Malate can be formed from maleate (*cis* isomer of fumarate) with maleate hydratase (malease; EC 4.2.1.31). This enzyme has been purified from rabbit kidneys (11) and has also been detected in several *Pseudomonas* spp. (17,27,34).

Maleate can be used as a carbon and energy source by microorganisms (24) and is an intermediate in one of the degradation pathways of gentisate (7,17) and in the degradation of the pyridine ring (1,5). Two degradation pathways for maleate have been described (see Fig. 1). In the first pathway, maleate is transformed into fumarate by maleate *cis-trans*-isomerase (24). Fumarate is then further degraded in the citric acid cycle. In the second degradation pathway, maleate is directly hydrated to D-malate (17,27). D-Malate is degraded by several microorganisms via an inducible, NAD^+ dependent D-malic enzyme (14,16,18-20,33). This enzyme catalyzes the oxidative decarboxylation of D-malate to pyruvate and CO_2. D-Malate can also be cleaved by a coenzyme A- and ATP-dependent enzyme to acetyl-coenzyme A and glyoxylate (32). D-α-Hydroxy acid dehydrogenase from yeasts (8) and animal mitochondria (38) and succinate dehydrogenase from pig hearts (10) were reported to transform D-malate into oxaloacetate, but it is not certain if these enzymes also play a role in the *in vivo* degradation of D-malate. Besides these three pathways, an $NADP^+$-dependent degradation pathway for D-malate has also been suggested (18).

In this report, we describe a screening procedure to select strains exhibiting malease activity but devoid of maleate *cis-trans*-isomerase activity. These strains should theoretically be capable of stoichiometric formation of D-malate from maleate. One strain, *Pseudomonas* sp. strain NCIMB 9867, was studied in more detail especially with respect to the enantiomeric composition of the D-malate formed.

MATERIALS AND METHODS

Isolation of microorganisms

Soil samples (5 g) collected from different polluted sites were incubated in 30 ml of mineral salts medium (15) containing 2 g of maleate per liter in serum bottles (130 ml) under different conditions. For standard conditions, the pH of the medium was adjusted to 7.0 and the enrichment culture was incubated statically at 30°C. For denitrifying conditions, 5 g of sodium nitrate per liter was added to the medium, the serum bottle was completely filled with the medium, and a syringe was placed in the rubber septum to allow simple monitoring of N_2 evolution. For low pH, the medium was adjusted to pH of 4 with phosphoric acid. For the selective isolation of enterobacteria, 1.5 g of bile salts per liter and 5 g of NaCl per liter were added to the medium. For the selective isolation of yeasts, 100 mg of streptomycin per liter and 200 mg of chloramphenicol per liter were added to the medium. For conditions of high temperature, the enrichment cultures were incubated at 45°C or 58°C.

The enrichment cultures were incubated for 2 weeks after which 5 ml was transferred to fresh medium and the new enrichment culture was incubated for another 2 weeks. After a second transfer and 2 week incubation period, a loopful was streaked onto mineral salts medium agar plates containing 2 g of maleate per liter.

Colonies were streaked to purity and maintained on yeast extract-glucose agar slants.

Culture collection strains were obtained from the culture collections of both the Department of Microbiology and the Department of Food Science, Wageningen Agricultural University, Wageningen, The Netherlands. *Pseudomonas* sp. strain NCIMB 9867 was obtained from the National Collections of Industrial and Marine Bacteria, Aberdeen, Scotland.

Medium and cultivation

Strains were cultivated in 300-ml Erlenmeyer flasks containing 75 ml of rich medium. Rich medium contained the following (per liter of demineralized water): 10 g of casein peptone, 7.5 g of yeast extract, 7.5 g of malt extract, 5 g of glucose and 2 g of maleate. The pH of the medium was adjusted to 7.0 with sodium hydroxide. Cells were cultivated for 30 h at 30°C on a reciprocal shaker oscillating at 1 Hz with an amplitude of 10 cm.

Cells of *Pseudomonas pseudoalcaligenes* used to determine if maleate was oxidized and degraded by intact cells were grown on the mineral salts medium containing 3 g of dicarboxylic acid per liter (pH 7.0) as sole carbon source. Final cell densities and growth rates on dicarboxylic acids were determined by monitoring the optical density at 660 nm. In these growth experiments, the substrate concentrations were 1 g of dicarboxylic acid per liter or 1 g of dicarboxylic acid plus 1 g of maleate (each per liter)(pH 7.0).

The growth substrate range of *P. pseudoalcaligenes* NCIMB 9867 was determined by cultivating this strain on mineral salts medium containing 1 g of substrate per liter.

Permeabilization of cell suspensions

The cells were permeabilized by the method of Miozzari et al. (23). The strains used in the screening were harvested (10 min at 16,000 × *g*) and washed once with 50 mM potassium phosphate buffer (pH 7.0). The cells were then resuspended in 2.5 ml of 50 mM potassium phosphate buffer (pH 7.0) with 0.1% (wt/vol) Triton X-100 and stored overnight at -20°C.

Enzyme assays with permeabilized cells

To determine malease and maleate *cis-trans*-isomerase activities, 2.5 ml of permeabilized cells (100 to 350 mg [dry weight]) was diluted in a 35-ml serum bottle with 7.5 ml of double-strength mineral salts medium containing 50 mM Tris-HCl (pH 7.0) and 20 mM maleate. The serum bottles were closed with rubber septa and incubated statically in a water bath at 30°C. Because of the high cell densities, these cell suspensions quickly became anaerobic. Samples were taken from the incubation mixtures after 0.5, 1.5, and 4 h, and the supernatants were analyzed for maleate, malate, and fumarate by high-performance liquid chromatography (HPLC).

HPLC analysis

Malate, fumarate and maleate were analyzed by HPLC by using the method described by Skelly (30). The dicarboxylic acids were separated on a C_{18} column (200 by 3 mm; Chrompack, Middelburg, The Netherlands) at room temperature. The mobile phase was 2 mM octylamine-25 mM potassium phosphate buffer (pH 7.0) in distilled water. The flow rate was 0.4 ml/min, and the organic acids were detected at 210 nm by using a variable wavelength detector. The column had to be equilibrated for at least 3 h with the mobile phase before a reproducible separation of the organic acids was achieved.

Dry weight

The cell suspension (1 ml) was put in a glass tube with a predetermined weight. The tube was placed overnight in a stove at 130°C. The glass tube was allowed to cool in a desiccator containing dried kiesel gel. The weight of the tube was again determined. Dry weights were corrected for the dry weight of the screening buffer which was determined in the same way.

Determination of L-malate

L-Malate concentrations were determined enzymatically. To 1.6 ml of 50 mM glycylglycine-10 mM glutamate buffer (pH 10.0) were added 100 μl of supernatant sample diluted with distilled water resulting in L-malate concentrations lower than 2 mM, 200 μl of 20 mM NAD^+, 50 μl of glutamate-oxaloacetate transaminase (~10 U), and 50 μl of L-malate dehydrogenase (~15 U). The absolute absorption increase after the addition of L-malate dehydrogenase was determined at 340 nm, and the L-malate concentration was calculated by using L-malate standards.

Respiration experiments

Dicarboxylic acid-dependent oxygen uptake experiments were performed as described previously (15) by determining the difference in oxygen uptake rates of whole cells before (endogenous oxygen uptake rate) and after the addition of substrate (final concentration, 0.33 mM).

Production of malate

Malate used for the determination of optical rotation and enantiomeric purity was produced from either maleate, maleic anhydride, or fumarate by permeabilized cells of *P. pseudoalcaligenes* grown on the rich medium. Ten grams (dry weight) of permeabilized cells were suspended in 30 ml of substrate solution (1.0 M) which had been adjusted to pH 7.0 with sodium hydroxide. This suspension was incubated for 3 days in a static water bath at 30°C. The suspension was then spun down (15 min at 27,000 × *g*) and the supernatant was used to determine the optical purity and the optical rotation of the malate formed.

Optical rotation measurements

The optical rotation of enzymatically produced malate was assayed by the method described by Krebs and Eggleston (21). Supernatant was made up to a total volume of 10 ml with 5 ml of 20% (wt/vol) $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$, 1 ml of 50% (vol/vol) acetic acid, 1 ml of 1.25 M sodium citrate, and deionized water. The mixture was centrifuged when precipitation was observed. The optical rotation, (final malate concentration in polarimeter, approximately 50 mM) was measured at room temperature at 589 nm in a 10.0-cm cuvette with a Perkin-Elmer 241 polarimeter.

Determination of the enantiomeric purity

The D-malate concentration of a 500-times-diluted supernatant sample was determined by using the enzymatic D-malic acid test kit of Boehringer with D-malate standards. A correction was made for the 1.3% L-malate determined enzymatically to be present in the commercially available D-malate. The L-malate concentration was determined in the undiluted sample (containing ~500 mM malate). The total absorption increase in the enzymatic L-malate assay was not influenced by the presence of these high concentrations D-malate.

Chemicals

L-Malate dehydrogenase (for analytical purposes, solution in glycerol), glutamate-oxaloacetate transaminase and NAD^+ were from Boehringer. Octylamine, maleate, and glycylglycine were from Aldrich. Fumarate, maleic anhydride, ammonium molybdate and D-malate were from Janssen Chimica. Triton X-100, L-malate and sodium citrate were from Merck. Casein peptone and yeast extract were from GIBCO and malt extract and agar (no. 3) were from Oxoid.

RESULTS

Screening

Two hundred microorganisms isolated on maleate under different environmental conditions (see materials and methods) and 116 microorganisms from culture collections were screened for their ability to convert maleate into D-malate. No effort was made to identify the isolated strains.

Cells used in the screening were permeabilized with Triton X-100. A concentration of 0.1% was found to be optimal for most types of microorganisms. Fumarate accumulation during incubation of permeabilized cells with maleate under anaerobic conditions was used as the selection criterion to discriminate between the two known maleate degradation pathways (Fig. 1). In Table 1, the distribution of the capacity of permeabilized cells of

Table 1. Distribution of the different maleate-transforming activities among various microorganisms from culture collections

Genera	Total no. of strains tested	No. of strains exhibiting:		
		No maleate consumption	Maleate consumption	
			Fumarate accumulation	No fumarate accumulation
Bacteria				
Acinetobacter	1			1
Actinoplanes	1			1
Arthrobacter	3		1	2
Bacillus	4	1	1	2
Brevibacterium	4			4
Citrobacter	1	1		
Corynebacterium	4		1	3
Enterobacter	2		2	
Escherichia	2	2		
Hyphomicrobium	1	1		
Klebsiella	1			1
Lactobacillus	2	2		
Lactococcus	1	1		
Leuconostoc	2	2		
Micrococcus	1			1
Mycobacterium	5			5
Nocardia	11			11
Proteus	1			1
Pseudomonas	11		3	8
Rhodococcus	3			3
Serratia	1		1	
Streptococcus	1	1		
Streptomyces	4			4
Xanthobacter	2			2
Yeasts				
Arxula	1	1		
Candida	4	1		3
Hansenula	1			1
Kluyveromyces	1	1		
Octosporus	1	1		
Pichia	2	1		1
Rhodotorula	10	3	2	5
Saccharomyces	3	1	1	1
Schizosaccharomyces	1			1
Sporobolomyces	2	1		1
Stephanoascus	1	1		
Trichosporiella	1		1	
Trichosporon	3		1	2
Yarrowia	1			1

Table 1. (Continued)

Genera	Total no. of strains tested	No. of strains exhibiting:		
		No maleate consumption	Maleate consumption	
			Fumarate accumulation	No fumarate accumulation
Fungi				
Aspergillus	3	1		2
Botrytis	1	1		
Dipodascus	1		1	
Exophilia	1			1
Mortriella	1		1	
Penicillium	3			3
Phanerochaete	1			1
Pleurotus	1			1
Rhizopus	1			1
Trichoderma	1	1		

microorganisms from culture collections to degrade maleate and the apparent distribution of the maleate degradation pathways are shown. The data in Table 1 indicate that the maleate-hydrating activity (no fumarate accumulation) is more widely distributed than maleate *cis-trans*-isomerase activity. The strains which did not accumulate fumarate showed a stoichiometric formation of malate from maleate.

Of the 315 strains screened, 55 strains which were isolated on maleate and 74 strains from culture collections consumed maleate without any significant (less than 0.1 mM) accumulation of fumarate.

Determination of the enantiomeric composition of the malate formed by the screened microorganisms

After this initial screening, the enantiomeric composition of the malate formed by the microorganisms which did not accumulate fumarate was determined. This was done by determining the total malate concentration by HPLC and by determining the L-malate concentration by using L-malate dehydrogenase. The stereochemical composition of the malate formed by 88 strains (55 strains isolated on maleate and 33 strains from culture collections with the highest maleate degrading activity) was determined. These strains all produced D-malate with an enantiomeric purity of at least 97%.

Strains which accumulated fumarate during incubation with maleate formed a much higher percentage of L-malate than strains which did not. The 15 fumarate-accumulating strains tested produced malate which contained between 20 and 90% L-malate. When the L-malate concentration, as determined by the enzymatic assay, is plotted on a graph against the concentration of fumarate, as determined by HPLC, a straight line could be drawn through

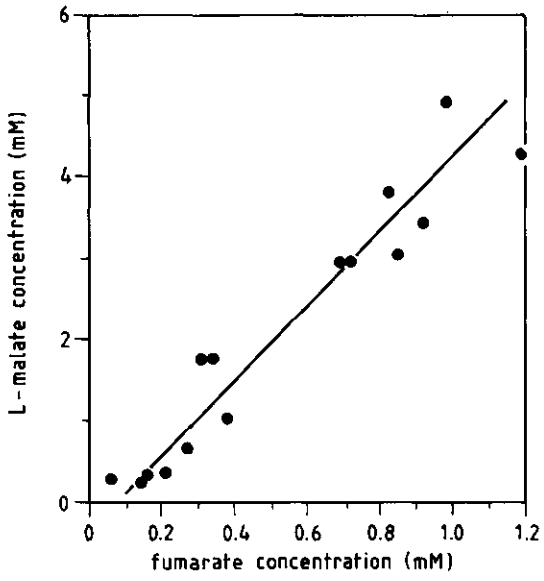

Figure 2. Relationship between L-malate and fumarate accumulated from maleate by permeabilized cells.

these points (Fig. 2). The slope of the line obtained by linear regression analysis was 4.6 ($r(x,y) = 0.96$).

Description of *Pseudomonas* sp. strain NCIMB 9867

Pseudomonas sp. strain NCIMB 9867 was selected for further studies because it had one of the highest specific malease activities and did not show as many contaminants on the HPLC chromatogram as most of the other screened microorganisms did.

This cream-colored *Pseudomonas* sp. was further characterized. It was a Gram-negative motile rod (0.3 to 0.5 by 1.3 to 2.9 μm) with one polar flagellum. It was oxidase positive and did not produce a fluorescent pigment. The strain did not contain arginine dihydrolase, urease, or β-galactosidase activity. It was able to reduce nitrate to nitrite but was not able to denitrify. The strain did not hydrolyze esculin, gelatin, or Tween 80. It could grow at 41°C and did not ferment glucose. It could utilize fructose, L-arginine, L-serine, L-phenylalanine, β-alanine, itaconate, mesaconate, fumarate, citraconate, succinate, glutarate, glycerate, L-malate, D-malate, β-hydroxybutyrate, glycerol, ethanolamine or betaine as the sole source of carbon and energy for growth. Glucose, sucrose, maleate, malonate, acrylate, crotonate, 2,5-dimethylphenol and *m*-cresol were not utilized. On the basis of these results strain NCIMB 9867 was identified as a *P. pseudoalcaligenes* strain according to *Bergey's Manual of Systematic Bacteriology* (25).

Although permeabilized cells of *P. pseudoalcaligenes* could convert maleate into D-malate and Hopper et al. (17) showed that gentisate is degraded via maleate in this microorganism, this strain was not able to grow on maleate as the sole source of carbon and energy, presumably because of its inability to transport maleate into growing cells. Of the other

screened strains which did not accumulate fumarate during maleate degradation, not one of the 33 strains with the highest maleate degrading activity from the culture collections and only 11 of the 55 strains isolated on maleate were actually able to grow on maleate.

Intact cells of *P. pseudoalcaligenes* grown on several dicarboxylic acids (fumarate, succinate, L-malate, D-malate, itaconate, mesaconate or citraconate) did not oxidize or degrade maleate, although maleate was converted into D-malate by permeabilized cells grown on these substrates.

In growth experiments with dicarboxylic acids (fumarate, succinate, L-malate, D-malate, itaconate, mesaconate, and citraconate), no increase in the optical density was observed when maleate was added in comparison with a control without maleate. The presence of maleate did not influence the growth rates of *P. pseudoalcaligenes* on these dicarboxylic acids.

Characterization of D-malate production by *P. pseudoalcaligenes*

When permeabilized cells of *P. pseudoalcaligenes* were incubated with 15 mM maleate under anaerobic conditions, maleate was degraded within 1 h to a level below the detection limit (<0.01 mM), with a stoichiometric formation of D-malate. No fumarate (detection limit of fumarate, ~ 0.002 mM) or L-malate could be detected. D-Malate consumption was not observed during the time of the experiment (5 h). Under aerobic conditions, a similar yield of D-malate from maleate was observed.

Maleic anhydride, which chemically hydrates to maleic acid and which is more then two times cheaper then maleic acid, was also completely transformed into D-malate by permeabilized cells of *P. pseudoalcaligenes*.

The optical rotation of the malate formed from maleate, maleic anhydride and fumarate by *P. pseudoalcaligenes* was determined after complexation with molybdate and citrate (Table 2). The specific optical rotation of the products of the hydratase reactions was in agreement with the specific rotation of the expected products. Also, an increase in optical rotation due to the presence of citrate, specific for malate (21), was observed. The observed specific optical rotations indicate an enantiomeric purity which is close to 100% for the malate formed.

The enantiomeric excess of the D-malate formed from maleate and maleic anhydride was

Table 2. Specific optical rotations ($[\alpha]^{25}_D$) of the products formed from the unsaturated acids by *P. pseudoalcaligenes* after complexation with molybdate and citrate

Product	$[\alpha]^{25}_D$
From maleate	- 111.8
From maleic anhydride	- 111.9
From fumarate	+111.9
D-Malate (Janssen Chimica)	- 111.6
L-Malate (Merck)	+111.0

determined more precisely by using commercially available L-malate dehydrogenase and D-malic enzyme. An enantiomeric purity of 99.97% was determined for the D-malate formed from maleate, and an enantiomeric purity of 99.996% was determined for the D-malate formed from maleic anhydride.

DISCUSSION

In this report, the production of D-malate from maleate was demonstrated for a number of culture collection strains and environmental isolates.

Two enzymes are known that transform maleate: (i) malease yielding D-malate, and (ii) maleate *cis-trans*-isomerase, yielding fumarate, and as a consequence, the undesired L-malate is formed by the action of the citric acid cycle enzyme fumarase (Fig. 1). As we wanted to select for strains containing only the first enzyme, a simple screening method was used in which incubations of permeabilized cells with maleate were analyzed by HPLC for malate and fumarate accumulation. The microorganisms used in the screening were permeabilized with Triton X-100 since the uptake of dicarboxylic acids (e.g., fumarate, L-malate, D-malate, and succinate) into the microbial cell is known to be dependent on specific transport mechanisms (28). Because we wanted to select microorganisms which do not produce maleate *cis-trans*-isomerase under any circumstances, maleate, which has been reported to be an inducer of maleate *cis-trans*-isomerase (35), was included in the growth medium.

Accumulation of fumarate during incubation of permeabilized cells with maleate was shown to be indicative for the presence of the unwanted maleate *cis-trans*-isomerase activity. The fumarase reaction has an equilibrium constant (K_{eq} = [L-malate]/[fumarate]) of 4.5 under the assay conditions used (2), and, as a consequence, both fumarate and L-malate will accumulate under anaerobic conditions. As fumarate can be detected with a 70-times-higher sensitivity than malate, low levels of maleate *cis-trans*-isomerase activity could already be detected.

Fumarate was shown to accumulate at about 22% of the L-malate concentration (Fig. 2) in experiments were the L-malate concentration was determined separately. This ratio is in good agreement with the equilibrium constant for fumarase. The enantiomeric composition of the malate formed can therefore be estimated fairly accurately from one HPLC run in which the total malate and fumarate concentrations are determined.

The enantiomeric composition of the malate formed by some of the fumarate-accumulating microorganisms showed a remarkable variation (between 20 and 90% L-malate was detected). Apparently all of these strains containing maleate *cis-trans*-isomerase also contained malease activity. Rahatekar et al. (27) already reported a *Pseudomonas* strain that contained both malease and maleate *cis-trans*-isomerase activity.

All strains degrading maleate without a significant accumulation of fumarate, produced D-malate with an enantiomeric purity of at least 97%. These strains probably only contain malease activity for the degradation of maleate. When maleate is degraded via malease, it is transformed into D-malate, which is not oxidized any further under anaerobic conditions. Theoretically, D-malate could be converted into L-malate via oxidation to oxaloacetate and a subsequent reduction to L-malate. D-Malate dehydrogenase has to our knowledge, however, not yet been described in bacteria. Furthermore, no interconversion of D- and L-malate has been reported in the literature even when the enantiomers are degraded by a common pathway (18,31-33). Fumarate formation from D-malate under anaerobic conditions is therefore not very likely to occur. This was also confirmed by the fact that we were able to select malease-containing microorganisms which exclusively accumulated D-malate from maleate.

Malease activity appeared to be widespread among bacteria, yeasts, and fungi (Table 1). Of the culture collection strains tested, at least 63% contained malease activity and no maleate *cis-trans*-isomerase activity. This percentage was much lower (28%) in the strains isolated on maleate. All strains reported in the literature and most of our own isolates capable of growth on maleate as the sole source of carbon and energy contained the undesired maleate *cis-trans*-isomerase activity. Therefore, the selection of maleate-utilizing strains for the production of D-malate from maleate was not very worthwhile.

The percentage of microorganisms containing malease activity was much higher than we expected, indicating that the enzyme may play a role in central metabolism, especially since most of these microorganisms were unable to grow on maleate. Maleate is known to be an intermediate in the aerobic degradation pathway of nicotinic acid, and, therefore, malease might play a role in the salvaging of pyridine nucleotides under conditions of nutrient depletion (22). The aerobic degradation pathway for nicotinic acid described by Behrman and Stanier (1) involves maleate conversion into fumarate by maleate *cis-trans*-isomerase; however, on the basis of the results in Table 1, the hydratation of maleate to D-malate is much more likely to occur in most microorganisms.

Only 20% of the malease-positive, maleate-*cis-trans*-isomerase-negative strains were able to grow on maleate as the sole source of carbon and energy. In addition, most of the malease-positive, maleate-*cis-trans*-isomerase-negative strains which were isolated from enrichment cultures with maleate also did not grow on maleate. Possibly, the enrichment cultures still contained a large number of microorganisms which were unable to grow on maleate but which could grow on impurities in the agar plates. Evans et al. (13) found similar results. Of 32 strains positive for phenylalanine ammonia-lyase that were isolated on *trans*-cinnamic acid, not one strain was able to grow on *trans*-cinnamic acid as the sole source of carbon and energy.

Pseudomonas sp. strain NCIMB 9867, which was selected for further studies, was previously reported to be a *Pseudomonas alcaligenes* by Poh and Bayly (26). Strain NCIMB 9867 does not, however, contain arginine dihydrolase activity and was able to grow on

fructose, β-hydroxybutyrate, mesaconate, itaconate, glycerate, glutarate, L-serine, L-phenylalanine, glycerol, ethanolamine and betaine and is therefore reclassified as *P. pseudoalcaligenes* according to *Bergey's Manual of Systematic Bacteriology* (25). Growth on 2,5-dimethylphenol and *m*-cresol, as reported by Hopper et al. (18), was not observed. Poh and Bayly (26) already found that spontaneous mutants which had lost the ability to grow on these two substrates were isolated readily, and they suggested that a plasmid is involved in the degradation of these two compounds.

P. pseudoalcaligenes did not grow on maleate, although it was able to grow on 3-hydroxybenzoate and gentisate, compounds which are known to be degraded through maleate and D-malate by this organism (17). Even when *P. pseudoalcaligenes* was grown on other dicarboxylic acids, which might induce a transport mechanism also capable of maleate transportation (28), intact cells were not able to oxidize or degrade maleate. The most likely explanation for the inability of *P. pseudoalcaligenes* to grow on maleate, therefore, is that it is not capable of synthesizing a transport mechanism for maleate.

Maleate is degraded by permeabilized *P. pseudoalcaligenes* to undetectable levels under anaerobic conditions with a concomitant formation of D-malate. The molar yield is close to 100%. From the equilibrium constant of the fumarase reaction (2) and the free energy for the maleate *cis-trans*-isomerase reaction (9), a K_{eq} ([D-malate]/[maleate]) of 275 can be calculated (30°C). This equilibrium constant corresponds to a maximum theoretical yield of 99.6%. This conversion is significantly higher than that observed for the fumarase reaction (82%)(2) and that observed for the mesaconate (2-methylfumarate) hydratase reaction (85%)(4). Citraconate (2-methylmaleate) can, however, be converted to D-citramalate, with a yield which is also close to 100% (34).

The stoichiometric formation of D-malate from maleate, even under aerobic conditions, indicates that there is no D-malate degrading activity present in permeabilized cells of this strain. Hopper et al. (18) already reported that there was no D-malate degrading activity detectable in cell extracts of this strain.

The stereospecificity of the malease reaction is very high, and the small amount of L-malate which is formed (0.03%) can be accounted for almost completely by the contamination with fumarate (0.02%) of the maleate used. The contaminating fumarate is converted into L-malate by fumarase, resulting in a reduced enantiomeric purity. By using maleic anhydride, which contains less fumarate, D-malate with an enantiomeric purity of 99.996% was produced. The enantiomeric purity of the D-malate produced with the malease reaction is very good compared with that of the commercially obtained D-malate, which was 98.7%.

Subsequent work will focus on the optimization of D-malate production from maleate with the selected *P. pseudoalcaligenes* strain.

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CHAPTER 4

INDUCTION OF MALEATE HYDRATASE IN *PSEUDOMONAS PSEUDOALCALIGENES*

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SUMMARY

The highest malease activity (2.0 μmol·min^{-1}·[mg protein]$^{-1}$) in *P. pseudoalcaligenes* was observed when grown on 3-hydroxybenzoate. Growth on gentisate also resulted in an enhanced malease activity. The specific malease activity of cells grown on 3-hydroxybenzoate was constant during the logarithmic phase, but dropped rapidly as soon as growth ceased.

Maleate hydratase (malease; EC 4.2.1.31) catalyzes the stereospecific hydration of maleate to D-malate, a versatile chiral synthon for organic synthesis (8). After an intensive screening we have selected *Pseudomonas pseudoalcaligenes* NCIMB 9867 for further studies (9). Recently, several other groups have also studied this microbial bioconversion using other biocatalysts (1,5,6).

In this report we describe the effect of the growth conditions on the induction of malease activity in *P. pseudoalcaligenes*.

Effect of growth substrate on the induction of malease

P. pseudoalcaligenes was tested for growth (30°C) in a mineral salts medium at pH 7 (2) with different carbon sources (1 g/l). Growth substrates tested were unsaturated acids, hydroxy acids, organic acids, amino acids, alcohols, sugars and aromatic compounds. The cultures in which growth was observed were used to inoculate [0.5-1% (v/v)] a new culture (250 ml of liquid medium in a 1-liter Erlenmeyer) of which the doubling time was determined by following the increase in OD_{660} with a spectrophotometer. Cells were harvested during the log-phase at approximately 80% of the maximal obtainable OD_{660}, washed and permeabilized with Triton X-100 (9). The malease activity of these cells was determined (9) using 500 mM Na_2-maleate in 200 mM Tris-buffer (pH 7.5) as the substrate (Table 1).

Although permeabilized cells of *P. pseudoalcaligenes* can convert maleate into D-malate, maleate can not serve as a growth substrate for this strain (Table 1), probably because it lacks a transport mechanism for maleate (9). Malease activity in *P. pseudoalcaligenes* was inducible and cells grown on 3-hydroxybenzoate had the highest specific malease activity. Also growth on gentisate resulted in an enhanced malease activity (Table 1). Growth on other substrates resulted in a constitutive level of malease activity in the range of 100-200 $nmol \cdot min^{-1} \cdot (mg\ protein)^{-1}$.

Table 1. Malease activity and doubling times of cells grown on various carbon sources

Growth substrate	Malease activity ($nmol \cdot min^{-1} \cdot [mg\ protein]^{-1}$)	Doubling time (h)
Maleate	No growth	-
D-Malate	50	4.9
L-Malate	110	1.1
Citraconate	160	3.3
Fumarate	100	0.9
Mesaconate	140	1.4
Itaconate	120	1.7
Gentisate (2,5-dihydroxybenzoate)	750	4.7
3-Hydroxybenzoate	2000	2.4
4-Hydroxybenzoate	210	2.0
L-Phenylalanine	180	3.9

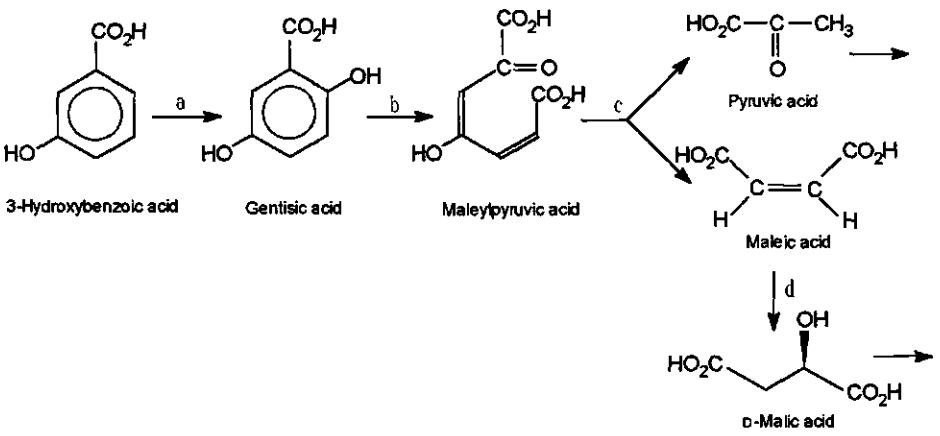

Figure 1. Degradation pathway of 3-hydroxybenzoate in *P. pseudoalcaligenes* NCIMB 9867 [from Hopper et al. (3,4)]. a, 3-Hydroxybenzoate-6-hydroxylase; b, gentisate 1,2-dioxygenase; c, maleylpyruvyl hydrolase; d, malease.

3-Hydroxybenzoate is degraded via gentisate and maleate to D-malate by *P. pseudoalcaligenes* NCIMB 9867 (3,4)(Fig. 1). In view of this degradation pathway for 3-hydroxybenzoate, the induction of malease by 3-hydroxybenzoate and gentisate is not surprising. It was shown previously (7) that specific activities of the earlier enzymes in the 3-hydroxybenzoate degradation pathway of this strain were also higher in 3-hydroxybenzoate grown cells than in cells grown on gentisate.

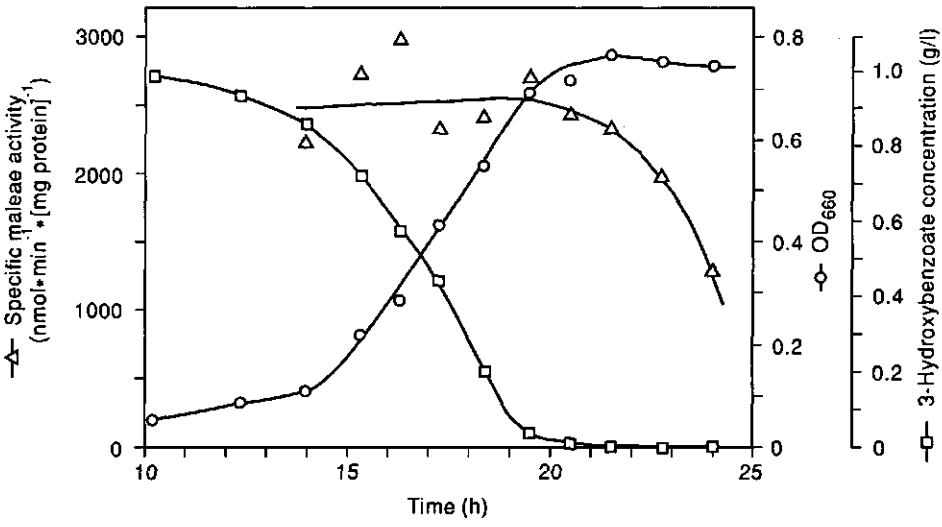

Figure 2. Malease activity during growth of *P. pseudoalcaligenes* on 3-hydroxybenzoate. ●, OD660; ▲, specific malease activity; ■, 3-hydroxybenzoate concentration.

Malease activity as a function of the growth phase

In a first attempt to determine the malease activity of *P. pseudoalcaligenes* grown on several substrates, it was observed that the malease activity varied considerably between different cultures. Therefore, *P. pseudoalcaligenes* was grown on 1 g/l 3-hydroxybenzoate (1 liter medium in a 5-liter Erlenmeyer, 30°C). Samples were taken at different times to determine the OD660, the 3-hydroxybenzoate concentration [analyzed by HPLC at 280 nm on a reversed phase (C_{18}) column using 5% methanol and 10 mM potassium phosphate buffer (pH 7.0) as the mobile phase] and the specific malease activity (Fig. 2). The specific malease activity remained constant during the logarithmic growth phase, but dropped rapidly as soon as all the substrate was consumed, at the end of the logarithmic phase. This drop in specific malease activity could not be prevented by the addition of the protein synthesis inhibitor chloramphenicol or the protease inhibitor α-toluenesulfonyl fluoride.

Conclusions

The highest malease activity was observed in *P. pseudoalcaligenes* when it was grown on 3-hydroxybenzoate. However, at the end of the logarithmic phase the malease activity dropped rapidly. Consequently, to obtain cells with maximal malease activity for the production of D-malate, care should be taken to harvest cells before 3-hydroxybenzoate is exhausted.

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CHAPTER 5

PURIFICATION AND CHARACTERIZATION OF MALEATE HYDRATASE FROM *PSEUDOMONAS PSEUDOALCALIGENES*

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SUMMARY

Maleate hydratase (malease) from *Pseudomonas pseudoalcaligenes* has been purified. The purified enzyme (98% pure) catalyzes the stereospecific addition of water to maleate and citraconate (2-methylmaleate) forming D-(+)-malate and D-(+)-citramalate, respectively. 2,3-Dimethylmaleate was also a substrate for malease. The stability of the enzyme was dependent on the protein concentration and the addition of dicarboxylic acids. The purified enzyme (89 kDa) consisted of two subunits (57 and 24 kDa). No cofactor was required for full activity of this colorless enzyme. Maximum enzyme activity was measured at pH 8 and 45°C. The K_m for maleate was 0.35 mM, and that for citraconate was 0.20 mM. Thiol reagents, such as *p*-chloromercuribenzoate and iodoacetamide, and sodium dodecyl sulphate completely inhibited malease activity. Malease activity was competitively inhibited by D-malate (K_i = 0.63 mM) and D-citramalate (K_i = 0.083 mM) and by the substrate analog 2,2-dimethylsuccinate (K_i = 0.025 mM). The apparent equilibrium constants for the maleate, citraconate, and 2,3-dimethylmaleate hydration reactions were 2050, 104 and 11.2, respectively.

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INTRODUCTION

Hydratases are commercially interesting enzymes since they can hydrate unsaturated compounds, forming optically pure alcohols (100% yield and 100% enantiomeric excess are theoretically possible). An example is the industrial production of L-malate from fumarate with immobilized cells of *Brevibacterium flavum* (9).

Since hydration does not involve a net oxidation or reduction, no expensive cofactors should be required or have to be regenerated. Not all hydratases are cofactor independent, however. In fact, most of the hydratases described so far require one or more cofactors.

Cofactors reported for hydratases are pyridoxal phosphate [for amino acid dehydratases (25)], coenzyme A [e.g., for crotonase (1)], metal ions [e.g., for enolase (6)], enzyme-bound pyridine nucleotide (NAD^+) [e.g., for CDP-glucose-4,6-dehydratase (20)], iron-sulfur clusters [e.g., for aconitase (2)], pyrroloquinoline quinone [for nitrile hydratase (19)], and adenosylcobalamin (coenzyme B_{12})[e.g., for propanediol dehydratase (26)]. These cofactors have a function in substrate binding (including water)(e.g., iron-sulfur clusters, pyrroloquinoline quinone and metal ions), in polarization of the substrate (e.g., coenzyme A and metal ions), in temporary binding of H^+ or OH^- during acid-base catalysis (e.g., iron-sulfur clusters), in stabilization of carbocation intermediates (e.g., pyridoxal phosphate), in converting the substrate after it is bound to the enzyme into a substrate which can be dehydrated (enzyme-bound NAD^+), or as a producer of radicals (coenzyme B_{12}).

Maleate hydratase (malease; EC 4.2.1.31) purified from rabbit kidney (10) catalyzes the hydration of maleate to D-(*R*)-malate. It contains an iron-sulfur cluster and is dependent on a divalent metal ion and a sulfur compound for activity.

Malease is also present in microorganisms (27) and D-malate production from maleate was studied in more detail in *Pseudomonas pseudoalcaligenes* NCIMB 9867. This strain produced D-malate with an enantiomeric purity of more than 99.9%. To further assess the potential of this malease as an industrial biocatalyst, characterization of the microbial enzyme with respect to cofactor requirements, substrate specificity, and stability was required.

In this report we describe the purification and characterization of maleate hydratase from *P. pseudoalcaligenes*.

MATERIALS AND METHODS

Organism and growth conditions

P. pseudoalcaligenes NCIMB 9867 was grown in a 30-liter fermenter with 20 liters of mineral salts medium (13) containing 1 g of 3-hydroxybenzoate per liter at 30°C and pH 7. When all the 3-hydroxybenzoate was consumed, as judged by the drop in the oxygen consumption rate, an additional amount of 3-hydroxybenzoate

(20 g) was added. This two-stage addition of substrate was used to prevent toxicity problems. Cells were harvested immediately after all the 3-hydroxybenzoate was consumed (as judged by the decrease in the oxygen consumption rate). The broth was cooled to 10°C and cells were concentrated with a Pellicon cross-flow filtration unit (Millipore) at 0°C to a volume of 2 liters. The cells were then collected by centrifugation (4°C, 10 min at 16,000 × *g*) and washed with 50 mM phosphate buffer (pH 7.0). The pellet was resuspended in 100 ml of the same buffer and stored at -20°C in 7-ml aliquots.

Purification of malease

All purification steps were performed at 4°C, and the pH of the buffers used was 7.0 unless specified otherwise.

(i) Preparation of cell extract

Three 7-ml aliquots of the frozen cell suspension were thawed and disrupted by sonication (6 min; 30% duty cycle; output control, 2.3) with a Branson Sonifier 250. Cell debris was removed by centrifugation at 20,000 × *g* for 20 min. The supernatant was used as the cell extract (~420 mg of protein).

(ii) Anion-exchange chromatography

The cell extract was applied to a DEAE-Sepharose CL-6B column (5.5 by 30 cm) equilibrated with 10 mM phosphate buffer containing 20 mM DL-malate. The column was washed with 400 ml of the same buffer (flow rate, 2.3 ml·min^{-1}; collected fraction volume, 23 ml), and subsequently the enzyme was eluted with a 0 to 500 mM linear gradient of NaCl in the same buffer (total volume, 2 liters). Malease eluted at an NaCl concentration of 40 mM. Active fractions were pooled. Protein was precipitated by bringing the pooled fractions to 80% ammonium sulphate saturation. After 15 min at 0°C, the precipitate was collected by centrifugation (15 min at 27,000 × *g*). The pellet was resuspended in 4 ml of 50 mM phosphate buffer.

(iii) Gel filtration

The solubilized precipitate was applied to a Sephacryl S300 column (3 by 65 cm) equilibrated with 25 mM phosphate buffer containing 50 mM DL-malate and eluted with the same buffer (flow rate, 0.4 ml·min^{-1}; collected fraction volume, 4 ml). Fractions containing malease were pooled and concentrated by ultrafiltration with an Amicon ultrafiltration unit, using a membrane with a cutoff of 30.000 under helium at a pressure of 4×10^5 Pa to a volume of approximately 4 ml.

(iv) Hydroxyapatite

The concentrate from the gel filtration step was applied to a hydroxyapatite column (1.2 by 15 cm) equilibrated with 10 mM phosphate buffer containing 10 mM DL-malate and eluted with the same buffer (flow rate, 0.2 ml·min^{-1}; collected fraction volume, 2.5 ml). The fractions containing malease were pooled and concentrated by ultrafiltration under helium at a pressure of 4×10^5 Pa to a volume of about 4 ml.

(v) Mono Q

Samples of 1 ml from the hydroxyapatite step were applied to a Mono Q column (0.6 by 6 cm) operated with a fast protein liquid chromatography system (Pharmacia) at room temperature. The column was equilibrated with 10 mM Tris-HCl buffer (pH 8.0) containing 20 mM DL-malate. The enzyme was eluted using a 0 to 50 mM linear gradient of NaCl in the same buffer (flow rate, 1 ml·min^{-1}; gradient in 11 min; collected fraction volume, 1 ml). Malease eluted at an NaCl concentration of 25 mM. Fractions exhibiting malease activity were pooled.

Assay of malease and citraconase activities

Malease and citraconase activities were determined spectrophotometrically by monitoring the decrease in the absorbance due to the disappearance of substrate. The absorption of the hydroxy acid formed is negligible compared with the absorption of the unsaturated acid. Activity in the fractions was routinely measured as follows. Fifty microliters of enzyme, 100 μl of 10 mM maleate or citraconate, and 1.85 ml of 50 mM Tris-HCl (pH 7.5) were incubated at 30°C and the decrease in absorption (240 nm) was monitored for at least 3 min (for extinction coefficients, see Table 1). Specific activities were determined at 270 nm by using initial substrate concentrations of 5 mM in the activity assay and are expressed in units (micromoles per minute) per milligram of protein. The effects of inhibitors and ions were studied by adding 200 μl of a 10 mM solution to the activity assay mixture (the total volume was held at 2 ml by adding less buffer).

Protein determination

Protein was determined by the method of Bradford (5) with bovine serum albumin (BSA) as the standard.

Stability of malease

Cell extract was diluted 1:1 with 50 mM phosphate buffer (pH 7) containing the additive. These mixtures

Table 1. Extinction coefficients (ε) of maleate and citraconate at different wavelengths

Wavelength (nm)	ε^a	
	Maleate	Citraconate
230	3,300	5,400
235	2,600	4,100
240	1,900	2,900
245	1,400	1,900
250	900	1,100
255	560	650
260	350	350
265	210	190
270	120	100
275	70	55
280	40	30

[a] In liters·mole^{-1}·centimeter^{-1}.

were stored at 4°C, and malease activity was measured each day.

Determination of molecular weight

The molecular weight of the native protein was determined by gel filtration on a Sephacryl S300 column as described above ("Purification procedure, (iii)"). The column was calibrated by using a Pharmacia low-molecular-weight calibration kit containing phosphorylase *b* (molecular weight, 94,000), BSA (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and α-lactalbumin (14,400).

The molecular weights of the subunits were determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). A 12.5% (wt/vol) separation slab gel was prepared by the method of Laemmli (15). The reference proteins were the same as used for the determination of the molecular weight of the native protein. Proteins were stained with Coomassie brilliant blue G.

Determination of amino acid composition

Purified malease was hydrolyzed in 6 N HCl for 16 h at 105°C. HCl was removed by evaporation with a rotary evaporator at 50°C, and the resulting residue was used for analysis on a Biotronic LC 6000E amino acid analyzer equipped with a separation program for physiological solutions. No modifications for cysteine or methionine were performed, and tryptophan was destroyed by the method used, so these amino acids could not be determined.

Determination of kinetic constants

Specific activities were determined spectrophotometrically at initial substrate concentrations of 0.25, 0.5, 1, 2.5, 5, and 10 mM. Depending on the substrate concentration, the wavelength was adjusted to obtain an absorbance of between 0.8 and 1.2 at the start of the activity assay. The decrease in absorption was monitored at 30°C.

The extinction coefficients of maleate and citraconate between 220 and 280 nm were determined in 50 mM phosphate buffer at pH 7 and are given in Table 1. The extinction coefficients did not vary significantly between pHs 5.5 and 9.

The initial activities at the different substrate concentrations were set out in Lineweaver-Burk plots, and the best line through the points was obtained by linear regression. K_M and V_{max} for maleate and citraconate were calculated from these Lineweaver-Burk plots.

The inhibition constants were determined by measuring the initial activities at the above-mentioned maleate concentrations in the presence of inhibitor at a concentration that inhibited the malease activity at 0.5 mM maleate around 30 and 60%. The inhibition constants were calculated from the change in the slope of the Lineweaver-Burk plot.

Substrate specificity

The activity of purified malease towards other compounds was determined spectrophotometrically (240 nm, 30°C), either by monitoring a possible increase in absorbance for the hydroxy acids, the amino acid, and the mercapto acid or by monitoring a decrease in absorbance for the unsaturated acids. The substrate concentrations of the unsaturated compounds were chosen to give absorbance values of between 0.5 and 1.0 at the beginning of the incubation. The amount of malease used in determining the substrate specificity was varied to allow the detection of activities as low as 1% of the malease activity with maleate. With the tartrates and acetylene dicarboxylate as potential substrates, L-malate dehydrogenase was added to the assay to determine if oxaloacetate was formed. The activity assay contained 100 μl malease (~3 U), 100 μl of 100 mM substrate, 100 μl of 5 mM NADH, 5 μl of L-malate dehydrogenase (30 U), and 1.7 ml of 50 mM Tris-HCl (pH 7.5). A possible decrease in absorption was monitored at 340 nm and 30°C.

Apparent equilibrium constant

To 1.7 ml of 25 mM potassium phosphate buffer (pH 7) containing 40 mM maleate, D-malate, citraconate, D-citramalate, or 2,3-dimethylmaleate in a 30-ml serum bottle, 300 μl of malease (~10 U) was added. The serum bottles were incubated in a water bath at 25°C. The maleate-malate, citraconate-citramalate, and 2,3-dimethylmaleate concentrations in the mixtures were monitored by high-performance liquid chromatography (HPLC) until equilibrium was reached.

HPLC analysis

Malate, citramalate, maleate, citraconate, and 2,3-dimethylmaleate were analyzed by HPLC as described before (27). The dicarboxylic acids were separated on a C_{18} column with 2 mM octylamine and 25 mM potassium phosphate buffer (pH 7.0) as the mobile phase.

Optical rotation

The optical rotation of the malate and citramalate produced was assayed as described previously (27).

Chemicals

L-Malate dehydrogenase (for analytical purposes, solution in glycerol) and NADH were from Boehringer. Octylamine, D-citramalate, and 2,3-dimethylmaleic anhydride were from Aldrich. Maleate, DL-malate, and D-malate were from Janssen Chimica. DEAE-Sepharose CL-6B, Sephacryl S300, and Mono Q were obtained from Pharmacia, and hydroxyapatite was from Bio-Rad.

2,3-Dimethylmaleate was prepared from 2,3-dimethylmaleic anhydride by adding sodium hydroxide. Isopropylmaleate was isolated from the culture medium of *Candida maltosa* G587, a kind gift of R. Bode, according to the method described by Bode et al. (4). All other chemicals were of analytical grade (at least 98% pure).

RESULTS

Malease stability

Because considerable losses in malease activity were observed during initial attempts to purify malease, the stability of this enzyme was studied first. Addition of DL-malate (50 mM) to the phosphate buffer (pH 7) stabilized malease. This stabilization was concentration dependent; higher DL-malate concentrations resulted in a better stabilization of malease activity, and D- and L-malate were equally effective. Succinate also stabilized malease, although it was less effective than DL-malate.

Therefore, phosphate buffer (pH 7) containing DL-malate was used during the different purification steps. Because DL-malate has an ionic strength which is three times higher than

that of NaCl, the concentrations of buffer and DL-malate were varied depending on the purification step.

The stability of purified malease was also dependent on the protein concentration. In the absence of DL-malate, a diluted malease preparation (0.008 mg·ml^{-1}) lost 80% of its activity after 30 min at 30°C, while the activity of the undiluted malease preparation (0.32 mg·ml^{-1}) remained constant. Addition of 3 mg of BSA per ml to the diluted malease preparation still resulted in a 60% loss of malease activity after 30 min at 30°C.

Purification of malease

The purification scheme for the malease of *P. pseudoalcaligenes* is presented in Table 2. Malease was purified ~53-fold, with an overall yield of ~30%. SDS-PAGE revealed two distinct bands, corresponding to proteins with molecular weights of 24,000 and 57,000, and

Table 2. Purification of malease from *P. pseudoalcaligenes*

Purification step	Total protein (mg)	Specific activity (U·[mg protein]$^{-1}$)	Ratio malease to citraconase activity	Purification (fold)	Yield (%)
Cell extract	423	2.0	1.78	1	100
Anion exchange	33.7	27.7	1.72	13.6	108
Gel filtration	14.8	38.3	1.71	18.8	66.0
Hydroxyapatite	7.2	72.2	1.83	35.5	60.3
Mono Q	2.4	108	1.81	53.2	30.2

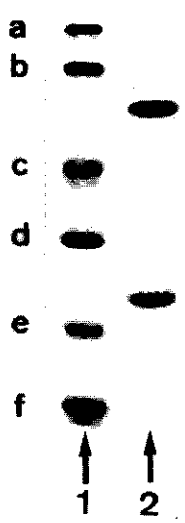

Figure 1. SDS-PAGE of malease from *P. pseudoalcaligenes*. Lane 1, molecular weight markers: a, phosphorylase *b* (94,000); b, BSA (67,000); c, ovalbumin (43,000); d, carbonic anhydrase (30,000); e, soybean trypsin inhibitor (20,100); f, α-lactalbumin (14,400). Lane 2, 20 μg of malease.

showed that the enzyme was ~98% pure (Fig. 1). The molecular weight of the native protein was 89,000 as determined by gel filtration. The enzyme consists of two subunits in an $\alpha\beta$ composition.

The absorption spectrum of this colorless protein did not give any indications of the presence of prosthetic groups.

From Table 2 it can be calculated that malease represents ~2% of the total soluble cellular protein. By using the molecular weight determined with SDS-PAGE (81,000), a turnover number of 150 molecules of maleate per molecule of malease per second could be calculated for the purified enzyme.

Amino acid composition

The amino acid composition of malease from *P. pseudoalcaligenes* is shown in Table 3. The amino acid compositions of malease from rabbit kidney (10) and of the cofactor-independent fumarase from *Escherichia coli* (28) are also shown.

Temperature optimum and pH optimum

Malease activity was maximal at approximately 45°C (Fig. 2). At temperatures above 45°C, inactivation of malease was observed during the time of the activity assay (3 min).

Malease has a quite broad pH optimum around 7.5 (Fig. 3). Succinate and phosphate buffer (50 mM) inhibited malease activity (Fig. 3; see also Table 5). At pH values above 9,

Table 3. Amino acid composition of malease

Amino acid	Amount[a] in:		
	P. pseudoalcaligenes malease	Rabbit kidney malease (10)	*E. coli* fumarase (*fumC*)(28)
Glutamate + glutamine	100	100	100
Aspartate + asparagine	110	173	88
Threonine	71	76	49
Serine	58	73	55
Proline	107	173	37
Glycine	134	116	67
Alanine	140	100	112
Valine	129	78	57
Isoleucine	84	57	43
Leucine	145	86	102
Tyrosine	23	149	8
Phenylalanine	62	70	22
Lysine	53	73	47
Histidine	33	30	29
Arginine	105	81	41

[a] The amino acid compositions of the purified enzymes are shown as a percentage of the glutamate-plus-glutamine content.

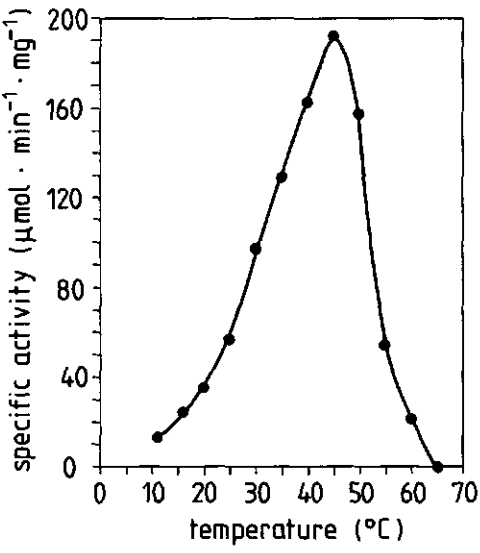

Figure 2. Specific activity of malease as a function of temperature (50 mM Tris-HCl, pH 7).

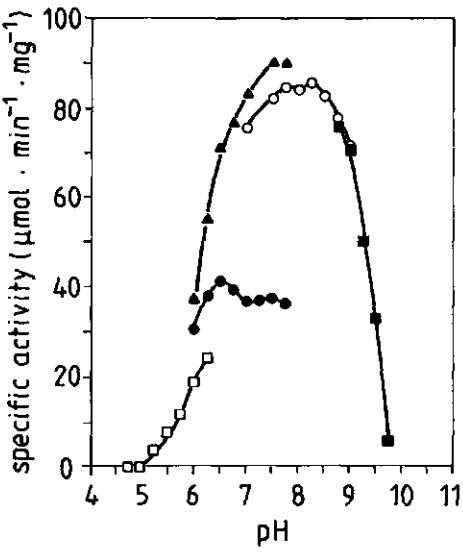

Figure 3. Specific activity of malease as a function of pH (30°C). □, 50 mM succinate buffer; ●, 50 mM phosphate buffer; ▲, 50 mM imidazole buffer; ○, 50 mM Tris-HCl buffer; ■, 50 mM glycine buffer.

inactivation of malease occurred during the time of the spectrophotometric assay.

Substrate specificity

The substrate analogs citraconate and 2,3-dimethylmaleate were also substrates for malease from *P. pseudoalcaligenes*. Citraconate and 2,3-dimethylmaleate (1 mM) were hydrated at, respectively, 54 and 0.8% of the rate of maleate hydration. The ratio of malease and citraconase activities remained constant during the purification procedure (Table 2), indicating that no additional citraconase is present in the cell extract.

The product of malease activity with maleate is D-malate (27), and the product of citraconate had the same optical configuration as commercially available D-citramalate (analyzed by optical rotation).

Malease showed less than 1% of the maleate-hydrating activity with the following compounds: fumarate, mesaconate, itaconate, dihydroxymaleate, maleatedimethylester, acetylenedicarboxylate, acrylate, methacrylate, crotonate, *cis*-3-chloroacrylate, *trans*-3-chloroacrylate, 2-chloroacrylate, 2-pentenoic acid, 3,3-dimethylacrylate, tiglic acid, glutaconate, *cis*-aconitate, *trans*-aconitate, 2-isopropylmaleate, L-malate, L-citramalate, D-tartrate, L-tartrate, *meso*-tartrate, DL-aspartate, and DL-mercaptosuccinate.

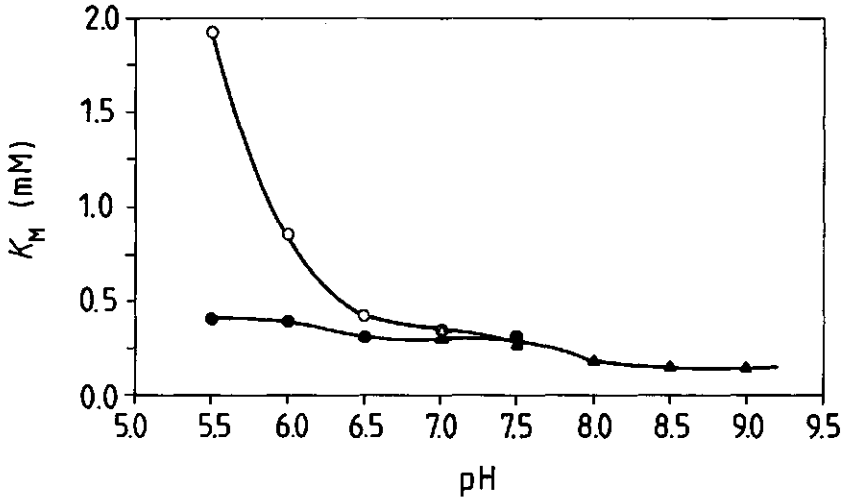

Figure 4. Michaelis-Menten constant (K_M) as a function of pH (30°C). Open symbols, K_ms based on the total maleate concentration; closed symbols, K_Ms based on the concentration of dianionic maleate. Circles, 50 mM imidazole buffer; triangles, 50 mM Tris-HCl buffer.

Kinetics

The K_M for maleate was determined at different pH values. Lineweaver-Burk plots and Eadie-Hofstee plots resulted in comparable values for K_M and V_{max}. The apparent K_M for maleate depended strongly on the pH (Fig. 4). Recalculation of the K_M values on the basis of the concentrations of the dianionic form of maleate [pKa_2 = 6.23 (23)] gave values which varied much less with the pH (Fig. 4), indicating that only maleic acid present in the dianionic form is a substrate for malease.

When the K_M of malease for maleate was determined in 50 mM phosphate buffer (pH 7.0) instead of in imidazole or Tris-HCl buffer, a much higher K_M (1.9 mM) was found while, the V_{max} was two times lower.

The K_M of malease for citraconate was 0.20 mM in Tris-HCl buffer (pH 7.5). Correction for the amount of citraconate present in the dianionic form [pKa_2 = 6.15 (23)], did not significantly change this value.

Effects of ions

The effects of several ions on malease activity were tested (Table 4). No stimulation of malease activity was observed with any of the salts tested. $NiCl_2$, $CuSO_4$ and $Fe_2(SO_4)_3$ inhibited malease activity to some extent, while $HgCl_2$ and $ZnSO_4$ completely inhibited malease activity.

The monovalent ions KCl, LiCl, CsCl, NH_4Cl, and NaCl (all at 10 mM concentration) did not have any significant influence on malease activity. NaCl at concentrations higher then 10

Table 4. Effect of ions on malease activity

Salt[a]	Relative activity (%)
None	100
$HgCl_2$	0
$CaSO_4$	97
$ZnSO_4$	0
$CoSO_4$	98 [b]
$BaCl_2$	99
$NiCl_2$	85
$MnSO_4$	96
$CuSO_4$	40 [b]
$MgSO_4$	98
$FeSO_4$	97 [b]
$Fe_2(SO_4)_3$	33 [b]

[a] All ions were at 1 mM final concentration.
[b] Determined by HPLC because of complex formation between maleate and the cation, which disturbed the spectrophotometric assay.

mM inhibited malease activity; 50, 100, and 200 mM NaCl inhibited malease activity by 8, 21, and 38%, respectively.

Inhibitors

A variety of enzyme inhibitors were tested for their ability to inhibit malease activity. The thiol reagents iodoacetamide and *p*-chloromercuribenzoate (at, respectively, 1 and 0.1 mM) completely inhibited malease activity, while iodoacetate (1 mM) inhibited malease activity only slightly (4%). The carbonyl reagents hydroxylamine, phenylhydrazine, and semicarbazide (all at 1 mM) did not inhibit malease activity. The chelating agents EDTA, *o*-phenanthroline, nitrilotriacetate, and α,α'-dipyridyl (all at 1 mM) also did not inhibit malease. The reducing agents dithiothreitol, cysteine, 2-mercaptoethanol, and glutathione (all at 1 mM) did not affect malease activity. SDS (1 mM) completely inhibited malease activity, while KCN (1 mM) did not cause any inhibition.

When malease was preincubated with one of the reducing agents for 30 min at 30°C in the presence of 1 mM $FeSO_4$ [which is the activation mixture as reported for the related iron-sulfur cluster-containing carbon-2-substituted maleate hydratases (10)], a decrease of malease activity was observed.

Inhibitory effect of various substrate analogs and substrates

D-Malate (Fig. 5), D-citramalate, and also some product and substrate analogs were tested for their abilities to inhibit malease activity with maleate as the substrate. The inhibition constants (K_i), representing the dissociation of the enzyme-product complex in the case of the products D-malate and D-citramalate or the dissociation of the enzyme-inhibitor complex in

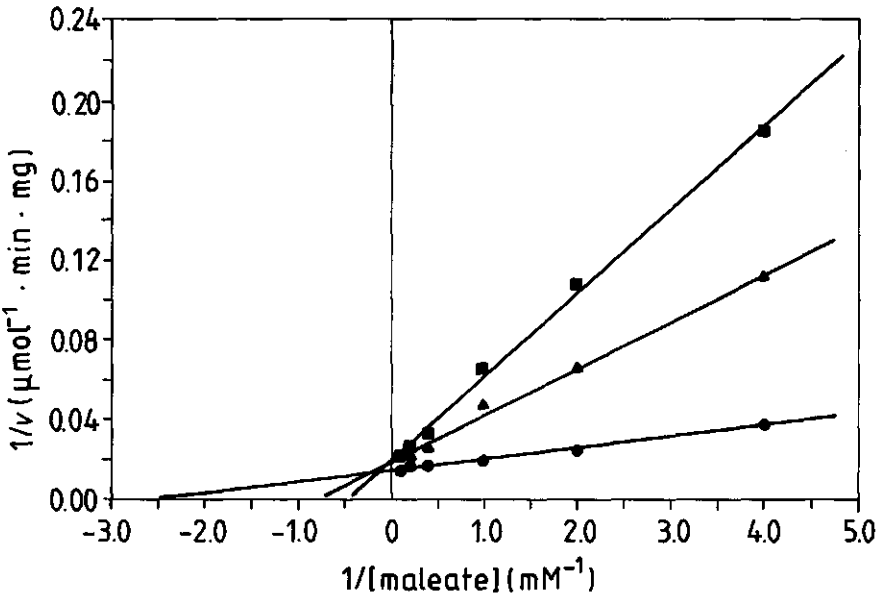

Figure 5. Competitive inhibition of malease by D-malate (50 mM Tris-HCl, pH 7, 30°C). ●, no D-malate; ▲, 2 mM D-malate; ■, 4 mM D-malate.

Table 5. Competitive inhibitors of malease

Inhibitor	K_i (mM)
Succinate[a]	6.3
2-Methylsuccinate[a]	0.76
2,2-Dimethylsuccinate[a]	0.025
L-Malate[a]	8.2
D-Malate[b]	0.63
L-Citramalate[a]	0.65
D-Citramalate[b]	0.083
L-Tartrate[a]	0.70
D-Tartrate[a]	10
Itaconate[a]	0.74
DL-Mercaptosuccinate[a]	0.88

[a] K_i is for dissociation of the enzyme-inhibitor complex.
[b] K_i is for dissociation of the enzyme-product complex.

the case of product and substrate analogs, are given in Table 5. Dicarboxylic acids (C_4) with two substituents at C-2 (e.g., 2,2-dimethylsuccinate and D-citramalate) were the most effective competitive inhibitors of malease (Table 5). The L enantiomers of malate and citramalate were also competitive inhibitors of malease activity (Table 5).

Equilibrium constant

The equilibrium constant of the maleate hydration reaction was determined. Under standard biochemical conditions [25°C, pH 7.0, and an ionic strength of 0.1 M (18)], the apparent equilibrium constant (K_{app} = [hydroxy acid]$_{total}$/[unsaturated acid]$_{total}$) was 2050 ± 100, starting either from maleate or from D-malate.

The apparent equilibrium constants for hydrating citraconate (starting either from citraconate or from D-citramalate) and 2,3-dimethylmaleate were 104 ± 6 and 11.2 ± 0.5, respectively.

DISCUSSION

This report describes the purification and characterization of maleate hydratase (malease) from *P. pseudoalcaligenes*. This strain was previously selected for the production of D-malate (27), which can be used as a synthon (starting compound for chemical synthesis) in the fine-chemicals industry. Special attention was paid to the substrate specificity and possible cofactor requirements of malease and to the apparent equilibrium constant of the hydration reaction, since these factors are of prime importance for the commercial use of such enzymes.

Malease from *P. pseudoalcaligenes* does not require the addition of cofactors for full enzyme activity. The enzyme, unlike malease from rabbit kidney (10) and unlike aconitate hydratase, homoaconitate hydratase, and isopropylmaleate hydratase (11), does not contain an iron-sulfur cluster. Oxidation of the iron-sulfur cluster in these enzymes results in the loss of one of the irons and in loss of activity (2). Subsequent incubation with iron and a sulfur compound results in reincorporation of iron in the iron-sulfur cluster and restoration of activity. These brownish enzymes are completely inhibited by metal-complexing agents at concentrations higher than the metal ion concentration present in the activity assay. Although citraconase (2-methylmaleate hydratase)(22) and 2,3-dimethylmaleate hydratase (14) were only partially purified, they had the same characteristics as the other reported iron-sulfur hydratases (reactivation by a metal ion and a sulfur compound and complete inhibition by metal-complexing agents), suggesting that these enzymes also contain an iron-sulfur cluster. Malease from *P. pseudoalcaligenes*, however, is not activated (it is even inactivated) by incubation with iron and a sulfur compound, and it is not strongly inhibited by chelating agents (and thiol reagents), suggesting that it is another type of enzyme different from malease from rabbit kidney.

A similar situation with two different lyases catalyzing the same reaction exists with fumarases. These enzymes catalyze the hydration of the *trans* isomer of maleate. One type is cofactor independent, while the other type contains an iron-sulfur cluster (24).

Malease from *P. pseudoalcaligenes* also differs from the enzyme from rabbit kidney because it is dimeric rather than monomeric. Also, its amino acid composition is quite different from that of the rabbit enzyme (Table 3), and high NaCl concentrations inhibit rather than stimulate (7) the enzyme. The bacterial enzyme has a much higher affinity for maleate than the rabbit enzyme (K_M = 0.35 versus 10 mM) and, in contrast to the rabbit enzyme, also catalyzes the hydration of citraconate and 2,3-dimethylmaleate.

Malease from *P. pseudoalcaligenes* is also quite different from the cofactor-independent fumarase from *E. coli* (24), which has four identical subunits and a completely different amino acid composition (Table 3).

The enzyme apparently catalyzes the hydration only of substrates containing two carboxyl groups in the *cis* position (maleate, citraconate, and 2,3-dimethylmaleate). Substrates having the *cis* configuration, but containing a methyl or a chloro group instead of a second carboxyl group were not hydrated. The fact that two carboxyl groups are necessary for activity suggests that these two groups are bound in the reaction center of the enzyme by bases and that when only one acid group is present, the substrate cannot be bound in the right configuration for hydration.

Because the enzyme is very unstable at pH values below 5 and above 9, the K_M could not be determined in these pH regions. Therefore, we are unable to say anything about the nature of the active groups in the reaction center, except that histidine is probably not involved as is the case with fumarase (16). With fumarase, large (10-fold) changes in the K_M are observed between pHs 6 and 8 (16).

The equilibrium constants for the maleate hydration (K_{app} = 2050) and the citraconate hydration (K_{app} = 104) reactions are much higher than the equilibrium constants for the hydration of the *trans* analogs [for the fumarate hydration reaction, K_{app} = 4.4 (3), and for the mesaconate hydration reaction, K_{app} = 5.7 (8)]. This is in contrast with the group contribution theory, which does not correct for *cis* or *trans* configuration at the double bound (17), thus giving the same equilibrium constants for the hydration of the *cis* and the *trans* compounds. However, the K_{app} for the hydration of *cis*-crotonyl coenzyme A (K_{app} = 5.5) is also higher than the K_{app} for the *trans* isomer (K_{app} = 3.5)(21). The K_{app} for the 2,3-dimethylmaleate hydration reaction was 11.2 (25°C). Previously the K_{app} for this reaction was reported to be 2.3 at 35°C (14).

Unfortunately, both potential commercial products of the hydration reaction, D-malate and D-citramalate, inhibit malease from *P. pseudoalcaligenes* (Table 5). For application of this enzyme on a commercial scale, it may therefore be necessary to (continuously) remove the product of the reaction, for instance, by precipitation with Ca^{2+} (12).

Subsequent work will focus on the thermodynamics of the maleate and citraconate hydration reactions. Also, the optimization of malease induction in *P. pseudoalcaligenes* and further optimization of the reaction conditions for the production of D-malate and D-citramalate will be studied.

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CHAPTER 6

THERMODYNAMICS OF THE MALEATE AND CITRACONATE HYDRATION REACTIONS CATALYZED BY MALEASE FROM *PSEUDOMONAS PSEUDOALCALIGENES*

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SUMMARY

Malease from *Pseudomonas pseudoalcaligenes* catalyzes the hydration of both maleate and citraconate to D-malate and D-citramalate, respectively. The K_{app} for these hydration reactions were 2050 and 104, respectively, under standard biochemical conditions (25°C, pH 7.0, I=0.1). The influence of the pH (6.0-8.5) on K_{app} was determined. The Gibbs-free-energy changes under standard biochemical conditions for the hydration of the dianionic acids were calculated to be -19.28 kJ·mol^{-1} and -11.65 kJ·mol^{-1}, respectively. From the obtained data together with data from the literature, the Gibbs free energy of formation of maleate^{2-} and citraconate^{2-} were calculated to be -588.91 kJ·mol^{-1} and -600.56 kJ·mol^{-1}, respectively. The influence of the temperature (10°C - 40°C) on K_{app} was determined for both hydration reactions. The enthalpy change ($\Delta H°'$) and entropy change ($\Delta S°'$) under standard biochemical conditions for the maleate^{2-} ($\Delta H°'$= -18.07 kJ·mol^{-1}, $\Delta S°'$= 2.94 J·mol^{-1}·K^{-1}) and citraconate^{2-} ($\Delta H°'$= -22.55 kJ·mol^{-1}, $\Delta S°'$= -35.92 kJ·mol^{-1}·K^{-1}) hydration reactions were calculated. The reaction rate of malease from *P. pseudoalcaligenes* was studied for both hydration reactions as a function of temperature. From these studies, the Gibbs free energies of activation for the maleate and citraconate hydration reactions catalyzed by malease from *P. pseudoalcaligenes* were calculated to be 62.21 kJ·mol^{-1} and 63.43 kJ·mol^{-1}, respectively.

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INTRODUCTION

Biological formation of optically active synthons for the fine-chemicals industry is a major area in biotechnology (10). To this end, lyases (e.g. hydratases, ammonia-lyases) are very interesting enzymes. They do not require cofactor recycling (since addition does not involve a net reduction or oxidation), are quite stable, show an almost absolute stereospecificity/stereoselectivity and may have a theoretical yield of 100% compared to only 50% for enantiomeric resolutions.

Lyases have already been applied on a commercial scale for some decades. L-Malate has been produced by the Japanese firm Tanabe Seiyaku since 1974 in a continuous process using immobilized *Brevibacterium flavum* cells containing high fumarase activity (6).

Maleate hydratase (malease) catalyzes the stereospecific hydration of maleate ("*cis*-fumarate") to D-(*R*)-malate (Fig. 1). This enzyme, which is rather common in microorganisms (25), was purified from *Pseudomonas pseudoalcaligenes* (26). In contrast to malease from rabbit kidney (9), malease from *P. pseudoalcaligenes* also catalyzes the stereospecific hydration of citraconate to D-(*R*)-citramalate at considerable rates (Fig. 1). Both products of these hydration reactions can be used as versatile optically pure synthons in the fine-chemicals industry (16,17,20).

From an economic point of view, a yield of 100% on the substrate basis is desired. The thermodynamic constants, the Gibbs-free-energy change, the enthalpy change and the entropy change under standard biochemical conditions ($\Delta G^{\circ\prime}$, $\Delta H^{\circ\prime}$ and $\Delta S^{\circ\prime}$, respectively) of a reaction allow predictions of the theoretical maximal conversion under specific reaction conditions. These data can also be used to select the reaction temperature. At higher temperatures, enzymes catalyze the conversion with a higher specific activity, however, exothermal reactions (negative $\Delta H^{\circ\prime}$) have a reduced yield at

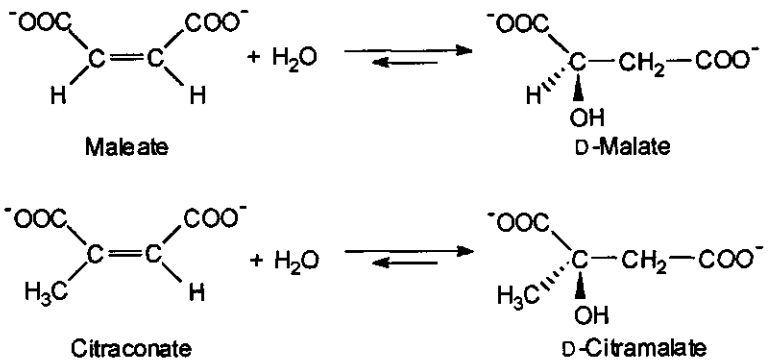

Figure 1. Maleate and citraconate hydration reactions.

higher temperatures. Especially when downstream processing is a cost-determining step, it can be economically more favourable to perform the process at a lower temperature to obtain a higher yield.

Here we report on the thermodynamic constants involved in both the maleate and citraconate hydration reactions catalyzed by malease from *P. pseudoalcaligenes*.

MATERIALS AND METHODS

Enzyme

Malease was purified from the cell extract of cells of *P. pseudoalcaligenes* NCIMB 9867 grown on 3-hydroxybenzoate as described previously (26).

Apparent equilibrium constant

K_{app} was determined by adding 300 μl malease (~10 U) to 1.7 ml 200 mM potassium phosphate containing 200 mM maleate, D-malate, citraconate or D-citramalate, adjusted to pH 7.0 with sodium hydroxide, in a 30-ml serum bottle. The serum bottle was placed in a static waterbath at the desired temperature. For experiments at other pH values, a 100 mM potassium phosphate-100 mM Hepes mixture was used which was adjusted to the required pH with sodium hydroxide. The maleate/malate and citraconate/citramalate concentrations in the mixtures were determined by HPLC until equilibrium was reached.

HPLC analysis

Malate, citramalate, maleate and citraconate were analyzed by HPLC as described previously (25). The dicarboxylic acids were separated on a reverse-phase (C_{18}) column using 2 mM octylamine-25 mM potassium phosphate (pH 7.0) as the mobile phase.

Determination pKa_2 of dicarboxylic acids

225 mg of maleate, 218 mg of citraconate, 32 mg of D-malate or 34 mg of D-citramalate were dissolved in 60 ml water containing 13 g of $KCl \cdot l^{-1}$. The pH profiles resulting from titration of these solutions with 1 M NaOH were used to determine the pKa_2 values of these compounds.

Assay of malease and citraconase activity

The malease activity was determined spectrophotometrically following the rate of the decrease in absorbance at 270 nm due to the disappearance of substrate. At this wavelength, the absorption of the hydroxy acid formed is negligible compared to the absorption of the unsaturated acid. Hydratase activity was measured as follows. 50 μl enzyme (~0.2 U), 100 μl 100 mM maleate or citraconate and 1.85 ml 50 mM Tris-HCl (pH 7.0) were incubated at different temperatures and the rate of the decrease in absorbance was monitored at 270 nm for at least 3 min [ε maleate = 120 $l \cdot mol^{-1} \cdot cm^{-1}$ and ε citraconate = 100 $l \cdot mol^{-1} \cdot cm^{-1}$ (26)]. The exact temperature of the incubation was measured directly in the optical cell. The K_M of the maleate and citraconate hydration reactions at different temperatures were determined as described previously (26).

Protein determination

Protein was determined by the method of Bradford (2) using bovine serum albumin as a standard.

Chemicals

Octylamine and D-citramalate were purchased from Aldrich. Maleate and D-malate were from Janssen Chimica. Citraconate was obtained from Fluka.

RESULTS

Apparent equilibrium constant

The apparent equilibrium constants (K_{app}, see Eqn [1]) of the maleate and citraconate hydration reactions were determined under standard biochemical conditions [25°C, pH 7.0, I = 0.1 M, excluding the water concentration (11)];

$$K_{app} = \frac{[\text{hydroxy acid}]_{total}}{[\text{unsaturated acid}]_{total}} . \qquad [1]$$

The K_{app} for the maleate hydration reaction was 2050 ± 100 starting either with maleate or D-malate. The K_{app} for the citraconate hydration reaction (starting either with citraconate or D-citramalate) was 104 ± 6.

From the K_{app} the maximal obtainable yield (Y), at a specific pH and temperature, as a percentage of the substrate concentration can be calculated according to Eqn [2] (see Table 1);

$$Y = \frac{K_{app}}{K_{app} + 1} . \qquad [2]$$

K_{app} as function of pH

At pH 7.0, a significant amount (~14% in the case of maleate) of, in particular, the unsaturated acids are present in the monoanionic form due to the high pKa_2 values of these compounds. The Gibbs free energies of formation ($\Delta_f G°'$) of the different forms (dianionic, monoanionic and protonated dicarboxylic acid) differ (5) and substrate and product have different pKa_2 values. Consequently the $\Delta G°'$ of these hydration reactions, and thus the K_{app}, will be strongly influenced by pH.

The experimentally determined K_{app} for both the maleate and citraconate hydration reactions were indeed pH dependent (Fig. 2) and levelled off at higher pH values. The equilibrium constant (K'_{eq}) for the hydration of the dianionic substrate to the dianionic product can now be determined; $K'_{eq} = K_{app}$ at high pH (see Table 1).

Table 1. K_{app}, K'_{eq}, $\Delta G°'$ and Y for the hydration reactions performed by malease

	K_{app}	K'_{eq} [a]	$\Delta G°'$ (kJ·mol^{-1})	Y (%)
Maleate^{2-} + H_2O ⇄ D-malate^{2-}	2050 ± 100	2400	-19.28	99.96
Citraconate^{2-} + H_2O ⇄ D-citramalate^{2-}	104 ± 6	110	-11.65	99.1

[a] K'_{eq} was extrapolated from Fig. 2a and b at high pH.

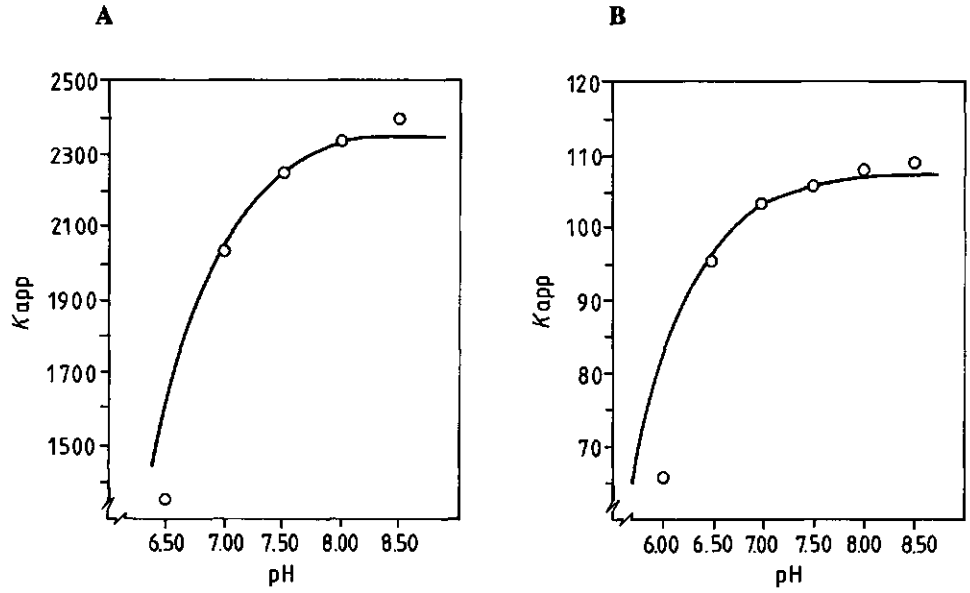

Figure 2. Effect of pH on K_{app}. (A) Maleate hydration reaction. (B) Citraconate hydration reaction. Open points are measured data (25°C). The solid line represents the K_{app} calculated using Eqn [3], with the value for pH 7.5 on the calculated line. Maleate hydratase reaction; pKa_2 maleate, 6.23 (23); pKa_2 D-malate, 5.11 (24). Citraconate hydratase reaction; pKa_2 citraconate, 5.67; pKa_2 citramalate, 5.06.

K'_{eq} for the hydration of the dianionic substrates to the dianionic products can also be calculated using Eqn [3] (1; only the influence of the pKa_2 is taken into account);

$$K_{app} = K'_{eq} \frac{(1 + [H^+]/Ka_{2\text{hydroxy acid}})}{(1 + [H^+]/Ka_{2\text{unsaturated acid}})}, \qquad [3]$$

with;

$$K'_{eq} = \frac{\gamma_{\text{hydroxy acid}}}{\gamma_{\text{unsaturated acid}}} \frac{[\text{hydroxy acid}^{2-}]}{[\text{unsaturated acid}^{2-}]}, \qquad [4]$$

in which γ is the activity coefficient.

Since only the γ of sodium maleate as a function of the ionic strength (I) is known (15), we were not able to correct for the change in K'_{eq} due to the change of the activity coefficients. However, the experimentally determined K_{app} did not significantly change when increasing I from 0.1 mol·l^{-1} to 1 mol·l^{-1}. This indicates that the change in activity coefficient due to the change in ionic strength is similar for both the hydroxy acid and the unsaturated acid, and this effect was therefore neglected. In view of the large K_{app}, especially for the maleate hydration reaction, subsequent experiments were performed at a substrate concentration of 170 mM (I = 0.89 mol·l^{-1}), thus enabling a more accurate

determination of K_{app}.

Available data for the pKa_2 of the dicarboxylic acids show quite a large variation (18,23,24) and the pKa_2 of D-citramalate could not be obtained from the literature. Therefore, the pKa_2 values of the dicarboxylic acids were determined at the ionic strength that was used in the experiments. The pKa_2 for maleate, D-malate, citraconate and D-citramalate were determined to be 5.82, 4.73, 5.67 and 5.06 respectively. The pKa_2 values, as determined for citraconate and D-citramalate could describe the pH dependency of the K_{app} (Fig. 2b) but for the maleate hydration reaction the literature data (23,24) described the pH dependency of K_{app} best (Fig. 2a).

Gibbs-free-energy change of the hydration reactions and Gibbs free energy of formation of maleate^{2-} and citraconate^{2-}

$\Delta G^{\circ\prime}$ for the hydration reactions can be calculated from the K'_{eq} according to Eqn [5] (Table 1);

$$\Delta G^{\circ\prime} = - R\,T \ln K'_{eq}\,, \qquad [5]$$

with R the gas constant and T the absolute temperature (K).

The $\Delta_f G^{\circ\prime}$ of maleate^{2-} and citraconate^{2-} were calculated from the $\Delta G^{\circ\prime}$ of these two hydration reactions and the $\Delta_f G^{\circ\prime}$ of malate^{2-}, citramalate^{2-} and H_2O obtained from literature (Table 2).

Determination of the enthalpy and entropy change

The influence of the temperature on the K_{app} was studied. The K_{app} was found to decrease at higher temperatures for both hydration reactions (Fig. 3).

The effect of temperature on the pKa_2 of both the unsaturated and hydroxy acids could not be obtained from literature, but it is known that the pKa_2 of the related compound, malonate, is only marginally influenced by temperature (24). Therefore, the relationship

Table 2. $\Delta_f G^{\circ\prime}$ of maleate^{2-} and citraconate^{2-}

Compound	$\Delta_f G^{\circ\prime}$ (kJ·mol^{-1})	Ref
D-Malate^{2-}	-845.38 [a,b]	14
H_2O	-237.19	14
Maleate^{2-}	-588.91	This manuscript
D-Citramalate^{2-}	-849.40 [a]	4
Citraconate^{2-}	-600.56	This manuscript

[a] Assuming that the $\Delta_f G^{\circ\prime}$ of both stereoisomers is the same (14).
[b] Calculated on the basis of the equilibrium constant for fumarase and with the $\Delta_f G^{\circ\prime}$ of fumarate^{2-} at an ionic strength of 0.1 (1).

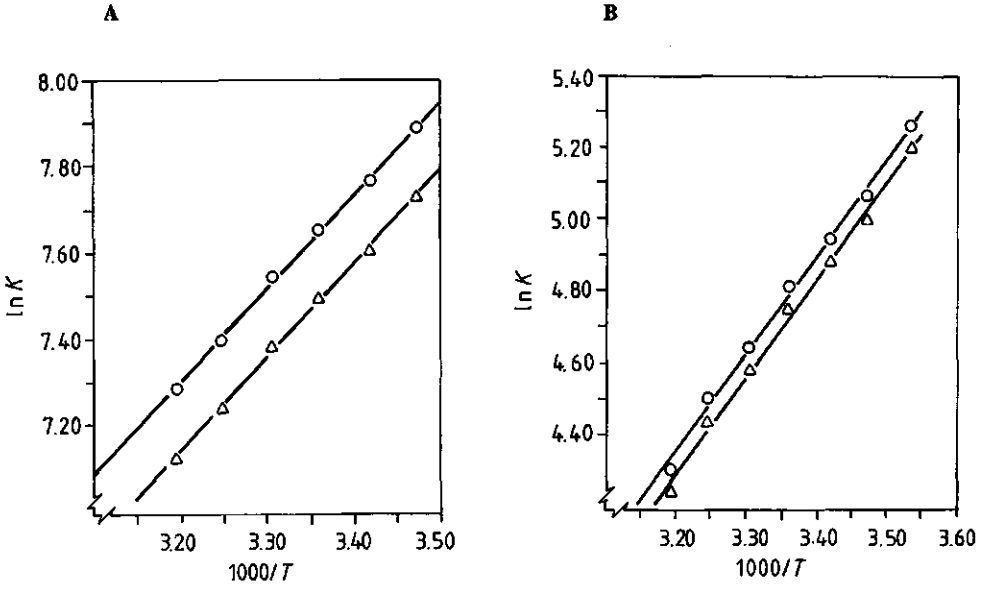

Figure 3. Van't Hoff plots of K_{app} and K'_{eq}. **(A)** Maleate hydration reaction. **(B)** Citraconate hydration reaction. K'_{eq} was determined with the K'_{eq}/K_{app} ratio given in Table 1. ▵, K_{app}; ○, K'_{eq}.

between the K_{app} and K'_{eq} at pH 7.0 is assumed to be independent of temperature. The K'_{eq} as function of temperature was calculated with the K'_{eq}/K_{app} ratio given in Table 1.

$\Delta H°'$ and $\Delta S°'$ for the hydration of the dianionic forms of both maleate and citraconate can be calculated from these data using the van't Hoff equation [6];

$$\frac{\mathrm{d} \ln K'_{eq}}{\mathrm{d} T} = \frac{\Delta H°'}{R T^2} . \qquad [6]$$

$\Delta H°'$ and $\Delta S°'$ can assumed to be independent of temperature for small temperature ranges (<50 K)(5) and Eqn [6] can then be integrated to Eqn [7];

$$\ln K'_{eq} = \frac{-\Delta H°'}{R T} + \frac{\Delta S°'}{R} . \qquad [7]$$

From a plot of ln K'_{eq} versus 1/T (Fig. 3), $\Delta H°'$ and $\Delta S°'$ of both hydration reactions were determined (Table 3). These data are compared with data derived from literature for related hydration reactions (Table 3).

In the literature, there are no data available for the hydration of maleate or citraconate under non-catalyzed conditions. There are, however, data known for the acid-catalyzed maleic acid hydration reaction, thus for the hydration of protonated maleate (12). Here,

Table 3. $\Delta G^{\circ\prime}$, $\Delta H^{\circ\prime}$, $\Delta S^{\circ\prime}$ and Y of the maleate and citraconate hydration reactions and of some related hydration reactions (25°C)

Reaction	$\Delta G^{\circ\prime}$ (kJ·mol^{-1})	$\Delta H^{\circ\prime}$ (kJ·mol^{-1})	$\Delta S^{\circ\prime}$ (J·mol^{-1}·K^{-1})	Y (%)	Ref
Maleate^{2-} + H_2O ⇄ D-malate^{2-}	-19.28	-18.07	2.94	99.96	This manuscript
Citraconate^{2-} + H_2O ⇄ D-citramalate^{2-}	-11.65	-22.55	-35.92	99.1	This manuscript
Fumarate^{2-} + H_2O ⇄ L-malate^{2-}	- 3.69	-16.55	-43.15	81.6	c.f.[a] (1)
Mesaconate^{2-} + H_2O ⇄ L-citramalate^{2-}	- 4.31			85.1	c.f. (4)
cis-Crotonyl-CoA + H_2O ⇄ D-β-hydroxy-butyryl-CoA	- 4.21			84.5	c.f. (19)
trans-Crotonyl-CoA + H_2O ⇄ L-β-hydroxy-butyryl-CoA	- 3.07			77.5	c.f. (19)
cis-Aconitate^{3-} + H_2O ⇄ citrate^{3-}	- 7.61			95.6	c.f. (22)
cis-Aconitate^{3-} + H_2O ⇄ (2*R*,3*S*)-isocitrate^{3-}	- 1.50			64.7	c.f. (22)
Maleic acid + H_2O ⇄ DL-malic acid	-22.02	-36.75	-49.41	99.99	c.f. (12)[b]
Maleic acid + H_2O ⇄ D- or L-malic acid	-20.32	-36.75	-55.27	99.97	c.f. (12)[b,c]

[a] c.f. = calculated from.

[b] The reaction was performed at temperatures between 160°C and 200°C and at 40% substrate concentration.

[c] The $\Delta G^{\circ\prime}$ derived from the equilibrium constants as mentioned in this article were recalculated for optically pure malic acid using Eqn [8].

however, racemic malic acid is formed.

Although the enthalpy of formation ($\Delta_f H^{\circ\prime}$) for the racemic mixture and the optically pure compound are the same, the entropy of formation ($S^{\circ\prime}$) of racemic mixtures is larger than for optically pure compounds. The relation in $\Delta_f G^{\circ\prime}$ for a racemic mixture and an optically pure compound is given by Eqn [8] (14);

$$\Delta_f G^{\circ\prime}{}_{DL} = \Delta_f G^{\circ\prime}{}_{D \text{ or } L} - R\,T \ln 2\,. \qquad [8]$$

The data presented by Kása and Deák (12) were corrected for the formation of optically pure malic acid (Eqn [8]) and these corrected data for $\Delta G^{\circ\prime}$ and $\Delta S^{\circ\prime}$ are given in Table 3.

Reaction rate as a function of the temperature

The specific activity of both the maleate and citraconate hydration reactions catalyzed by malease of *P. pseudoalcaligenes* were determined as function of temperature [Fig. 4; see also (26)]. At low temperatures, the hydration rate of citraconate was highest while at temperatures >18°C the maleate hydration reaction was faster (Fig. 4).

The enthalpy of activation ($\Delta H^{\neq}$) and the entropy of activation ($\Delta S^{\neq}$) of the rate-determining step of the substrate/product-enzyme complex of the maleate and citraconate

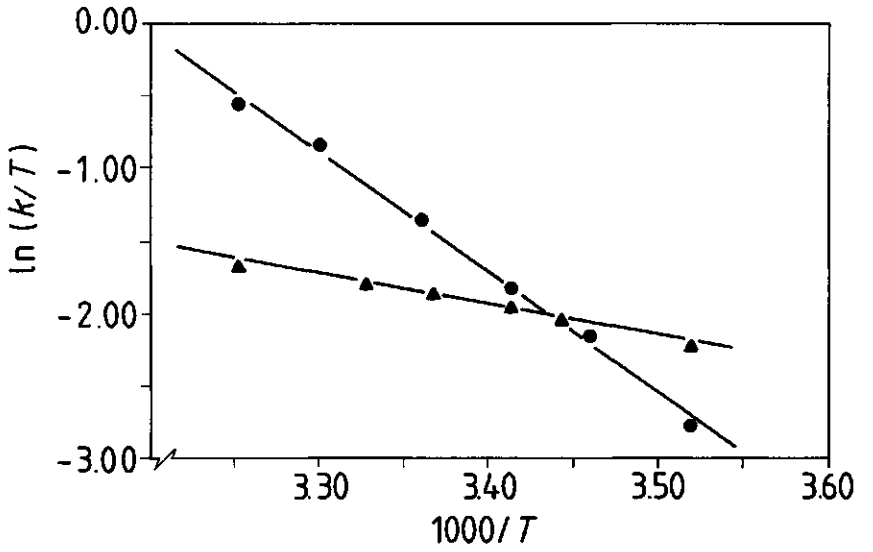

Figure 4. Eyring plot of the rate constants. ●, Maleate hydration reaction; ▲, citraconate hydration reaction.

hydration reactions can be calculated from these studies with the Eyring equation [9];

$$k = \frac{k_B T}{h} \quad e^{\Delta S^{\neq}/R} \quad e^{-\Delta H^{\neq}/R\,T} \quad , \qquad [9]$$

in which k is the rate constant (s^{-1}), k_B is the Boltzman constant and h is the Planck constant.

k is calculated using Eqn [10], which is valid at substrate concentrations >> Michaelis-Menten constant (K_M).

$$k = v\,m\,, \qquad [10]$$

in which v is the specific activity ($mol \cdot s^{-1} \cdot g^{-1}$) and m is the molecular mass of the enzyme [$g \cdot mol^{-1}$; based on the molecular mass for malease as determined by SDS-PAGE (81 kDa)(26) and assuming one catalytic site/molecule].

The K_M is slightly dependent on the temperature [K_M = 0.32 mM (35°C) and 0.41 mM (11°C) for maleate and K_M = 0.19 mM (35°C) and 0.23 mM (11°C) for citraconate] but does not influence v significantly at the used substrate concentration.

$\Delta H^{\neq}$ and $\Delta S^{\neq}$ for both hydration reactions were determined from a plot of ln (k/T) versus $1/T$ (Fig. 4, Table 4).

Table 4. $\Delta H^{\neq}$, $\Delta S^{\neq}$, $\Delta G^{\neq}$ and E_a (25°C, pH 7) of the maleate and citraconate hydration reactions catalyzed by malease from *P. pseudoalcaligenes* and the acid-catalyzed maleate hydration

Reaction	$\Delta H^{\neq}$ (kJ·mol^{-1})	$\Delta S^{\neq}$ (J·mol^{-1}·K^{-1})	$\Delta G^{\neq}$ (kJ·mol^{-1})	E_a (kJ·mol^{-1})	Ref
Enzymatic reaction					
Maleate^{2-} + H_2O ⇄ D-malate^{2-}	68.66	21.63	62.21	71.10	This manuscript
Citraconate^{2-} + H_2O ⇄ D-citramalate^{2-}	17.60	-153.8	63.43	20.07	This manuscript
Fumarate^{2-} + H_2O ⇄ L-malate^{2-}	29.59	-83.3	54.43	32.00	c.f.[a] (3)
Chemical reaction					
Maleic acid + H_2O ⇄ D-malic acid or L-malic acid	81.56	-76.51	104.36		c.f. (12)[b,c]

[a] c.f. = calculated from.
[b] The reaction was performed at temperatures between 160°C and 200°C and at 40% substrate concentration.
[c] Recalculated for the formation of optically pure malic acid using Eqn [8].

Now, the Gibbs free energy of activation ($\Delta G^{\neq}$) of the maleate hydration reaction can be calculated using Eqn [11](Table 4);

$$\Delta G^{\neq} = \Delta H^{\neq} - T\,\Delta S^{\neq} . \qquad [11]$$

The activation energy (E_a) for the hydration reactions was calculated with the Arrhenius equation [12];

$$\ln k = \ln A - \frac{E_a}{R\,T} , \qquad [12]$$

where A is the frequency factor (Eqn [13]);

$$A = \frac{k_B\,T}{h}\; e^{\Delta S^{\neq}/R} . \qquad [13]$$

From plots of ln k versus $1/T$, E_a for both hydration reactions were determined (Table 4). The relationship between E_a and $\Delta H^{\neq}$ is given by Eqn [14];

$$E_a = \Delta H^{\neq} + R\,T . \qquad [14]$$

The calculated E_a and $\Delta H^{\neq}$ did indeed show this relationship (Table 4).

The $\Delta G^{\neq}$, $\Delta H^{\neq}$ and $\Delta S^{\neq}$ for the fumarate hydration reaction, catalyzed by fumarase from pig hearts, were calculated from the data presented by Brant et al. (3; Table 4). The $\Delta G^{\neq}$, $\Delta H^{\neq}$ and $\Delta S^{\neq}$ for the acid-catalyzed hydration of maleic acid were calculated from the data of Kása and Deák (12), after correction (Eqn [8]) for the formation of optically

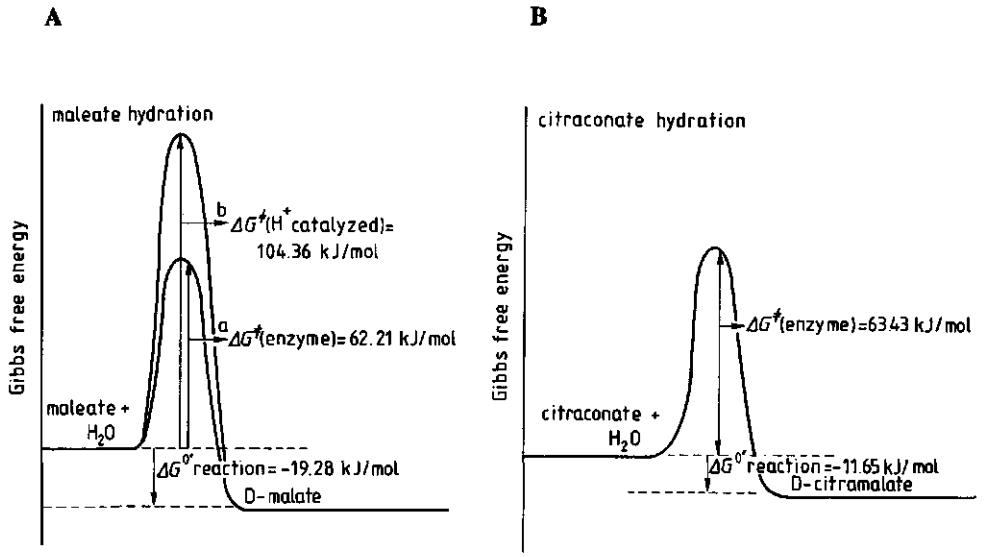

Figure 5. Schematic energy diagram for the maleate and citraconate hydration reactions. (A) Maleate hydration. (a) Reaction catalyzed by malease from *P. pseudoalcaligenes*. (b) Acid-catalyzed reaction. (B) Citraconate hydration reaction.

pure malic acid. The $\Delta G^{\neq}$ for the acid-catalyzed maleate hydration reaction is higher than for the malease catalyzed maleate hydration reaction (Table 4, Fig. 5).

The energy diagrams for the maleate and citraconate hydration reactions catalyzed by malease from *P. pseudoalcaligenes* are shown in Fig. 5.

DISCUSSION

In this report the thermodynamics of the maleate and citraconate hydration reactions catalyzed by malease from *P. pseudoalcaligenes* are described. From an economic point of view, a yield of 100% on substrate basis is most advantageous. Knowledge concerning the thermodynamic constants is necessary to calculate the maximal achievable yield and also to predict the effect of the reaction temperature on the yield.

According to the group-contribution theory, which views a compound as composed of functional groups and sums amounts contributed by each group to the Gibbs energy of formation of the compound (13), hydration reactions with the general formula $>C=C<$ + H_2O ⇄ -HOCH-CH_2-, as for the maleate and citraconate hydration reactions, should have a similar $\Delta G°'$ value. Using the data of Mavrovionitis (13) a $\Delta G°'$ of -12.18

$kJ \cdot mol^{-1}$ can be calculated for the hydration of a $>C=C<$ double bond. This value is in the same order of magnitude as the $\Delta G^{\circ\prime}$ obtained for the citraconate hydration reaction (Table 3). However, the maleate hydration reaction has a lower $\Delta G^{\circ\prime}$ (Table 3) while the fumarate hydration reaction has a higher $\Delta G^{\circ\prime}$, demonstrating the limitations of the group-contribution theory in predicting $\Delta_f G^{\circ\prime}$ values. Probably groups further away from the site of reaction also greatly influence the $\Delta_f G^{\circ\prime}$.

The equilibrium constants for the maleate hydration (K'_{eq} = 2400) and the citraconate hydration reactions (K'_{eq} = 110) are much higher than the equilibrium constants for the hydration of the *trans* analogs of these substrates (Table 2). For the fumarate hydration reaction, K'_{eq} = 4.4 (1) and for the mesaconate hydration reaction, K'_{eq} = 5.7 (4). Also, the K'_{eq} for the hydration of *cis*-crotonyl-CoA (K'_{eq} = 5.5) is higher than the K'_{eq} for the *trans* isomer (K'_{eq} = 3.5)(19). This suggests that the *cis* configuration or *trans* configuration around the double bound has a significant effect on the $\Delta_f G^{\circ\prime}$ of the unsaturated compounds.

Since the pKa_2 values of maleate and citraconate, especially, are quite high (6.23 and 5.67, respectively) a significant amount (~ 14% in the case of maleate) of the unsaturated acids is present in the monoanionic form at physiological pH (pH 7.0). This leads to a discrepancy between the apparent equilibrium constant and the equilibrium constant for the hydration of the dianionic substrate due to a difference in $\Delta_f G^{\circ\prime}$ of the different forms of these acids (5).

The maleate and citraconate hydration reactions are exothermal reactions (negative $\Delta H^{\circ\prime}$; Table 3). The negative enthalpies of these hydration reactions imply that at higher temperatures, in which case the specific activity of the enzyme is higher, the K_{app} decreases and thus also the overall yield of the reaction decreases. However, the K_{app} for the maleate and citraconate hydration reactions are so high that the yield does not drop significantly at increased temperatures (99.96% yield at 25°C compared to 99.84% at 75°C and 99.76% at 100°C for the maleate hydration reaction).

The fumarate hydration reaction, however, shows a significant drop in yield (for example, increasing from 25°C to 75°C, the yield will drop from 83% to 60%). The fact that the commercial process of L-malate production is, however, performed at 55°C, the temperature with the maximal productivity, can be explained by the simple recovery of the remaining substrate by precipitation with HCl (7).

For enzymes catalyzing reactions showing a significant drop in yield at higher temperatures, it is therefore not always worthwhile isolating enzymes from thermophilic microorganisms. Although enzymes from thermophilic organisms are generally more (thermo-)stable, these enzymes often have a low specific activity at temperatures below 40°C. For specific cases, an evaluation has to be made for the application of thermophilic enzymes; higher productivity due to a higher specific activity [although most enzymes of thermophilic origin do, in general, not show a higher specific activity at these high temperatures compared with enzymes from a mesophilic microorganisms at lower

temperatures (8)] and a higher (thermo-)stability of the enzyme will have to be compared with a lower yield, possibly resulting in much higher down-stream processing costs.

The $\Delta S^{\neq}$ of the activated complex can be used to characterize the rate-limiting step of the reaction. When $\Delta S^{\neq} \sim 0$, as is the case for the maleate hydration reaction (Table 4), the rate-determining step of the reaction has a monomolecular character. This suggests that the dissociation of D-malate from the enzyme is the rate-determining step in the maleate hydration reaction. When $\Delta S^{\neq} << 0$, as is the case for the citraconate hydration reaction (Table 4), the rate-determining step of the reaction has a bimolecular character. This suggests that the formation of a water(H^+/OH^-)-citraconate complex is the rate-determining step in the citraconate hydration reaction.

Unfortunately, neither the temperature optimum nor the $\Delta G^{\neq}$ have been reported for purified malease from rabbit kidney (9) or for purified citraconase (21). It is, therefore, not possible to compare the $\Delta G^{\neq}$ of these enzymes with the $\Delta G^{\neq}$ of malease from *P. pseudoalcaligenes*. It would be interesting to determine if, for example, an iron-sulphur cofactor (as is present in the two enzymes mentioned) would be more efficient (lower activation energy) than the cofactor-independent malease from *P. pseudoalcaligenes* (26).

In conclusion, due to the high values of the K_{app} for both the maleate and citraconate hydration reactions, application of malease on a commercial scale will not be limited in this respect. The fact that both hydration reactions are exothermal is not crucial because of the large K_{app}. Both hydration reactions can, therefore, be performed at higher temperatures and the isolation of a malease from thermophilic origin might be advantageous, especially with respect to enzyme stability.

Subsequent work will focus on the further optimization of D-malate production from maleate with the pertinent malease from *P. pseudoalcaligenes*.

NOMENCLATURE

A	Frequency factor	(s^{-1})
E_a	Activation energy	($kJ \cdot mol^{-1}$)
h	Planck constant	(6.63×10^{-34} J·s)
I	Ionic strength	($mol \cdot l^{-1}$)
k	Rate constant	(s^{-1})
k_B	Boltzmann constant	(1.38×10^{-23} $J \cdot K^{-1}$)
Ka_2	Acid constant	($mol \cdot l^{-1}$)
K_{app}	Apparent equilibrium constant	(-)
K'_{eq}	Equilibrium constant	(-)
K_M	Michaelis-Menten constant	(mM)
m	Molecular mass	($g \cdot mol^{-1}$)
R	Gas constant	(8.314 $J \cdot mol^{-1} \cdot K^{-1}$)
$S^{\circ\prime}$	Entropy under standard biochemical conditions	($J \cdot mol^{-1} \cdot K^{-1}$)
T	Absolute temperature	(K)

v	Specific activity	$(mol \cdot s^{-1} \cdot g^{-1})$
Y	Yield	(%)
γ	Activity coefficient	(-)
$\Delta G^{\circ\prime}$	Gibbs-free-energy change under standard biochemical conditions	$(kJ \cdot mol^{-1})$
$\Delta G^{\neq}$	Gibbs free energy of activation	$(kJ \cdot mol^{-1})$
$\Delta_f G^{\circ\prime}$	Gibbs free energy of formation under standard biochemical conditions	$(kJ \cdot mol^{-1})$
$\Delta H^{\circ\prime}$	Enthalpy change under standard biochemical conditions	$(kJ \cdot mol^{-1})$
$\Delta H^{\neq}$	Enthalpy of activation	$(kJ \cdot mol^{-1})$
$\Delta_f H^{\circ\prime}$	Enthalpy of formation under standard biochemical conditions	$(kJ \cdot mol^{-1})$
$\Delta S^{\circ\prime}$	Entropy change under standard biochemical conditions	$(J \cdot mol^{-1} \cdot K^{-1})$
$\Delta S^{\neq}$	Entropy of activation	$(J \cdot mol^{-1} \cdot K^{-1})$
ε	Molar absorption coefficient	$(l \cdot mol^{-1} \cdot cm^{-1})$

ENZYMES

Maleate hydratase or malease	(EC 4.2.1.31)
Citraconate hydratase or citraconase or (*R*)-2-methylmalate dehydratase	(EC 4.2.1.35)
Fumarate hydratase or fumarase	(EC 4.2.1.2)
Mesaconate hydratase or mesaconase or (*S*)-2-methylmalate dehydratase	(EC 4.2.1.34)
Aconitase	(EC 4.2.1.3)
Crotonase or enoyl-CoA hydratase	(EC 4.2.1.17)

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CHAPTER 7

PERMEABILIZATION AND LYSIS OF *PSEUDOMONAS PSEUDOALCALIGENES* CELLS BY TRITON X-100 IN RELATION TO D-MALATE PRODUCTION

Mariët J. van der Werf, Sybe Hartmans and Will J.J. van den Tweel

SUMMARY

Triton X-100 was very effective in diminishing the transport barrier for maleate in *Pseudomonas pseudoalcaligenes* cells. Intact cells did not show any malease activity, whereas Triton X-100 treated cells did show malease activity. Incubation of cells with Triton X-100 also resulted in an increase in the protein concentration of the supernatant, indicating the occurrence of lysis. Permeabilization and lysis were time dependent: longer periods of incubation resulted in higher malease activities of the cell suspensions and in more protein in the supernatant. These permeabilization and lysis rates were dependent on the Triton X-100 concentration and on the biomass concentration.

D-Malic acid is an α-hydroxy acid with potential commercial interest (13). One of the ways of producing D-malate bio(techno)logically is from maleate using maleate hydratase (malease; EC 4.2.1.31) (1,9,10,15).

We have studied the production of D-malate from maleate with malease from *Pseudomonas pseudoalcaligenes* (14,15,16,17). The specific malease activity in this strain is very high but the microorganism was not able to grow on maleate, probably because it lacks a transport mechanism for maleate (15). As maleate can not diffuse freely over the cell membrane (6) this barrier needs to be overcome to allow hydration of maleate by the cytoplasmic enzyme malease (16). Therefore, cells of *P. pseudoalcaligenes* were treated with Triton X-100 (14,15) to overcome the maleate transport problem. Triton X-100 is a mild detergent (5) very effective in permeabilizing microbial cells (7,15). It solubilizes cell-membranes (5), but it can also induce autolytic enzymes (2,11), suggesting that besides permeabilization also lysis can take place.

Here we describe the permeabilization and lysis of *P. pseudoalcaligenes* cells by Triton X-100. Permeabilization is the damaging of cell-membranes in such a way that the morphology of the cells remains intact but allowing the diffusion of low-molecular-weight molecules into and out of the cell (3). Cell-lysis implicates a complete disintegration of the cell wall and as a result high-molecular-weight molecules can diffuse into the supernatant (5).

Permeabilization and lysis in time

P. pseudoalcaligenes NCIMB 9867 was grown at 30°C in a mineral salts medium (4) with 1 g of 3-hydroxybenzoate per liter as the carbon-source (Chapter 4). Cells were harvested just before the end of the logarithmic phase, washed (15) and resuspended in 50 mM potassium phosphate buffer (pH 7.0) containing the indicated amount of Triton X-100. Subsequently, these cells were incubated at room temperature and the malease activity of the cell suspension (as a measure for permeabilization plus lysis) and of the supernatant (as a measure for lysis) were determined as described before (Chapter 4).

When cells of *P. pseudoalcaligenes* were incubated in the absence of Triton X-100, no malease activity could be measured (Fig. 1). Upon prolonged incubation only a very moderate increase in malease activity, probably due to autolysis, could be measured.

When cells were incubated in the presence of 0.5% Triton X-100, a rapid increase of the malease activity of the cell suspension was observed, indicating permeabilization or lysis of the cells. The maximal malease activity was reached already after 40 minutes of incubation with 0.5% Triton X-100 (Fig. 1). No inactivation of malease activity was observed upon prolonged incubation.

Incubating the cells with 0.5% Triton X-100 also resulted in an increase of the protein concentration in the supernatant, indicating lysis (Fig. 1). The increase in protein concentration in the supernatant was proportional to the increase in malease activity in the supernatant (Fig. 1). Lysis due to incubation with Triton X-100 is not a general phenomenum. For example, incubations of *Escherichia coli* with 0.5% Triton X-100 resulted

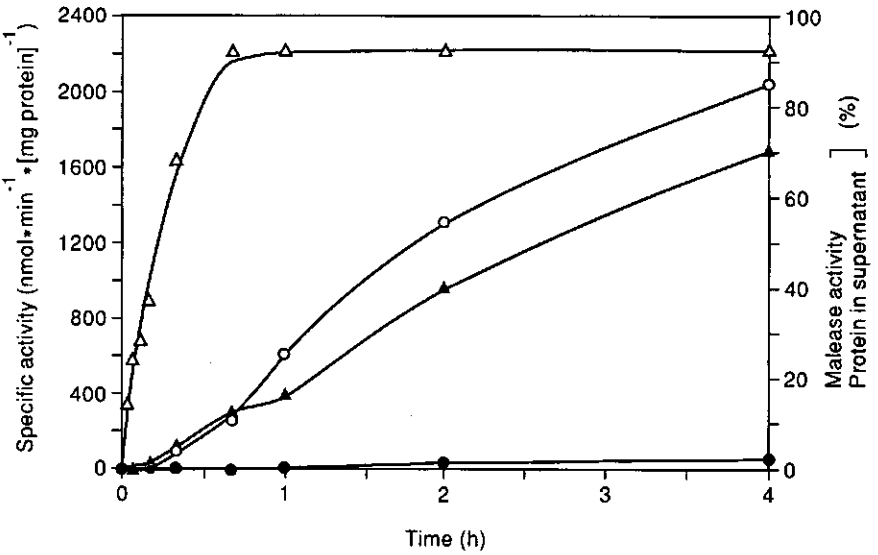

Figure 1. Permeabilization and lysis of *P. pseudoalcaligenes* cells (1.8 mg protein/ml) by Triton X-100. ●, Specific malease activity of the cell suspension in the absence of permeabilizing agent; △, specific malease activity of the cell suspension in the presence of 0.5% Triton X-100; ○, malease activity in the supernatant as percentage of the total malease activity; ▲, protein concentration in the supernatant as percentage of the total protein concentration.

in a maximal release of only 5% of the total protein into the supernatant even after incubation for 38 hours (8), suggesting that no lysis took place. However, when cells of *Streptococcus faecalis* or *Clostridium acetobutylicum* were incubated with Triton X-100 a fast autolysis was observed (2,11).

Although permeabilization and lysis of *P. pseudoalcaligenes* cells occurred simultaneously, these processes were independent, with permeabilization occurring much faster than lysis (Fig. 1).

Effect of Triton X-100 concentration and the amount of cells on the permeabilization plus lysis rate

The permeabilization plus lysis rate was dependent on the Triton X-100 concentration: the higher the Triton X-100 concentration the faster the maximal malease activity of the cell suspension was achieved (Fig. 2). Lysis, i.e. the malease activity in the supernatant, also occurred faster with higher Triton X-100 concentrations (unpublished results).

The amount of cells present in the incubation mixture also influenced the permeabilization plus lysis rate: the more cells, the longer it took before the maximal specific malease activity of the cell suspension was reached (Fig. 3). This suggests that not only the Triton X-100 concentration, but also the ratio Triton X-100/cells influences the permeabilization plus lysis rate.

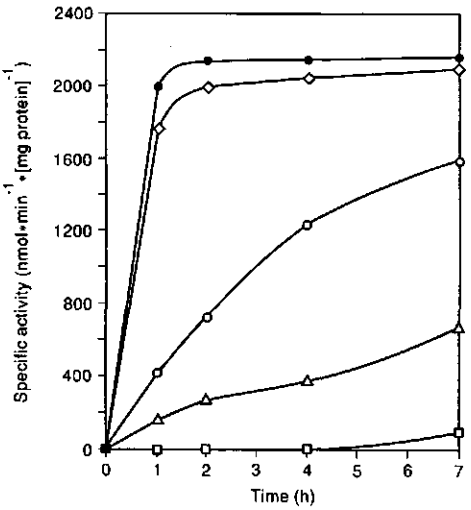

Figure 2. Effect of the Triton X-100 concentration and of time on permeabilization plus lysis (2.9 mg protein/ml). □, 0%,; △, 0.025%; ○, 0.05%; ◇, 0.1%; ●, 0.5% Triton X-100.

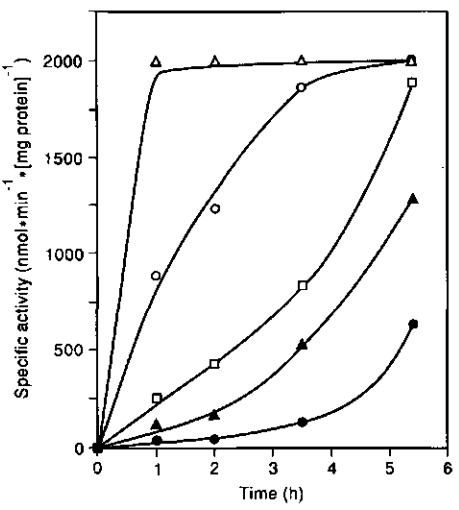

Figure 3. Effect of the amount of cells and of time on permeabilization plus lysis (in the presence of 0.05% Triton X-100). △, 1.15; ○, 2.3; □, 4.6; ▲, 6.9; ●, 11.5 mg/ml protein.

Conclusions

Triton X-100 is very effective in diminishing the transport barrier for maleate in *P. pseudoalcaligenes* cells. At appropriate concentrations of Triton X-100, maximal malease activities of cell suspensions could be measured within one hour. Furthermore no inhibition of malease activity by Triton X-100 was observed.

When permeabilized cells are to be used to produce D-malate in a continuous set-up, lysis should be avoided as the enzyme will be washed away. Protein leakage can possibly be prevented by minimizing lysis by treating the cells with autolytic enzyme inhibitors (2,11), by preventing cellular protein synthesis with chloramphenicol (2) or by using an autolytic-defective strain (2). Protein leakage can also be prevented by cross-linking the cells (12).

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CHAPTER 8

EFFECT OF THE MALEATE COUNTER-ION ON MALEASE ACTIVITY: PRODUCTION OF D-MALATE IN A CRYSTAL-LIQUID TWO-PHASE SYSTEM

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SUMMARY

Malease activity of permeabilized cells of *P. pseudoalcaligenes* was strongly affected by the maleate salt used as the substrate. With Na_2-maleate as the substrate, malease activity decreased strongly with increasing substrate concentrations. By using other counter-ions than sodium, the concentration of dissociated maleate, the "real" substrate for the enzyme, could be reduced significantly due to metal-substrate complex formation. Use of metal-ions which resulted in the formation of an insoluble metal-substrate complex, as Ca^{2+} and Ba^{2+}, further reduced the $maleate^{2-}$ concentration. The use of these metal-ions resulted in the formation of a crystal-liquid two-phase system, due to the low solubility of both the metal-substrate and the metal-product complex. Ca^{2+} was found to be the best counter-ion for the conversion of high concentrations (up to 160 g/l) of either maleate or citraconate by malease into D-malate and D-citramalate, respectively, with yields >99%. The use of this metal-ion gave the highest malease activities and no substrate inhibition could be observed using this counter-ion.

Submitted for publication

INTRODUCTION

An important factor which may prevent bioconversion processes from being commercialized is the fact that often product concentrations are too low. An increase in the product concentration can usually not be realized simply by using higher substrate concentrations, since this often leads to severe inhibition and/or inactivation of the biocatalyst. Higher product concentrations can, however, be obtained by using two-phase systems. In these systems one of the phases acts as a reservoir for product and/or substrate, allowing the accumulation of high product concentrations and minimizing substrate and product inhibition. Another factor which can severely hamper commercialization of a biocatalytic process is an unfavourable equilibrium constant. Also in this respect the use of two-phase systems might be advantageous (8,16). Since both product concentration and molar yield of the reaction have a great impact on downstream processing costs, the use of a two-phase system may lead to an economically, and also an environmentally, more attractive process.

The application of two-phase systems in biocatalysis has been widely studied (1,7,15,18). In most of these two-phase systems a water-immiscible solvent is used as the second phase and these systems are, therefore, mainly applied for the conversion of hydrophobic compounds.

The use of two-phase systems for the conversion of hydrophilic compounds, such as organic acids and amino acids, has not been studied extensively. A two-phase system which can be used in the case of hydrophilic compounds is the crystal-liquid two-phase system. In a crystal-liquid two-phase system product and/or substrate are also present as crystals due to their low solubility. This type of two-phase system has not been studied very well although

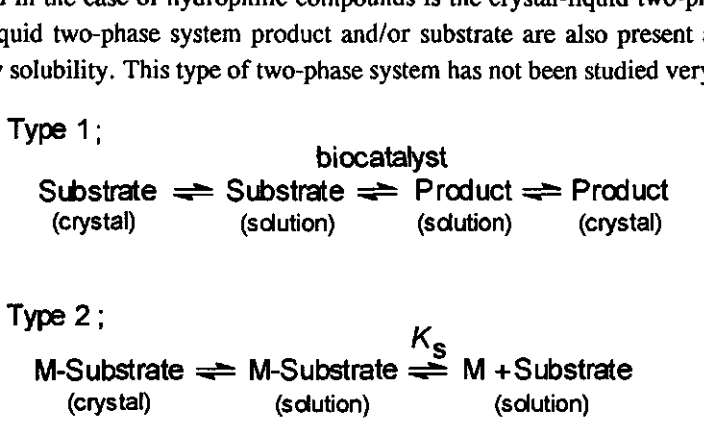

Figure 1. Crystal-liquid two-phase systems. Type 1, spontaneous crystallization; type 2, crystallization resulting from the addition metal counter-ions (M = metal).

it is being used on an industrial scale for some decades. Two types of crystal-liquid two-phase systems can be recognized (Fig. 1). The first type comprises systems in which the product crystallizes spontaneously during the reaction due to its low solubility (Fig. 1, Type 1). Examples of commercialized processes using such a system are the production of aspartame (20) and acrylamide (3). The other type comprises systems in which the solubility of the product is lowered specifically by the addition of, for instance, Cu^{2+} or Ca^{2+} giving rise to insoluble metal-complexes (Fig. 1, Type 2). Examples of commercialized processes of the second type of crystal-liquid two-phase systems are the production of D- or L-lactate from sugar and the conversion of Ca-fumarate into Ca-L-malate (13,27).

D-Malate, a chiral hydrophilic compound which can be used as a synthon by the fine-chemicals industry (23,24), can be produced from maleate with the lyase maleate hydratase (malease; EC 4.2.1.31). The advantage of using lyases for the production of optically active compounds is that these enzymes show an absolute stereospecificity and give a theoretical yield of 100% (31). Malease was already described for the first time in 1951 (22). Only recently, however, the first reports appeared in which this enzyme has been studied for the production of D-malate (2,17,19,28). We have studied the production of D-malate with malease from *Pseudomonas pseudoalcaligenes* and found that D-malate was a strong competitive inhibitor of the enzyme (29).

In this paper we describe the use of a crystal-liquid two-phase system to overcome both substrate and product inhibition to achieve high concentrations of D-malate from maleate with permeabilized cells of *P. pseudoalcaligenes*.

MATERIALS AND METHODS

Strain and growth conditions

P. pseudoalcaligenes NCIMB 9867 was obtained from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland. Cultures were grown in 5-liter Erlenmeyer flasks containing 1 liter of mineral salts medium (10) with 0.1% (w/v) 3-hydroxybenzoate and 0.1% (w/v) yeast extract adjusted to pH 7 with sodium hydroxide. The flasks were incubated at 30°C on a horizontal shaker oscillating at 1 Hz with an amplitude of 10 cm. Batch cultures were harvested at the end of the log-phase. Cells were concentrated and washed by centrifugation (28). The cells were permeabilized by freezing them overnight at -18°C in the presence of 0.15% Triton X-100 (28).

Solutions

Maleate solutions were prepared by adding maleic acid to 200 mM Tris after which the pH was adjusted to pH 7.5 with the indicated hydroxide salt. The pH of these solutions was checked again the other day, especially when precipitation was observed. Solutions used to determine the inhibition of malease by metal ions were made by adding different amounts of the metal chloride to 10 ml of a solution containing 100 mM maleate-50 mM Tris. After addition of the metal chloride and adjustment of the pH to 7.5 with sodium hydroxide, the total volume of this solution was made up to 20 ml with 100 mM maleate-50 mM Tris-HCl (pH 7.5). The effect of the addition of Ca^{2+} was studied by adding different amounts of $CaCl_2$ to 12 ml of a solution containing 2 M maleate-200 mM Tris adjusted to pH 7.5 with sodium hydroxide. After addition of $CaCl_2$, the total volume of

this solution was made up to 20 ml with 200 mM Tris-HCl (pH 7.5).

Determination of malease activity

To 4 ml of a substrate solution in a 30-ml serum bottle, 1 ml of a suspension containing permeabilized cells was added and this mixture was incubated in a shaking (200 rpm) water bath of 30°C. At t = 5, 10 and 15 minutes 500 μl samples were taken and mixed with 500 μl 6 N HCl in an Eppendorf vial to terminate the enzymatic reaction and to redissolve the precipitated substrate and product. The vials were centrifuged (5 min 13.000 rpm) and the supernatant was diluted 10 times with demineralized water. Subsequently, these samples were analyzed by HPLC.

The dissolved Ca-maleate and Ca-D-malate concentrations were determined in the supernatant obtained by centrifuging (5 min at 30 °C) samples from the incubation. Immediately after centrifugation 100 μl of the supernatant was mixed with 900 μl 0.6 N HCl in an Eppendorf vial to precipitate the protein. After centrifugation the soluble Ca-maleate and Ca-D-malate concentration were determined in the supernatant.

HPLC analysis

Malate, citramalate, maleate and citraconate were analyzed by HPLC as described before (28). The dicarboxylic acids were separated on a C_{18} column with 2 mM octylamine-25 mM potassium phosphate buffer (pH 7.0) as the mobile phase.

Protein determination

Protein was determined by the method of Bradford (5) using bovine serum albumin as the standard.

Chemicals

Octylamine, $Mg(OH)_2$ and D-citramalate were purchased from Aldrich. Maleate, D-malate, LiOH and $Ca(OH)_2$ were from Janssen Chimica. Citraconate was obtained from Fluka. NaOH, KOH, NH_4OH and $BaOH \cdot 8H_2O$ were purchased from Merck.

RESULTS

Effect of maleate counter-ion on malease activity

The malease activity of permeabilized cells of *P. pseudoalcaligenes* was determined with different maleate-salt solutions (Table 1). The maleate-salt solutions were prepared by using different metal hydroxides to adjust the pH of maleic acid solutions to a value of 7.5. $Ni(OH)_2$, $Co(OH)_2$, ZnO and $Al(OH)_3$ could not be used because these hydroxide salts could not adjust the pH of the maleic acid solutions to a value of 7.5 due to the fact that these salts were too weak bases and/or because the low solubility of these hydroxide salts prevented the formation of a homogeneous suspension.

Using $Ba(OH)_2$ or $Ca(OH)_2$ to adjust the pH of the maleate solution, a slurry was formed due to the low solubility of both Ba- and Ca-maleate (Fig. 2). With these substrate solutions it is, therefore, not correct to talk about a "substrate concentration", because not all substrate is dissolved. However, to be able to compare substrate and product concentrations and to be able to compare the concentrations of the different substrates, the "concentration" of these crystallized substrate solutions are also expressed in molar (M).

At low substrate concentrations, the initial malease activity was identical with all monovalent counter-ions tested (Table 1). With the bivalent metal-ions as the counter-ion the

Table 1. Initial malease activity with different maleate salts at increasing substrate concentrations

Counter-ion	Initial malease activity (nmol·min^{-1}·[mg protein]$^{-1}$)				
	Maleate concentration (M)				
	0.4	0.6	0.8	1.2	1.6
K^+	2400	2200	1700	700	350
Na^+	2400	2200	1700	700	400
Li^+	2400	2400	1900	1650	1000
NH_4^+	2400	2400	2200	1800	1300
Ba^{2+}	1400 [a]	1400 [a]	1400 [a]	[b]	[b]
Ca^{2+}	3200 [a]	3200 [a]	3200 [a]	3200 [a]	3200 [a]
Mg^{2+}	2800	2800	2550	2000	1400

[a] Precipitated substrate present.
[b] Formation of an inhomogeneous slurry due to incomplete wetting.

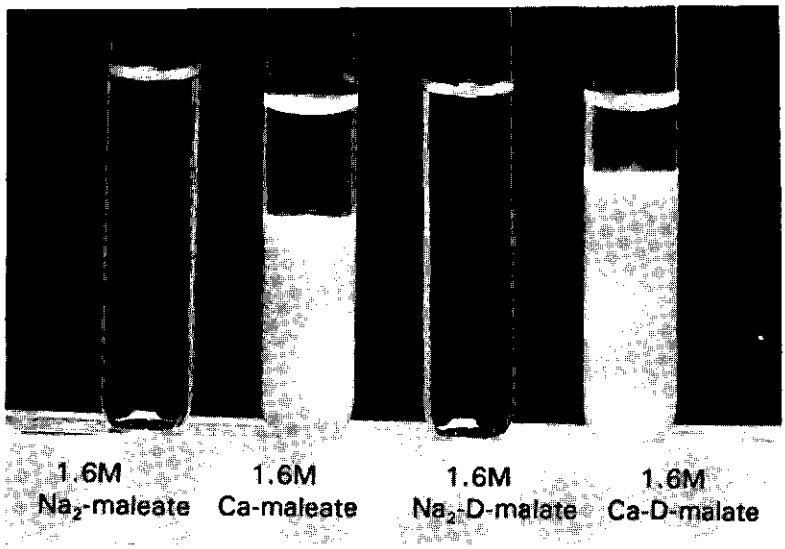

Figure 2. Solutions of 1.6 M Na_2- and Ca-maleate and of 1.6 M Na_2- and Ca-D-malate.

malease activity varied considerably. While Mg^{2+} and Ca^{2+} gave a higher malease activity compared to the monovalent counter-ions, the use of Ba^{2+} resulted in a lower malease activity. At higher substrate concentrations the malease activity decreased with all counter-ions except for Ba^{2+} and Ca^{2+} (Table 1). The malease activity measured with these two counter-ions was independent of the substrate "concentration" (Table 1).

Inhibition of malease activity at high dissolved substrate concentrations also varied depending on the counter-ions used. While malease activity at high substrate concentrations was strongly inhibited when Na^+ and K^+ were used as the counter-ions, malease activity was much less inhibited at these high substrate concentrations with NH_4^+ or Mg^{2+} as the counter-ion (Table 1).

Table 2. Effect of cat-ions on the relative malease activity with 100 mM Na_2-maleate as the substrate

Cat-ion	Relative malease activity (%)[a]				
	Cat-ion concentration (mM)				
	50	100	250	500	1000
K^+	100	100	100	91	75
Na^+	100	100	100	100	91
Li^+	100	100	100	100	77
NH_4^+	100	100	100	100	75
Ba^{2+}	100 [b]	64 [b]	42 [b]	0 [b]	0 [b]
Ca^{2+}	100	100	80	11	0
Mg^{2+}	100	100	84	22	0

[a] 100% is the malease activity in the absence of additional salt (= 2400 nmol·min^{-1}·[mg protein]$^{-1}$).
[b] Precipitated substrate and/or product present.

Inhibition of malease by metal-ions

The inhibition of malease by different concentrations of the counter-ions was determined at low maleate concentrations (100 mM; Table 2). The monovalent counter-ions did not significantly effect malease activity at the concentrations tested. Addition of divalent metal ions on the other hand, resulted in a strongly reduced malease activity (Table 2). Especially the addition of Ba^{2+} and Ca^{2+} resulted in low malease activities when high concentrations of these counter-ions were added. This is in contrast with the situation observed when high concentrations of these maleate salts were used as the substrate, and no decrease of malease activity was observed (Table 1).

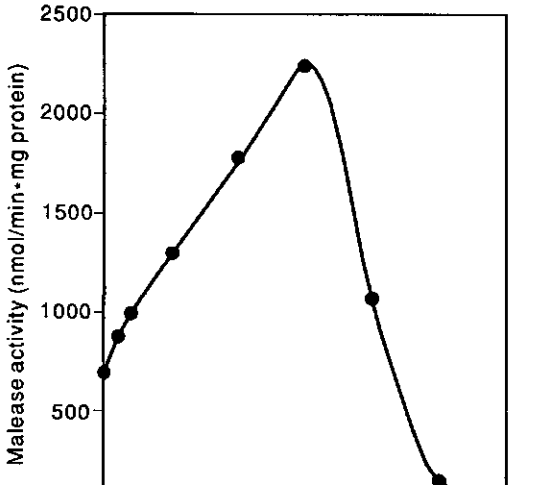

Figure 3. Effect of the addition of different $CaCl_2$ concentrations to a solution of 1.2 M Na_2-maleate on the initial malease activity. At a Ca^{2+} of 250 mM and higher a crystal-liquid two-phase system was formed.

Effect of adding Ca^{2+} to Na_2-maleate solutions on the initial malease activity

When 1.2 M Na_2-maleate is used as the substrate, a considerable inhibition of malease activity was observed (Table 1). The effect of adding different concentrations of Ca^{2+} to this substrate solution on the malease activity was determined (Fig. 3). The initial malease activity increased proportionally with the added Ca^{2+} concentration up to a concentration of 750 mM Ca^{2+} (Fig. 3). A further increase in the Ca^{2+} concentration resulted in a strong decrease of initial malease activity (Fig. 3).

Conversion of maleate to D-malate

D-Malate formation from different concentrations of maleate with either Na^+ or Ca^{2+} as the counter-ion were monitored in time (Fig. 4). With Na^+ as the counter-ion, complete conversion of maleate into D-malate was observed only at low substrate concentrations: after 24 hours, all the 1.2 M maleate was converted into D-malate, but only 50% of the 1.6 M maleate solution was converted into D-malate.

When Ca^{2+} was used as the counter-ion, a complete conversion of maleate into D-malate was observed with all three substrate concentrations (Fig. 4). Using 1.6 M maleate, almost all maleate was already converted after 4 hours, resulting in the formation of 1.57 M (210 g/l) D-malate.

Maleate and D-malate concentrations in solution during the conversion of Ca-maleate

Since the use of Ca^{2+} as the counter-ion results in a precipitation of substrate and product (Fig. 2, Table 1), the "total" and the dissolved maleate and D-malate concentrations were

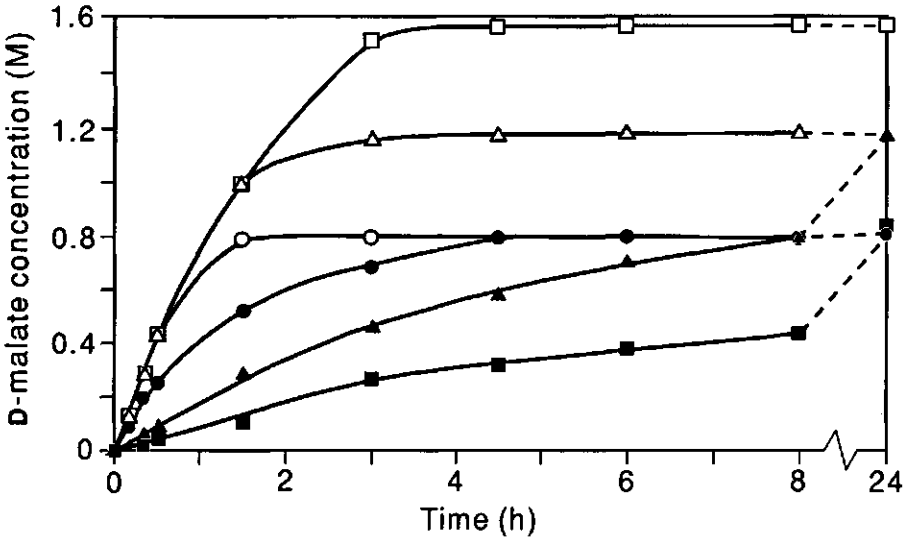

Figure 4. Conversion of various concentrations of Na_2- and Ca-maleate into D-malate with permeabilized cells (4.8 mg protein/ml). ●, 0.8 M Na_2-maleate; ▲, 1.2 M Na_2-maleate; ■, 1.6 M Na_2-maleate; ○, 0.8 M Ca-maleate; △, 1.2 M Ca-maleate; □, 1.6 M Ca-maleate.

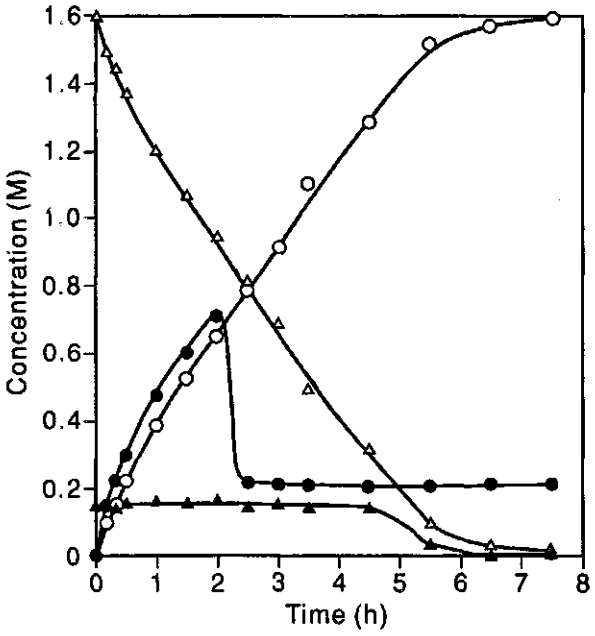

Figure 5. Conversion of Ca-maleate into Ca-D-malate by permeabilized cells (4 mg protein/ml). ○, total Ca-D-malate; ●, soluble Ca-D-malate; △, total Ca-maleate; ▲, soluble Ca-maleate.

monitored in time during the conversion of 1.6 M Ca-maleate (Fig. 5). The solubility of maleate under these conditions was 150 mM and that of D-malate was 210 mM. In the beginning of the conversion, however, supersaturation of Ca-D-malate was observed exceeding three times its solubility, after which crystallization started (Fig. 5).

The dissolved maleate concentration remained constant during the conversion, indicating a fast equilibrium between crystalline maleate and maleate in solution. Only at the end of the conversion, the release of maleate from the crystals became rate-limiting. When a magnetic

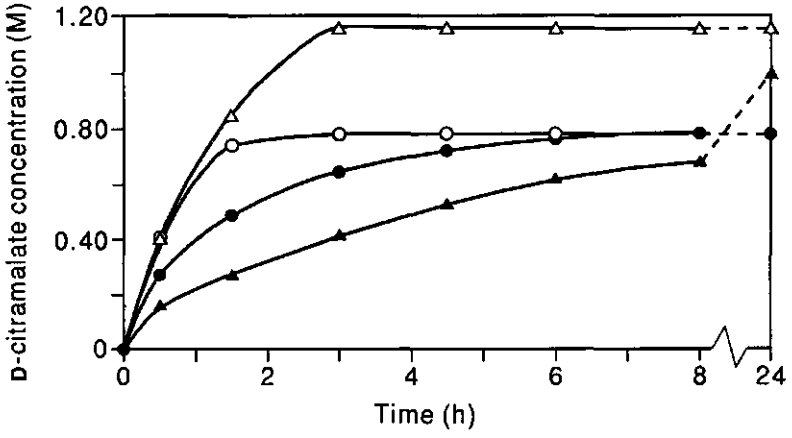

Figure 6. Conversion of various concentrations of Na_2- and Ca-citraconate into D-citramalate using permeabilized cells (4.8 mg protein/ml). ●, 0.8 M Na_2-citraconate; ▲, 1.2 M Na_2-citraconate; ○, 0.8 M Ca-citraconate; △, 1.2 M Ca-citraconate.

stirrer was placed inside the serum bottle, resulting in the formation of smaller crystals, the final yield was reached sooner.

D-Citramalate production

Citraconate is also a good substrate for malease from *P. pseudoalcaligenes* resulting in the formation of D-citramalate (29,30). The conversion of citraconate using either Na^+ or Ca^{2+} as the counter-ion into D-citramalate was also studied (Fig. 6). The same effects as described for the conversion of maleate using Na^+ and Ca^{2+} as the counter-ion were observed (Fig. 6). However, with this substrate, at a concentration of 1.6 M, an inhomogeneous slurry was formed, because not all Ca-citraconate crystals could be hydrated. This prohibits the use of this high a substrate concentration in the bioconversion. Using 1.2 M citraconate with Ca^{2+} as the counter-ion, in three hours the substrate was converted into 1.16 M (174 g/l) D-citramalate.

DISCUSSION

This paper describes the effect of the concentration and nature of the maleate counter-ion on the malease activity of permeabilized cells of *P. pseudoalcaligenes*. Large variations in malease activity were observed with the different maleate salts, especially at high substrate concentrations (Table 1).

In a maleate salt solution, both undissociated maleate-salt (metal-complex; M_x-maleate) and dissociated maleate^{2-} and metal ion (M^{y+}) are present. The ratio between the undissociated and dissociated maleate is given by the stability constant (K_s):

$$K_s = \frac{[M_x\text{-maleate}]}{[\text{maleate}^{2-}][M^{y+}]^x} . \qquad [1]$$

The percentage of the substrate present as dissociated maleate, depends on the metal ion used. Complex formation of a specific compound with a series of metal ions have shown to have stability constants which fall in the order (25,26):

$$K^+ < Na^+ < Li^+ < Ba^{2+} < Ca^{2+} < Mg^{2+} < Mn^{2+} < Co^{2+} < Ni^{2+} < Cu^{2+} > Zn^{2+} . \qquad [2]$$

Quite a large percentage of the organic acid salt is present in the undissociated form. For example with the K_s for K_2-, Na_2-, and Li_2-malate (14) calculations (for a malic acid solution in which the metal hydroxide is used to adjust the pH to 7) show that approximately 60% of the organic acid is present as the mono-salt-malate complex. When bivalent cations are used

as the counter-ion, an even higher percentage of the organic acid is present as metal-substrate complex. For instance, in Mg-malate and Ca-malate solutions over 95% is present as the undissociated-salt complex (14).

The inhibition of malease at high substrate concentrations with different maleate-salts (Table 1) is stronger when the K_s is lower (Eqn. [2], Table 1). This inhibition, therefore appears to correlate with the concentration of dissociated maleate.

The conversion of the soluble maleate salts can be described by the following equilibrium reactions, assuming that maleate^{2-} is actually the substrate transformed by the enzyme;

$$\underset{M_x\text{-maleate}}{\overset{K_s}{\rightleftarrows}} \; xM^{y+} + \underset{\text{maleate}^{2-}}{\overset{\text{malease}}{\rightleftarrows}} \; \underset{\text{D-malate}^{2-}}{} + xM^{y+} \underset{M_x\text{-D-malate}}{\overset{K_s}{\rightleftarrows}} \; . \qquad [3]$$

When Ca^{2+} or Ba^{2+} were used as the counter-ion, precipitation was observed (Fig. 2), due to the low solubility of these maleate- and D-malate-salt complexes. This precipitation is determined by the solubility product constant (K_{sp}) of the maleate salt;

$$K_{sp} = a_{\text{maleate}^{2-}} \; (a_{M^{y+}})^x \; , \qquad [4]$$

in which $a_{\text{maleate}^{2-}}$ and $a_{M^{y+}}$ are the activities of maleate^{2-} and of the metal-ion, respectively.

Since the used solutions are not dilute, this equation can in our case not simply be modified to the product of the concentrations (molalities). Because the activity coefficients of the Ba-maleate and Ca-maleate salts are not known from literature, it was not possible to simply calculate the K_{sp}.

The use of Ba^{2+} and Ca^{2+} results in the formation of a crystal-liquid two-phase system. In this system both dissolved and crystallized metal-substrate and metal-product complex are present;

$$\begin{array}{ccccccc} & & K_s & & \text{malease} & & K_s \\ M_x\text{-maleate (aq.)} & \rightleftarrows & xM^{y+} + \text{maleate}^{2-} & \rightleftarrows & \text{D-malate}^{2-} + xM^{y+} & \rightleftarrows & M_x\text{-D-malate (aq.)} \\ \uparrow\downarrow & & & & & & \uparrow\downarrow \\ M_x\text{-maleate (crystal)} & & & & & & M_x\text{-D-malate (crystal)} \; . \end{array} \qquad [5]$$

Due to the crystallization of the metal-organic acid complex the "real" substrate and product concentrations can be reduced even further than in solutions where only a soluble metal-organic acid complex is formed. In such a crystal-liquid two-phase system, the "real" substrate concentration remains constant even when the "total" substrate concentration is increased due to the maximal solubility of the metal-organic acid complex (Fig. 5). This is also reflected by the fact that the malease activity with maleate salts resulting in crystallization is independent of the "total" substrate concentrations (Table 1).

The formation of metal-substrate complexes is illustrated by the experiment described in

Fig. 3. When Ca^{2+} is added to a solution with an inhibitory Na_2-maleate concentration, an increase in malease activity was observed up to Ca^{2+} concentration of 750 mM (Fig. 3). This can be explained by the fact that Ca^{2+} binds stronger to maleate (larger K_s) than Na^+ thus reducing the "real" substrate concentration. When more than 750 mM Ca^{2+} is added, a decrease of the malease activity is observed. This can be explained either by the fact that free Ca^{2+} is present in such a high concentration that inhibition by free Ca^{2+} occurs, or because the "real" substrate concentration, approaches the K_m of malease for maleate^{2-} (29) due to metal-complexation of the maleate^{2-}.

Both the soluble metal-substrate complexes (12) and the insoluble metal-substrate/product complexes (6,21) can be used to improve a biocatalytic conversion. Crystal-liquid two-phase systems formed by the addition of specific counter-ions are already used on a commercial scale for some decades in organic acid producing bioconversions as for instance in the production of lactate and gluconate. In these fermentations $CaCO_3$ is added to neutralize the organic acid formed (6,21). In this way, a pH drop is prevented, thus maintaining the biocatalyst activity, prohibiting the formation of excess undissociated acid, which is very toxic (11). The fact that the use of Ca^{2+} also results in the formation of Ca-organic acid crystals thereby reducing the "real" substrate and/or product concentration and minimizing substrate and/or product inhibition, is only scarcely recognized.

The formation of a crystal-liquid two-phase system by the addition of specific counter-ions, is restricted to ionized compounds. This two-phase system shares, however, some of the major advantages with other two-phase systems. The use of the crystal-liquid two-phase system results in low substrate and product concentrations in the phase containing the biocatalyst (Fig. 5). In this way, both substrate (this report) and product inhibition, substrate and product toxicity (11) and the occurrence of side reactions (12) can be minimized. Also the yield of the reaction in metal-substrate complex systems can be influenced positively (4,9,12,13) when the substrate-metal complex is better soluble than the product-metal complex. Metal-substrate/product complex systems can also be effective in enhancing the optical purity of a bioconversion involving diastereoisomers (12), due to the fact that the stability constants of the metal-ion with the different diastereoisomers differs, mainly due to steric features (32).

In this report we have shown that the crystal-liquid two-phase system is a very dynamic system which can be used for the production of high concentrations of D-malate and D-citramalate. Ca^{2+} was found to be the best counter-ion for the conversion of high concentrations of maleate and citraconate (Table 1, Fig. 4 and 6). The use of this counter-ion resulted in the highest malease activity (Table 1). No substrate or product inhibition were observed during the conversion of high substrate concentrations. Yields of more than 99% were reached during the conversion of either maleate or citraconate (Fig. 4 and 6). The crystal-liquid two-phase system might also work out favourably in the bioconversion of many other ionized compounds catalyzed by for instance amino acid transaminases, halo acid dehalogenases, keto acid dehydrogenases and lipases.

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CHAPTER 9

CONVERSION OF 1 KILOGRAM OF MALEATE INTO D-MALATE BY MALEATE HYDRATASE FROM *PSEUDOMONAS PSEUDOALCALIGENES*

Mariët J. van der Werf, Sybe Hartmans and Will J.J. van den Tweel

SUMMARY

One kilogram of maleate (200 g/l) was converted by malease from *Pseudomonas pseudoalcaligenes* into 1.15 kg of D-malate using a crystal-liquid two-phase system. The yield of the reaction was 99.4% and the D-malate formed had an enantiomeric purity of 99.2%.

Maleate hydratase (malease; EC 4.2.1.31) catalyzes the enantiospecific addition of water to maleate forming D-malate, a versatile chiral synthon for organic synthesis (5). Recently, several research groups are studying this microbial conversion (1,3,4,7). After an intensive screening we have selected *Pseudomonas pseudoalcaligenes* NCIMB 9867 as the best malease containing biocatalyst (7). The use of Ca^{2+} as the counter-ion for maleate results in the formation of a crystal-liquid two-phase system at concentrations above 150 mM (6). In this system crystallized Ca-maleate complex is present, thus reducing the "real" substrate concentration and minimizing substrate and product inhibition. Under these conditions maleate concentrations of more than 150 g/l could be converted very efficiently into D-malate with a yield of more than 99% (6). In these studies, however, only small amounts of D-malate (0.5-1 g) were produced.

In this report we describe the conversion of 1 kg of maleate into D-malate by malease from *P. pseudoalcaligenes* using a crystal-liquid two-phase system.

Conversion of maleate into D-malate

P. pseudoalcaligenes NCIMB 9867 was grown at 30°C in a mineral salts medium (2) with 1 g of 3-hydroxybenzoate per liter as the carbon-source. Cells were harvested during the log-phase at approximately 80% of the maximal obtainable OD_{660}, washed and permeabilized with Triton X-100 (7).

The maleate hydratase reaction is cofactor and oxygen independent, and this bioconversion

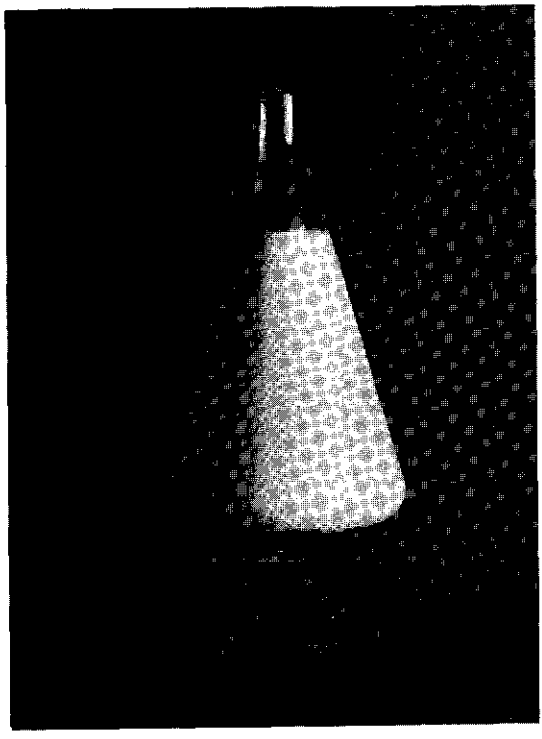

Figure 1. Conversion of 1 kg of maleate in a stirred 5-liter Erlenmeyer.

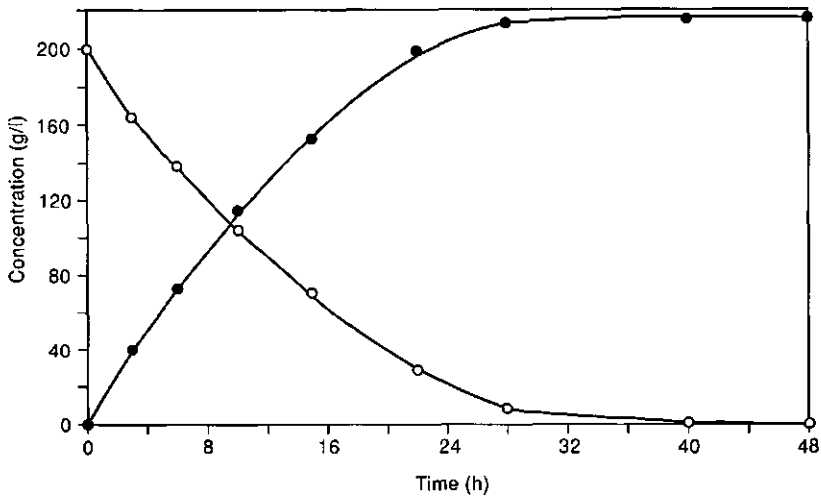

Figure 2. Conversion of 1 kg of maleate into 1.15 kg of D-malate by permeabilized cells of *P. pseudoalcaligenes* (1 g/l protein)

can, therefore, be performed in a simple stirred vessel (Fig. 1). 1.006 kg of maleic acid and 30.53 g of Tris were dissolved in 3 liter of demineralized water in a 5-liter Erlenmeyer flask stirred with a magnetic stirrer (500 rpm). Slowly (to prevent boiling of the solution, and to equilibrate the solution to prevent the addition of too much Ca-base) a total of 654.9 g $Ca(OH)_2$ was added to give a final pH of 7.5, resulting in the formation of a dense white slurry due to crystallization of calcium maleate complex (Fig. 1)(6). Permeabilized cells (final concentration: 1 g/l protein) were added and the solution was placed in a temperature controlled room (30°C) under stirring. The volume of the solution was adjusted to 5 liters with demineralized water resulting in a maleate concentration of 200 g/l.

Maleate and D-malate concentrations were followed in time (Fig. 2) and determined as described previously (6). After 48 hours, maleate was converted into D-malate with a yield of 99.4%. As the water addition reaction results in an increased molecular weight of the product, 1.15 kg of D-malate was produced from 1 kg of maleate (Fig. 2).

When, after 2 days, fresh cells were added to the incubation the yield did not increase any further, indicating that the maximal yield was reached. This yield of 99.4% is slightly lower than the maximal theoretical molar yield for the maleate hydration reaction of 99.96% (8). The enantiomeric purity was estimated to be 99.96% based on the 0.14 mM fumarate present during the conversion (7). This was in good agreement with the enzymatically determined (7) enantiomeric purity of 99.2%.

Upon prolonged incubation (up to 2 weeks) no degradation of the produced D-malate was observed.

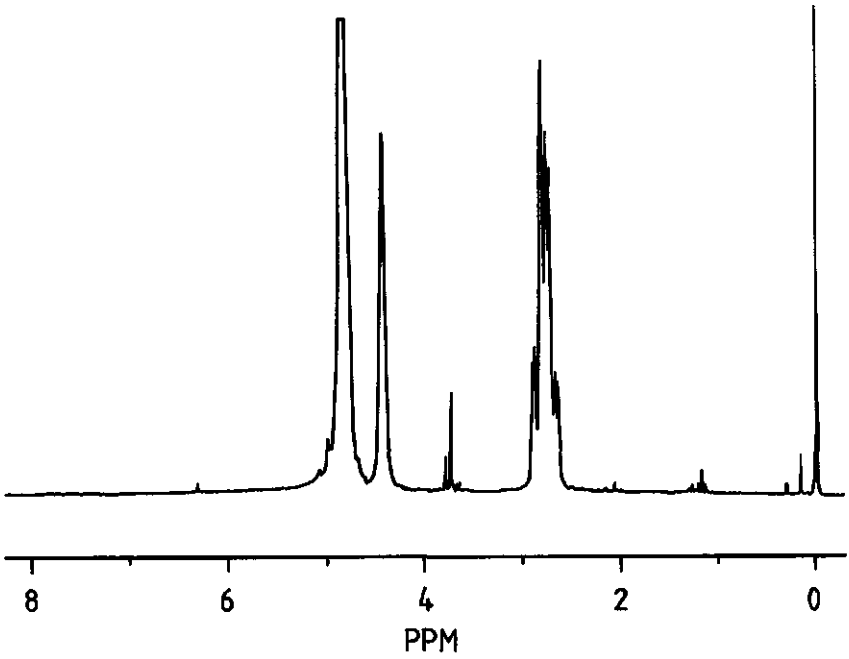

Figure 3. ^{1}H NMR spectra of the produced D-malate (in D_2O).

Analysis of the formed D-malate

The produced D-malate was purified by addition of H_2SO_4 to remove calcium which precipitated as $CaSO_4$. D-Malate was purified by recrystallization in ethanol. During the recrystallization an enhancement of the enantiomeric purity of D-malate was observed as L-malate could no longer be detected by the enzymatic method.

The identity of the product was confirmed by ^{1}H NMR (Fig. 3). From the fused peaks of the spectrum it could be concluded that still quite a lot of the D-malate was present as Ca^{2+}-complex.

Conclusions

The crystal-liquid two-phase system can be used to transform large quantities of maleate into D-malate in a very simple process. The use of $Ca(OH)_2$ functions both to adjust the pH of the solution and to form a crystal-liquid two-phase system thus preventing substrate and product inhibition.

Acknowledgments

We thank V.G.R. Steukers and B. Kaptein (both of the Bio-organic Chemistry Section, DSM Research, Geleen) for crystallizing the D-malate. We thank A. van Veldhuizen (Department of Organic Chemistry, Wageningen Agricultural University, Wageningen) for determining the ^{1}H NMR spectrum.

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CHAPTER 10

GENERAL DISCUSSION

In the past decade, the importance of biocatalysis for the production of optically pure fine chemicals has increased, and it is expected to increase even further in the near future. Advantages of enzyme-catalyzed reactions are their reaction-, regio- and stereospecificity. For the production of chiral compounds, enzymes that can transform a substrate for 100% into a 100% optically pure product are, both from an economical and an environmental point of view, most interesting. To date, however, mainly hydrolytic enzymes such as lipases, proteases and esterases are applied as biocatalysts for the production of chiral compounds (6,9). These enzymes are generally very stable and have a relaxed substrate specificity. However, a major disadvantage of these hydrolytic enzymes is that they generally do not show an absolute stereoselectivity: the optical purity of the product depends on the origin of the enzyme, the substrate (2), the solvent (17), the reaction conditions etc. (18). Furthermore, the maximal theoretical yield of these hydrolytic reactions is only 50%. Therefore, racemisation of the unwanted enantiomer is often necessary to make the process economically feasible and environmentally friendly (18).

Lyases, enzymes that catalyze addition and elimination reactions, are in principle better suited for the production of optically active compounds. These enzymes show an absolute stereochemistry (100% e.e.) and give a theoretical yield of 100% (Chapter 2). Another advantage of lyases is that no cofactor regeneration is necessary because addition or elimination reactions do not involve a net reduction or oxidation. In Japan lyases have already been applied on a commercial scale for some decades for the production of for instance L-malate and L-aspartate (22, Chapter 2).

This project was initiated to assess the availability and potential of lyases for the production of optically active compounds from prochiral substrates.

Selection of suitable biocatalysts

In collaboration with DSM research several potentially interesting addition reactions were selected. These reactions were the stereospecific addition of ammonia to α-methylcinnamate, acrylate, methacrylate and crotonate, and the stereospecific addition of water to maleate, acrylate, methacrylate, crotonate and 1,3-butadiene. Both microorganisms from culture collections and microorganisms isolated from enrichment cultures grown under different environmental conditions on the unsaturated prochiral compound, were screened for the presence of the enzyme of interest.

Screening for the presence of the ammonia-lyase activities described above was in all cases without success. Also other research groups have made efforts to isolate novel ammonia-lyase activities, unfortunately also without success (19).

No hydration of 1,3-butadiene was found but various microorganisms were identified that were able to hydrate the unsaturated acids. The enzymatic hydration of acrylate, methacrylate and crotonate was, with the exception of one microorganism, dependent on prior activation of the carboxylic group to its CoA-ester. Hydration reactions of these unsaturated compounds reported in the literature also involved the CoA-esters of substrate and product (5). In CoA-esters, the α-carbon atom has a stronger acidic character, thus weakening the α-C-H bond, resulting in a lower activation energy for the reaction (4,5). One microorganism which was able to hydrate acrylate directly was selected. Unfortunately, this strain lost this ability upon further investigation.

For the CoA-independent hydration of maleate to D-malate a very suitable biocatalyst was selected (Chapter 3).

D-Malate production from maleate with maleate hydratase from *Pseudomonas pseudoalcaligenes* and comparison with other malease containing biocatalysts

Maleate hydratase (malease; EC 4.2.1.31) from microbiological origin has been described before (8,16) but it had not been studied for the production of D-malate. We started our studies aimed at D-malate production from maleate by performing a broad screening for malease activity (Chapter 3). Malease activity appeared to be widespread amongst bacteria, yeasts, and fungi. Two selection criteria were used to select the best biocatalyst for further studies.

The first selection criterion was the optical purity of the produced D-malate. Hydratases produce hydroxy acids with an absolute enantiomeric purity (7, Chapter 2). However, the presence of other substrate or product transforming enzymatic activities might reduce the enantiomeric purity of the D-malate formed. One of these activities is maleate *cis-trans*-isomerase activity which transforms maleate into fumarate (15). By the action of the citric acid cycle enzyme fumarase, the unwanted L-malate is formed. Also the presence of malate racemase activity might result in a reduction of the enantiomeric purity of the D-malate formed. As far as we know no malate racemase activity has been described, but several other hydroxy acid racemases, as tartrate, mandelate and lactate racemase, have been described

(14). However, both malate racemase activity and maleate *cis-trans*-isomerase activity, would result in the formation of fumarate upon incubation of malease-containing cells with maleate. Therefore, accumulation of fumarate implicates that L-malate is also formed so that accumulation of fumarate was used as a negative selection criterion.

The other selection criterion was a stoichiometric production of D-malate from maleate. A stoichiometric conversion implicates that the selected microorganism is not able to degrade/transform the product, D-malate, or the substrate, maleate, into other compounds. *Pseudomonas pseudoalcaligenes* NCIMB 9867 was selected for further studies because it met these selection criteria and because it had one of the highest malease activities (Chapter 3).

Whitin one year after publication of our first manuscript, also three Japanese groups published about their work on the malease catalyzed D-malate production (Table 1). All three Japanese groups used the enantiomeric purity of the formed D-malate and the volumetric productivity as criteria for biocatalyst selection. Two biocatalysts (*Arthrobacter globiformis* and *Pseudomonas fluorescens* [see Table 1]) selected by the group of Nakayama did, however, contain maleate *cis-trans*-isomerase activity which resulted in large variations in the optical purity of the D-malate formed under different reaction conditions (11,13). Thereupon, this group selected the maleate-*cis-trans*-isomerase-negative strain, *Providencia stuarti* which produced optically pure D-malate (12)(Table 1).

Table 1. Comparison of malease containing microbial biocatalysts described in literature

	Biocatalyst					
	Pseudomonas pseudoalcaligenes NCIMB 9867 (This thesis)	*Arthrobacter* sp. MCI 2612 (1,23,25)	*Ustilago sphaerogena* S 402 (10)	*Arthrobacter globiformis* IFO 12137 (11)	*Pseudomonas fluorescens* IFO 3081 (13)	*Providencia stuarti* ATCC 25825 (12)
cis-trans-Isomerase activity	No	No	No	Yes	Yes	No
Enantiomeric purity (%)	99.97	100	99.55	90	99.5	100
Product degradation	No	Yes	Yes	Yes	Yes	No?
Molar yield (%)	99.4	72	49.6	62.3	57.8	78.8
Product conc. (g/l)	215	87	40.8	7.2	6.9	9.1
Specific activity (U/[mg protein])	3.2	0.148				
Productivity ($g \cdot l^{-1} \cdot h^{-1}$)	43 5 g/l (protein)	4.35 ? g/l	0.85 10 g/l (cells)	0.3 ? g/l	0.29 ? g/l	0.13 ? g/l
Cofactor dependent enzyme	No	Yes: Fe^{2+}, sulfur comp.	-	-	-	-
Act. pure enzyme (U/[mg protein])	100	6.5				
K_M (maleate) (mM)	0.35	3.85	-	-	-	-

All Japanese groups determined the productivity of the selected strains by measuring the D-malate concentration produced after a certain time. Often the maleate degradation was not compared to the D-malate produced, thus neglecting any product degradation and side-product formation which might take place. Indeed most of the selected biocatalysts also degraded D-malate (Table 1), requiring the need to terminate the reaction after a specific time to attain the highest yield. Consequently, the yields obtained with these strains were in all instances much lower than the maximal yield of 99.96% which can be calculated on the basis of the equilibrium constant for the maleate hydration reaction (Chapter 6)(Table 1). Furthermore, due to product degradation the real malease activity of these strains was not measured, but merely the difference between malease activity and product degradation activity. In this way strains with higher malease activities might be overlooked.

The induction of malease activity in *P. pseudoalcaligenes* was optimized and cells with a specific malease activity of 3.2 U·(mg protein)$^{-1}$ could be obtained (Chapter 4, Chapter 8). Also the group of Yamada/Asano optimized the induction of malease in their *Arthrobacter* sp. (1) and reported a specific malease activity of 0.15 U·(mg protein)$^{-1}$ (23). The other groups did not mention the specific malease activity of their selected biocatalysts. When, however, the volumetric productivities are compared, *P. pseudoalcaligenes* at 5 mg protein/ml had the highest productivity and also *Arthrobacter* sp. had a much higher productivity than those which could be achieved by using the other malease-containing biocatalysts (Table 1). In most cases, however, the biomass concentrations were not reported.

The malease activity of *P. pseudoalcaligenes* is high enough to allow commercialization of this process based on the comparison with the specific activity of fumarase in the commercialized fumarase process (Table 2) and genetic techniques should, therefore, not be necessary to further enhance the malease activity. However, when a genetically modified microorganism containing the malease gene can be obtained, the expensive and toxic 3-hydroxybenzoate might no longer be required as an inducer. Furthermore, the inactivation of malease activity at the end of the log-phase (Chapter 4) might be overcome. A genetically modified microorganism containing the malease gene might also allow the use of cheap growth substrates such as sugars, this in contrast to *P. pseudoalcaligenes* which is limited in its ability to grow on cheap growth substrates (Chapter 3). We have, therefore, made an effort to isolate the gene coding for malease activity, unfortunately without success.

The versatility of malease as a biocatalyst also depends on its substrate specificity. Therefore, the enzyme was purified from *P. pseudoalcaligenes* (Chapter 5). The enzyme has a rather narrow substrate specificity and only catalyzes, as does malease from *Arthrobacter* sp. (24), the hydration of α-substituted-*cis*-unsaturated-α,β-dicarboxylic acids (e.g. citraconate) (Chapter 5).

Based on the data in Table 1 it can be concluded that *P. pseudoalcaligenes* is the best malease containing biocatalyst described so far (Table 1). This strain produces D-malate with a high enantiomeric purity, with the highest yield and has the highest malease activity. Also the characteristics of purified malease from *P. pseudoalcaligenes* are better than those

determined for purified malease from *Arthrobacter* sp (23,24)(Table 1). It is cofactor independent, has a higher specific activity and a lower K_M for maleate.

The reaction conditions for D-malate production with *P. pseudoalcaligenes* were optimized. Permeabilized cells were used to avoid limitation of the reaction rate due to substrate uptake or product excretion (Chapter 7). Substrate and product inhibition (Chapter 5 and 8) could be avoided by using a crystal-liquid two-phase system (Chapter 8) and in this way product concentrations up to 215 g/l could be reached (Chapter 9).

For commercial application, it might be favourable to immobilize the biocatalyst as in this way both biocatalyst costs and the fixed expenses of the process can be reduced by continuous operation. Some initial experiments were performed in this area but there were two major problems. The enzyme was found to leak from the cells due to lysis (see also Chapter 7) and microbial growth occurred on the D-malate produced.

Comparison of the malease process with the commercialized fumarase process

The maleate hydratase reaction shows quite some resemblance with the commercialized fumarate hydratase reaction (Fig. 1). Therefore, we compared the malease process with the commercialized fumarase process to obtain an indication of the economic feasibility of the malease process (Table 2).

As can be seen from Table 2 the productivities of these processes and the prices of the substrates are comparable. The yield of the malease process is higher and no by-products are formed during the malease process. However, the half-life of malease has not yet been

Table 2. Comparison of the malease process with the commercialized fumarase process

	Biocatalytic process	
	D-Malate production with malease from *P. pseudoalcaligenes* (This thesis)	L-Malate production with fumarase from *Brevibacterium flavum* (Tanabe Seiyaku Co., Ltd., Osaka, Japan)(20)
Specific activity ($nmol \cdot min^{-1} \cdot [mg\ protein]^{-1}$)	3200	700 (21)
Mode of operation	Batch	Continuous
Permeabilized cells	Yes	Yes
Productivity	43 $g \cdot l^{-1} \cdot h^{-1}$	65 $g \cdot l^{-1} \cdot h^{-1}$
Cofactor requirements	None	None
By-products	None	Succinate
Maximal molar yield hydration reaction	99.96%	72.6% (50°C) (Chapter 6)(3)
Molar yield of the conversion	99.4%	70%
Half-life of enzyme activity	?	128 days (50°C)
Price of substrate ($\$ \cdot kg^{-1}$)	1.0	1.4
Price of product ($\$ \cdot kg^{-1}$)	175	8
Market	?	1000 $ton \cdot y^{-1}$

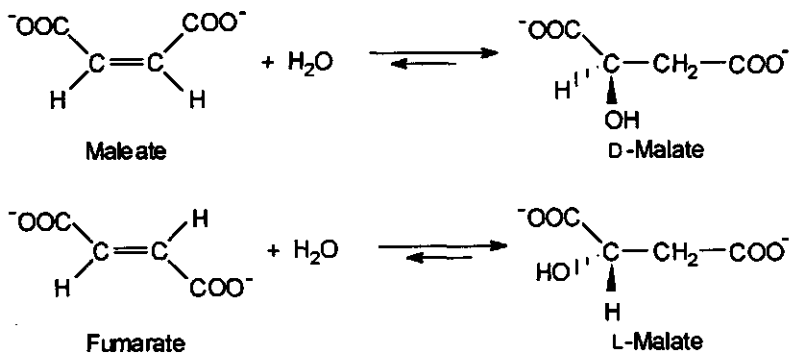

Figure 1. Maleate and fumarate hydration reactions catalyzed by malease and fumarase, respectively.

yet been thoroughly investigated, but it seems to be lower (~10 days in the presence of 100 mM DL-malate) than that of the immobilized fumarase of *Brevibacterium flavum*. D-Malate is, however, presently 20 times more expensive than L-malate and therefore the malease process does not have to be as efficient as the fumarase process to allow commercialization. Therefore, based on the data in Table 2, a process for the production of D-malate with malease from *P. pseudoalcaligenes* appears to be very promising.

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SUMMARY

The biological activity of a chiral compound with respect to its pharmaceutical and agrochemical activity, flavour and taste can vary dramatically for the different enantiomers. Especially when using chiral compounds for pharmaceutical or agrochemical applications, the presence of the "wrong" stereoisomer can have severe effects on patients or may cause an additional environmental load. The availability of (cheap) optically pure compounds is, therefore, of prime importance, especially for the pharmaceuticals industry.

There are several ways in which optically active compounds can be produced. One way is by using biocatalysts. During the last decade, the importance of biocatalysis for the production of optically pure fine-chemicals has increased, and it is expected to increase even further in the near future. Advantages of enzymes as catalysts are the fact that they work under mild reaction conditions and their reaction-, regio-, and stereospecificity. These last three aspects also imply that fewer side-products are produced which is positive in view of the increasing environmental concern. Enzymes that can transform a substrate for 100% into a 100% optically pure product, and which do not require cofactor recycling are, both from an economical and an environmental point of view, most suitable as biocatalysts.

D-Malate is an optically active compound which can be used as a synthon in organic synthesis, as a resolving agent and as a ligand in asymmetric catalysis (Chapter 1). D-Malate can be produced in several ways, but the approach using the lyase maleate hydratase (malease; EC 4.2.1.31), which catalyzes the hydration of maleate to D-malate, seems to be the most promising approach for commercialization (Chapter 1).

Lyases are enzymes that catalyze the cleavage of C-C, C-N, C-O and other bonds by elimination to produce double bonds or, conversely, catalyze the addition of groups to double bonds. These enzymes do not require cofactor recycling, show an absolute stereospecificity (100% e.e.) and can give a theoretical yield of 100%. Lyases are attracting increasing interest as biocatalysts for the production of optically active compounds, and have already found application in several large commercial processes (Chapter 2).

We have screened more than 300 microorganisms for the presence of malease activity (Chapter 3). Many strains (n = 128) could convert maleate to D-malate with an enantiomeric purity of more than 97%. Accumulation of fumarate during incubation of permeabilized cells with maleate was shown to be indicative for the presence of the unwanted maleate *cis-trans*-isomerase activity, which ultimately results in the formation of the unwanted L enantiomer of malate. The ratio in which fumarate and malate accumulated could be used to estimate the

enantiomeric composition of the malate formed (Chapter 3).

Pseudomonas pseudoalcaligenes NCIMB 9867 was selected for more detailed studies, because it contained one of the highest malease activities, the D-malate formed had an enantiomeric purity of more than 99.97%, it did not degrade D-malate and because it did not show any side-product formation (Chapter 3).

The highest malease activity in *P. pseudoalcaligenes* was observed when it was grown on 3-hydroxybenzoate (Chapter 4). Growth on gentisate also resulted in an enhanced malease activity. Both compounds are degraded via maleate in this microorganism. The specific malease activity of cells grown on 3-hydroxybenzoate was constant during the logarithmic phase, but dropped rapidly as soon as growth ceased.

Malease from *P. pseudoalcaligenes* was purified (Chapter 5). The purified enzyme (89 kDa) consisted of two subunits (57 and 24 kDa). No cofactor was required for full activity of this colorless enzyme. The stability of the enzyme was dependent on the protein concentration and the presence of dicarboxylic acids. Maximum enzyme activity was measured at pH 8 and 45°C. The purified enzyme also catalyzes the hydration of citraconate (2-methylmaleate) forming D-(+)-citramalate and of 2,3-dimethylmaleate at, respectively, 54 and 0.8% of the rate of maleate hydration (30°C). The K_M of malease for maleate was 0.35 mM, and for citraconate 0.20 mM. The products D-malate and D-citramalate and the substrate analog 2,2-dimethylsuccinate were strong competitive inhibitors of malease (Chapter 5).

Hydratases catalyze equilibrium reactions. To optimize the reaction conditions, data concerning the equilibrium constant are necessary to determine the maximal obtainable yield. In literature no data were available concerning the equilibrium of the malease catalyzed reactions. Therefore, we determined the equilibrium constants (K_{app}) for the malease catalyzed hydration reactions. The K_{app} for the maleate, citraconate, and 2,3-dimethylmaleate hydration reactions were 2050, 104 and 11.2, respectively, under standard biochemical conditions (25°C, pH 7.0, I=0.1) (Chapter 5 and 6). The equilibrium constants for the maleate and citraconate hydration reactions make yields of more than 99% possible.

As especially maleate has a high pKa_2, the influence of the pH (6.0-8.5) on the K_{app} was determined to describe the influence of the presence of the different forms of maleate (i.e. dianionic, monoanionic and diprotonated) on the equilibrium constant (Chapter 6). Also the influence of the temperature (10°C - 40°C) on K_{app} was determined. From these experiments the Gibbs-free-energy change ($\Delta G°'$), the enthalpy change ($\Delta H°'$), and entropy change ($\Delta S°'$) under standard biochemical conditions for the maleate^{2-} and citraconate^{2-} hydration reactions were calculated (Chapter 6). Also the effect of the temperature on the maleate and citraconate hydration rates of the purified enzyme was determined to calculate the activation energy. At low temperatures the hydration rate of citraconate was higher, while at temperatures above 18°C the maleate hydration reaction was faster (Chapter 6).

P. pseudoalcaligenes is not able to grow on maleate, probably because it lacks an uptake system for maleate (Chapter 3). Intact cells of *P. pseudoalcaligenes* do not show any

accumulation of D-malate from maleate but Triton X-100 treated cells showed accumulation of D-malate from maleate, indicating that these cells were permeabilized or lysed (Chapter 7). Incubation of cells with Triton X-100 also resulted in an increase in the protein concentration of the supernatant, indicating the occurrence of lysis. Permeabilization and lysis were time-dependent: longer incubations resulted in higher malease activities and more protein in the supernatant. The permeabilization and lysis rates were also dependent on the Triton X-100 and biomass concentration (Chapter 7).

Malease activity of permeabilized cells of *P. pseudoalcaligenes* decreased strongly when Na_2-maleate concentrations higher than 0.6 M were used as the substrate (Chapter 8). When other counter-ions than Na^+ were used, in some instances the malease activity was found to be affected much less by high substrate concentrations. When for instance Mg^{2+} was used as the counter-ion, a much larger percentage of maleate is present as metal-substrate complex, thereby reducing the "real" substrate concentration resulting in less substrate inhibition. When Ca^{2+} and Ba^{2+} were used as the counter-ion, the malease activity was not at all affected by increasing substrate concentrations. The use of these metal-ions resulted in the formation of a crystal-liquid two-phase system, due to the low solubility of the metal-substrate complex. In this situation, the "real" substrate concentration was independent on the total amount of substrate present. Ca^{2+} was the best counter-ion for the conversion of maleate into D-malate. The use of this metal-ion resulted in the highest malease activities and the absence of substrate inhibition at high substrate concentrations (Chapter 8). In this way, high concentrations (up to 160 g/l) of either maleate or citraconate were converted by malease of *P. pseudoalcaligenes* into D-malate and D-citramalate, respectively, with yields of more than 99%.

To determine the potential of the crystal-liquid two-phase system for the large scale conversion of maleate into D-malate, the conversion of one kilogram of maleate was studied (Chapter 9). At a substrate concentration of 200 g/l, permeabilized cells of *P. pseudoalcaligenes* (1 g/l protein) converted maleate within two days into 1.15 kg of D-malate with a yield of 99.4%.

During our studies on the D-malate production from maleate with malease from *P. pseudoalcaligenes*, also three Japanese groups published about their studies on this conversion, using other biocatalysts. *P. pseudoalcaligenes* was the best malease containing biocatalyst (Chapter 10). It produced D-malate at the highest product concentrations, with the highest yield and the highest specific activity. Comparing these data with the data for the already commercialized process for L-malate production catalyzed by fumarase, suggests that commercialization of the malease process is very promising (Chapter 10).

SAMENVATTING

De biologische activiteit van een chirale verbinding met betrekking tot zijn farmaceutische en agrochemische activiteit, geur en smaak kan sterk variëren tussen de verschillende enantiomeren. Vooral wanneer chirale verbindingen worden toegepast als farma- of agrochemicaliën kan de aanwezigheid van de "verkeerde" enantiomeer schadelijke gevolgen hebben voor patiënten of kan het een extra milieubelasting tot gevolg hebben. De beschikbaarheid van (goedkope) optisch zuivere verbindingen is daarom van essentieel belang, met name voor de farmaceutische industrie.

Er zijn verschillende manieren waarop optisch actieve verbindingen geproduceerd kunnen worden. Eén manier is met behulp van biokatalyse. In de afgelopen tien jaar is het belang van biokatalyse voor de produktie van optisch zuivere fijnchemicaliën sterk toegenomen en dit zal naar verwachting in de nabije toekomst nog verder toenemen. Voordelen van enzymen als katalysatoren zijn het feit dat zij werken onder milde reactiecondities en dat zij reactie-, regio-, en stereospecifiek zijn. Deze laatste drie factoren impliceren ook dat minder bijprodukten gevormd worden waardoor er een lagere belasting van het milieu optreedt. Enzymen die in staat zijn om een substraat voor 100% om te zetten in een 100% optisch zuiver produkt en waarbij geen cofactorregeneratie nodig is, zijn zowel vanuit een economisch als vanuit een milieu oogpunt het geschiktst om als biokatalysator toegepast te worden.

D-Appelzuur (D-malaat) is een optisch actieve verbinding die gebruikt kan worden als synthon in de organische synthese, voor racemaat scheidingen en als een ligand in asymmetrische katalyse (Hoofdstuk 1). D-Appelzuur kan op verschillende manieren geproduceerd worden, waarbij toepassing van het lyase maleïnezuurhydratase (malease; EC 4.2.1.31), dat de hydratatie van maleïnezuur (maleaat) tot D-appelzuur katalyseert, het meest geschikt lijkt voor commercialisering (Hoofdstuk 1).

Lyasen zijn enzymen die het verbreken van C-C, C-N, C-O en andere bindingen katalyseren door eliminatie, resulterend in de vorming van dubbele bindingen of, omgekeerd, de additie van groepen aan dubbele bindingen katalyseren. Deze enzymen zijn onafhankelijk van cofactorrecycling, zij hebben een absolute stereospecificiteit (100% e.e.) en kunnen een theoretische opbrengst van 100% opleveren. Er is een toenemende belangstelling voor lyasen als biokatalysator voor de produktie van optisch actieve verbindingen en zij worden reeds toegepast in verschillende grootschalige commerciële processen (Hoofdstuk 2).

Wij hebben meer dan 300 micro-organismen gescreend op de aanwezigheid van malease-

activiteit (Hoofdstuk 3). Vele stammen (n = 128) waren in staat om maleïnezuur om te zetten in D-appelzuur met een enantiomere zuiverheid van meer dan 97%. Ophoping van fumaarzuur tijdens de incubatie van gepermeabiliseerde cellen met maleïnezuur was een indicatie voor de aanwezigheid van de ongewenste maleïnezuur-*cis-trans*-isomerase-activiteit, welke uiteindelijk resulteert in de vorming van het ongewenste L-enantiomeer van appelzuur. De verhouding waarin fumaarzuur en appelzuur ophoopten kon gebruikt worden om de enantiomere samenstelling van het gevormde appelzuur te schatten (Hoofdstuk 3).

Pseudomonas pseudoalcaligenes NCIMB 9867 werd geselecteerd voor verder onderzoek omdat het één van de hoogste malease-activiteiten bevatte en omdat het gevormde D-appelzuur een enantiomere zuiverheid van meer dan 99.97% had. Bovendien was deze stam niet in staat om het gevormde D-appelzuur af te breken en werden geen bijprodukten gevormd (Hoofdstuk 3).

P. pseudoalcaligenes bevatte de hoogste malease-activiteit na groei op 3-hydroxybenzoaat (Hoofdstuk 4). Groei op gentisaat resulteerde ook in een verhoogde malease-activiteit. Beide verbindingen worden in dit micro-organisme via maleïnezuur afgebroken. De specifieke malease-activiteit van cellen gekweekt op 3-hydroxybenzoaat was constant gedurende de logaritmische groeifase, maar nam snel af wanneer de groei ophield.

Het malease van *P. pseudoalcaligenes* is gezuiverd (Hoofdstuk 5). Het gezuiverde enzym (89 kDa) bestaat uit twee subunits (57 and 24 kDa). Er was geen cofactor nodig voor volledige activiteit van dit kleurloze enzym. De stabiliteit van het enzym was afhankelijk van de eiwitconcentratie en van de aanwezigheid van dicarboxylzuren. De maximale enzymactiviteit werd gemeten bij pH 8 en 45°C. Het gezuiverde enzym katalyseerde ook de hydratatie van citraconaat (2-methylmaleïnezuur), resulterend in de vorming van D-(+)-citramalaat en van 2,3-dimethylmaleïnezuur met respectievelijk 54 en 0.8% van de maleïnezuurhydratatiesnelheid (30°C). De K_M van malease voor maleïnezuur was 0.35 mM, en die voor citraconaat 0.20 mM. De produkten D-appelzuur en D-citramalaat en de substraatanaloog 2,2-dimethylsuccinaat waren sterke competitieve remmers van malease (Hoofdstuk 5).

Hydratasen katalyseren evenwichtsreacties. Om de reactiecondities te optimaliseren is het nodig om de parameters die de evenwichtsconstante beïnvloeden te kennen om de maximale opbrengst te kunnen bepalen. In de literatuur waren geen gegevens bekend over het evenwicht van de door malease gekatalyseerde reacties. Daarom zijn de evenwichtsconstanten (K_{app}) van de door malease gekatalyseerde hydratatiereacties bepaald. De K_{app} voor de maleïnezuur-, citraconaat-, en 2,3-dimethylmaleïnezuurhydratatiereacties waren respectievelijk 2050, 104 and 11,2 onder standaard-biochemische condities (25°C, pH 7.0, I=0.1) (Hoofdstuk 5 en 6). De evenwichtsconstanten voor de maleïnezuur- en citraconaathydratatiereacties maken opbrengsten van meer dan 99% mogelijk.

Aangezien in het bijzonder maleïnezuur een hoge pKa_2 heeft, is de invloed van de pH (6.0-8.5) op de K_{app} bepaald om de invloed van de aanwezigheid van de verschillende vormen van maleïnezuur (dianion, monoanion en geprotoneerd) op de evenwichtsconstante te beschrijven

(Hoofdstuk 6). Ook de invloed van de temperatuur (10°C - 40°C) op de K_{app} is bepaald. Uit deze experimenten is de Gibbs-vrije-energieverandering ($\Delta G°'$), de enthalpieverandering ($\Delta H°'$) en de entropieverandering ($\Delta S°'$) onder standaard-biochemische condities voor de maleaat^{2-}- en citraconaat^{2-}-hydratatiereacties berekend (Hoofdstuk 6). Ook het effect van de temperatuur op de maleïnezuur- en citraconaathydratatiesnelheden, gekatalyseerd door het gezuiverde enzym, zijn bepaald om de activatie-energie te kunnen berekenen. Bij lage temperaturen was de hydratatiesnelheid van de citraconaatreactie hoger, terwijl bij temperaturen boven de 18°C juist de maleïnezuurhydratatiesnelheid hoger was (Hoofdstuk 6).

P. pseudoalcaligenes is niet in staat om op maleïnezuur te groeien, waarschijnlijk omdat dit micro-organisme een opnamesysteem voor maleïnezuur mist (Hoofdstuk 3). Intacte cellen van *P. pseudoalcaligenes* waren niet in staat om D-appelzuur uit maleïnezuur op te hopen, terwijl met Triton X-100 behandelde cellen wel D-appelzuur uit maleïnezuur konden ophopen, wat er op wijst dat de cellen gepermeabiliseerd of gelyseerd zijn (Hoofdstuk 7). Incubatie van cellen met Triton X-100 resulteerde ook in een toename van de eiwitconcentratie in het supernatant, wat er op wijst dat lysis heeft plaatsgevonden. Permeabilisatie en lysis zijn tijdsafhankelijk: hoe langer de cellen geïncubeerd werden met Triton X-100 des te hoger werd de malease-activiteit van de celsuspensie, en des te meer eiwit er zich in het supernatant bevond. De permeabilisatie- en lysissnelheden waren ook afhankelijk van de Triton X-100-concentratie en de biomassaconcentratie (Hoofdstuk 7).

De malease-activiteit van gepermeabiliseerde cellen van *P. pseudoalcaligenes* nam sterk af wanneer Na_2-maleïnezuurconcentraties van meer dan 0.6 M werden gebruikt als substraat (Hoofdstuk 8). Wanneer andere tegenionen gebruikt werden, werd in sommige gevallen de malease-activiteit veel minder beïnvloed door de hoge substraatconcentraties. Wanneer gebruik wordt gemaakt van bijvoorbeeld Mg^{2+} als tegenion, is een veel groter percentage maleïnezuur aanwezig als metaalsubstraatcomplex, waardoor de "echte" substraatconcentratie wordt gereduceerd, resulterend in een lagere substraatremming. Wanneer Ca^{2+} en Ba^{2+} als tegenion worden gebruikt, werd de malease-activiteit helemaal niet beïnvloed door toenemende substraatconcentraties. Gebruik van deze metaalionen resulteert in de vorming van een kristal-vloeistof-twee-fasen-systeem vanwege de lage oplosbaarheid van het metaalsubstraatcomplex. In dit geval is de "echte" substraatconcentratie onafhankelijk van de totale hoeveelheid substraat die aanwezig is. Ca^{2+} was het beste tegenion voor de conversie van maleïnezuur in D-appelzuur. Het gebruik van dit metaalion resulteert in de hoogste malease-activiteit doordat er geen substraatremming optreedt (Hoofdstuk 8). Op deze manier, kunnen hoge concentraties (tot 160 g/l) van zowel maleïnezuur als citraconaat worden omgezet, door malease van *P. pseudoalcaligenes*, in respectievelijk D-appelzuur en D-citramalaat, met opbrengsten van meer dan 99%.

Om het potentieel van het kristal-vloeistof-twee-fasen-systeem voor de grootschalige omzetting van maleïnezuur in D-appelzuur na te gaan is de omzetting van 1 kilogram maleïnezuur bestudeerd (Hoofdstuk 9). Met gepermeabiliseerde cellen van *P.*

pseudoalcaligenes (1 g/l) wordt maleïnezuur (200 g/l) binnen twee dagen omgezet in 1.15 kg D-appelzuur met een opbrengst van 99.4%.

Tijdens ons onderzoek naar D-appelzuurproduktie uit maleïnezuur met malease van *P. pseudoalcaligenes* hebben ook drie Japanse groepen gepubliceerd over hun studies naar dezelfde bioconversie, gebruikmakend van andere biokatalysatoren. Vergelijking van de verschillende biokatalysatoren liet zien dat *P. pseudoalcaligenes* de beste malease-bevattende biokatalysator was (Hoofdstuk 10). Deze biokatalysator bezat de hoogste specifieke activiteit en produceerde de hoogste D-appelzuurconcentraties met de hoogste opbrengst. Wanneer deze resultaten worden vergeleken met de gegevens van het reeds gecommercialiseerde proces voor L-appelzuurproduktie met fumarase, lijkt commercialisering van het malease met *P. pseudoalcaligenes* als biokatalysator zeer veelbelovend (Hoofdstuk 10).

ABBREVIATIONS

a	Activity
A	Frequency factor
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CoA	Coenzyme A
L-DOPA	L-3,4-Dihydroxyphenylalanine
e.e.	Enantiomeric excess
E_a	Activation energy
EC	Enzyme class
EDTA	Ethylenediaminetetraacetic acid
FAD	Flavin adenine dinucleotide
h	Planck constant
HPLC	High-performance liquid chromatography
I	Ionic strength
k	Rate constant
k_B	Boltzmann constant
Ka_2	Acid constant
K_{app}	Apparent equilibrium constant
K'_{eq}	Equilibrium constant
K_i	Inhibition constant
K_M	Michaelis-Menten constant
K_s	Stability constant
K_{sp}	Solubility product constant
m	Molecular mass
NAD^+	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NCIMB	National Collections of Industrial and Marine Bacteria
PAGE	Polyacrylamidegel electrophoresis
PLP	Pyridoxal phosphate
PQQ	Pyrroloquinoline quinone
R	Gas constant
$S^{\circ\prime}$	Entropy under standard biochemical conditions

SDS	Sodium dodecyl sulfate
T	Absolute temperature
TPP	Thiamine pyrophosphate
Tris	Tris(hydroxymethyl)aminomethane
U	Units
v	Specific activity
Y	Molar yield
$[\alpha]^{T}_{\lambda}$	Specific optical rotation
γ	Activity coefficient
$\Delta G^{\circ\prime}$	Gibbs-free-energy change under standard biochemical conditions
$\Delta G^{\neq}$	Gibbs free energy of activation
$\Delta_f G^{\circ\prime}$	Gibbs free energy of formation under standard biochemical conditions
$\Delta H^{\circ\prime}$	Enthalpy change under standard biochemical conditions
$\Delta H^{\neq}$	Enthalpy of activation
$\Delta_f H^{\circ\prime}$	Enthalpy of formation under standard biochemical conditions
$\Delta S^{\circ\prime}$	Entropy change under standard biochemical conditions
$\Delta S^{\neq}$	Entropy of activation
ε	Molar absorption coefficient
λ	Wavelength

CURRICULUM VITAE

Mariët van der Werf werd op 23 januari 1966 geboren te Maarssen. In 1984 behaalde zij het VWO diploma aan de Samenwerkingsschool te Waddinxveen. In hetzelfde jaar werd begonnen met de studie Biologie aan de Landbouwuniversiteit Wageningen. In november 1989 studeerde zij af met als afstudeervakken Industriële Microbiologie, Moleculaire Biologie en Proceskunde, en een 6 maands stage by ICI Biological Products (tegenwoordig Zeneca Bioproducts) te Billingham, Groot-Brittannië. In mei 1990 werd begonnen met een vierjarig promotieonderzoek bij de Sectie Industriële Microbiologie, Landbouwuniversiteit Wageningen. Van januari 1993 tot juli 1993 is het promotieonderzoek uitgevoerd bij de sectie Bio-organische Chemie, DSM Research, Geleen. Het resultaat van dit promotieonderzoek staat beschreven in dit proefschrift.

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