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The Use of Natural Fatty Acids for the Biotechnological Production of Natural Flavour Compounds: Application to Ethyl *trans*-2,*cis*-4-decadienoate

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Abstract: Natural flavour compounds can be obtained by a number of processes, one of the most important of which is the enzymatic conversion of natural substrates. The immobilised lipase from the yeast *Candida antarctica*, is capable of transesterifying Stillingia oil in the presence of ethanol, thereby producing a complex mixture of ethyl esters. The efficiency of the biotransformation step is high and lies in the region of 95%. By means of fractional distillation, the ethyl ester of *trans*-2,*cis*-4-decadienoic acid can be isolated from this mixture in approximately 5% yield and with a high degree of purity. The product thus obtained exhibits the sensory properties typical for this well-known character impact compound of the pear.

Keywords: *Candida antarctica* · Ethyl *trans*-2,*cis*-4-decadienoate · Lipase · Pear · Stillingia oil · Transesterification

Introduction

Natural flavours continue to be of significant importance in Western Europe and the USA and in both regions there are clear legal definitions as to what may or may not be considered natural. For example, in the EU Flavouring Directive 88/388/EEC the term 'natural' is allowed for a flavouring substance which is obtained 'by appropriate physical processes including distillation and solvent extraction, or enzymatic or microbiological processes from material of vegetable or animal origin either in the raw state or after processing for human consumption by traditional food preparation processes, including drying, torrefaction and fermentation.'

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Based on this definition, industrial processes have been developed to isolate key flavour ingredients from more or less complex natural mixtures using relatively simple physical techniques. Thus, natural L-menthol can be and is still obtained from peppermint oils *via* crystallisation and (+)-linalool by distilling coriander oil [1]. Furthermore, a combination of distillation and extraction steps can be successfully used to isolate and enrich the oxidative degradation products of fatty acids such as *trans*-2-hexenal and *cis*-3-hexenol from certain essential oils, such as peppermint oil [2] which have been obtained from fresh green enzymatically active plant tissue.

Flavour Biotechnology

Both plants and certain microorganisms have produced flavours and fragrances or components thereof since the beginning of time. Very early scientific reference to the flavour- and fragrance-producing capabilities of selected bacteria and fungi appeared in 1922 [3]. During the last 20 years, considerable progress has been made in this field and many of the larger flavour and fragrance companies are actively engaged in this area. Currently it is estimated that some 100–150 individual natural flavour components are being produced using biotechnological processes of various types and these materials belong to very many different classes of chemical compounds. Some are produced in multi-ten ton quantities and good examples of this would be ethyl acetate, n-butyric acid and 2-methylbutyric acid. The smaller volume category encompasses such products as γ-decalactone and vanillin, which may be produced to the extent of up to five tons per year. Even smaller volume and consequently more valuable materials include *trans*-2-hexenal, phenylethanol and n-hexanal [4]. The price of such small volume specialities can easily be in the range of $ 1000 to $ 10000/ kg depending upon supply and demand.

The aliphatic esters were probably the first class of flavour compounds investigated in depth for biotechnological production. The reason for this being that the alcohol and acid components required have been available in natural form for many years and that esters are key components in many, predominantly fruit flavours [5]. The esterification process was achieved in many cases by the judicious use of certain lipases [6][7] some of which are capable of synthesising esters

from their constituent acids and alcohols under certain conditions.

Some of the carboxylic acids required as starting materials can be obtained by the lipolytic cleavage of animal or vegetable fats followed by distillative purification. Yet other acids are accessable *via* the fermentative oxidation of the corresponding alcohols obtained from fusel oil. The most important acid obtained this way is 2-methylbutyric acid which can be produced in high yields by the oxidative bioconversion of 2-methylbutanol using *Acetobacter* species [8].

Fatty acids are also the substrates of choice when natural, flavour-active γ- and δ-lactones are the targets. Thus, the site-specific hydroxylation of medium chain fatty acids using the fungi *Monila fructicola* [9] or *Mucor circinelloides* [10] results in the formation of the corresponding γ- and/or δ-lactones. Certain yeasts can convert naturally occurring hydroxy fatty acids such as ricinoleic acid *via* multiple β-oxidation steps into the corresponding lactone, in this case γ-decalactone [11]. Still other enzymatic systems can convert unsaturated fatty acids such as linoleic and linolenic acid into C_6-aldehydes and their alcohols which are very valuable flavour compounds. Thus, *trans*-2-hexenal and *cis*-3-hexenol can be obtained from linolenic acid *via* the action of lipoxygenase and hydroperoxide lyase [12].

Ester Synthesis *via* Enzymatic Transesterification

Natural fats can also be good sources of unusual fatty acids. Such fatty acids can themselves exhibit interesting flavour properties or can be considered as starting materials for producing derivatives with valuable sensory properties.

Trans-2,*cis*-4-decadienoic acid is a relatively rare fatty acid, the ethyl ester of which has been known for many years as being the flavour character impact compound in pears [13]. Detailed analytical work subsequently showed that quite a number of related esters contribute to the overall flavour of Bartlett pears, *Pyrus communis* L. [14]. The acid moiety of these esters had, in the most cases, ten carbon atoms and either two or three carbon–carbon double bonds. The tropical Asiatic durian fruit also contains these uncommon C_{10}-esters which contribute significantly to the fruity aspect of the unusual flavour [15].

The results of a literature survey suggested that Stillingia oil, which has now become commercially available again, could be a reasonable source of *trans*-2,*cis*-4-decadienoic acid [16]. Stillingia oil is obtained from the kernels of the shrub *Sapium sebiferum* Roxb. and contains approximately 5% of this fatty acid in the form of mixed triglycerides. Initial trials designed to liberate the *trans*-2,*cis*-4-decadienoic acid from the triglycerides using purely physical processes, such as the action of superheated steam, were not successful. More or less complete cleavage of the triglycerides was achieved but the high reactivity of the acid itself prevented it from being isolated as such in quantities of any interest. Gas chromatographic analysis of the crude product showed that the *trans*-2,*cis*-4-decadienoic acid was present at levels of 0.2% or less. Furthermore, attempts to liberate the desired fatty acid by hydrolysis of the triglyceride bonds using esterases and lipases either failed completely or gave only very small yields of the acid. The esterases and lipases tested evidently had too low a specificity with respect to *trans*-2,*cis*-4-decadienoic acid.

Theoretically, the target compound might be accessible *via* an enzymatic transesterification of Stillingia oil in the presence of ethanol. Most of the lipases and esterases tested however, were not successful and exhibited only a very low specificity towards the bound *trans*-2,*cis*-4-decadienoic acid. Thus, the widely used lipases from *Mucor*, *Pseudomonas* and *Candida* which are well known for their ability to synthesise esters in general, were not able to produce ethyl decadienoate *via* transesterification. The data in Table 1 show that these enzymes were certainly active under the conditions chosen otherwise the ethyl ester of linolenic acid would not have been formed. Linolenic acid is the major acid present in Stillingia oil.

Further broader screening trials were undertaken in order to find more suitable enzymes and the results obtained are shown in Table 2. Again, most of the lipases showed very low activity towards decadienoic acid. Good yields were obtained only with lipase SP525 and Novozym 435, both obtained from Novozymes in Denmark. Novozym 435 is immobilised lipase B and lipase SP525 is free lipase B from *Candida antarctica*. These results clearly show that the ability to obtain ethyl *trans*-2,*cis*-4-decadienoate from Stillingia oil *via* transesterification with lipases is certainly the exception rather than the rule.

Optimisation of the Lipolytic Transesterification Reaction

The preliminary results shown in Table 2 indicate that the immobilised lipase Novozym 435 was in fact the better of the two lipase preparations from *Candida antarctica*. The further optimisation steps were performed using this enzyme and the results obtained are summarised in Table 3. Under the initial standard conditions shown in the footnote of Table 3, the optimal conditions for the transesterification were shown to be 45 °C for 2 days. Typical maximum values found by quantitative GC analyses were around 4.5% ethyl *trans*-2,*cis*-4-decadienoate which, taking in mind the approximate 20% dilution due to the added ethanol, corresponded to about 5.0–5.5% level of the acid in the original oil.

The optimum ethanol and enzyme levels were re-investigated under the optimised conditions found so far, whereby the amount of ethanol originally chosen for the trials was confirmed to be optimal. The velocity of the transesterification reaction was very much dependant

Table 1. Ability of commonly used lipases and esterases to produce ethyl *trans*-2,*cis*-4-decadienoate from Stillingia oil.

Formation of Ethyl ester of [a]	Ester Formation [GC %]		
	Enzyme 1[b]	Enzyme b[c]	Enzyme 3[d]
trans-2,*cis*-4-decadienoic acid	0.4	0.05	0.10
linolenic acid	65.8	57.5	65.2

[a]Typical reaction conditions: 10 g Stillingia oil, 1 g ethanol, 1 g enzyme, 20 °C, 3 days
[b]Enzyme 1: esterase 30,000 from *Mucor miehei* (Gist Brocades)
[c]Enzyme 2: lipase PS from *Pseudomonas* species (Amano)
[d]Enzyme 3: lipase B from *Candida cylindracea* (Biocatalysts)

upon the enzyme concentration (Fig.) as was to be expected. Bearing in mind the much longer reaction time required for complete turnover at lower enzyme concentrations, it was decided to continue using the 20% enzyme level in all further trials.

In order to determine the long-term stability of the enzyme under operational conditions, the transesterification procedure was repeated for many cycles whereby the immobilised enzyme was used repeatedly without any additional treatment. The data shown in Table 4 indicate that the enzyme can be used for very many cycles without registering any significant loss of activity. During these trials, it was decided to deliberately vary the incubation period between one and three days in order to verify the results shown in the Fig. obtained for a single cycle.

Properties of the Purified Natural Ethyl Decadienoate

In order to evaluate the sensory properties of the ethyl *trans*-2,*cis*-4-decadienoate thus produced, it was decided to scale-up the process and to purify the product. A scale-up factor of 50 was chosen which meant that a typical batch consisted of 5 kg Stillingia oil, 1 kg ethanol and 1 kg Novozym 435 all of which were stirred mechanically in a closed, thermostated glass vessel at 45 °C under a blanket of nitrogen. The use of a nitrogen blanket was a precautionary measure to prevent unwanted oxidation of the unsaturated fatty acids. The reaction period was deliberately lengthened to between three and five days per cycle in order to counteract any scaling-up problems with respect to the velocity of the reaction. The data in Table 5 indicate that this precaution was not necessary and also show that also under preparative conditions, the enzyme can be repeatedly used without losing any significant amount of activity. However, after every five cycles it was necessary to remove the enzyme from the glass vessel and to separate approximately 2 kg of a heavy, predominantly organic phase which was closely associated with the immobilised enzyme. Analyses showed that this phase consisted of about 90% glycerin and 10% water.

The crude product thus obtained was subjected to a distillative purification, whereby a relatively low boiling fraction of about 10% by weight of the amount employed was isolated. This preliminary fraction had a content of ethyl decadienoate of about 40% by weight and recovery of ester amounted to about 95%. This fraction was then subjected to fractional distillation and those fractions con-

Table 2. Results of screening of lipases for their ability to produce ethyl *trans*-2,*cis*-4-decadienoate from Stillingia oil.

	Lipase (Name or source)	Company	Ethyl *trans*-2,*cis*-4-decadienoate [GC area %] after	
			3 d	7 d
1	SP 523	Novo	0.35	1.15
2	SP 524	Novo	0.10	0.10
3	SP 525	Novo	5.60	5.55
4	*Candida cylindracea*	Biocatalysts	0.15	0.10
5	*Mucor miehei*	Biocatalysts	–	–
6	Pancreatin	Biocatalysts	–	–
7	*Pseudomonas fluorescens*	Biocatalysts	0.20	0.45
8	*Chromobacterium viscosum*	Biocatalysts	0.05	0.05
9	Novozym 435	Novo	6.50	8.00

Table 3. Optimal conditions for the transesterification catalysed by Novozym 435.

Parameter/Component	Optimum value
Temperature[a)]	45 °C
Reaction time[a)]	2 d
Ethanol	20 g
Novozym 435	20 g

[a)]Typical reaction batch: 100 g Stillingia oil, 20 g ethanol, 20 g Novozym 435 shaken under nitrogen in a 500 ml stoppered conical flask

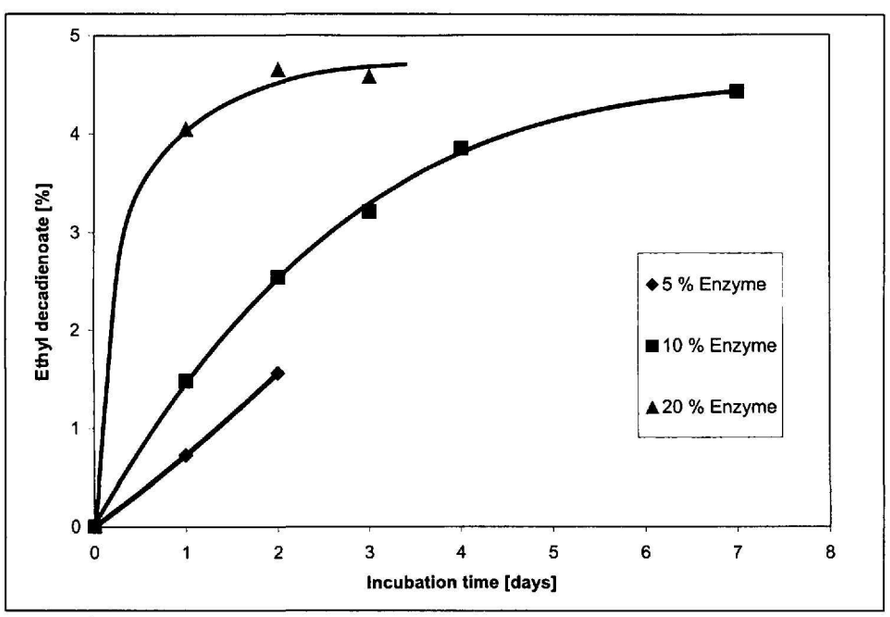

Fig. Effect of enzyme concentration (Novozym 435) upon rate of formation of ethyl decadienoate *via* transesterification of Stillingia oil.

taining approximately 90% or more of ethyl *trans*-2,*cis*-4-decadienoate were pooled. The typical composition of the isolated material is shown in Table 6 which indicates the presence of further compounds, all of which are ethyl esters of either isomeric acids or of related trienoic acids, which are also present in Stillingia oil and also contribute to the flavour of pears [17–19]. Sensory evaluation indicated that the combined fractions indeed exhibited the very clean and typical flavour of Williams pears and were equivalent in taste to the nature identical material.

The chemical synthesis of nature identical ethyl *trans*-2,*cis*-4-decadienoate is complicated and the cost of the material is at approximately $ 600/kg, relatively expensive. The enzymatic process described above is easy to perform and even though the concentration of *trans*-2,*cis*-4-decadienoic acid is very low in Stillingia oil, the process yields a final product which surprisingly competes favourably cost-wise with the nature identical material. This is an unusual situation since the vast majority of natural flavour compounds produced *via* biotechnology are many times more expensive than their nature identical counterparts.

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Table 4. Effect of repeated use of Novozym 435 upon yield of ethyl decadienoate.

Cycle	Incubation time [d]	Content of ethyl decadienoate [%]
1	1	4.5
2	1	4.2
3	1	4.2
4	2	4.6
5	2	4.2
6	2	4.3
7	2	4.5
8	3	4.4
9	3	4.0
10	1	4.1
11	1	4.2
12	3	4.8

Table 5. Efficiency of enzymatic transesterification performed under preparative conditions.

Cycle	Reaction Period [d]	Concentration Ethyl decadienoate [%]	Yield[a] [kg]
1	4	4.18	5.3
2	3	3.57	5.4
3	5	3.47	5.2
4	5	3.98	4.6
5	3	4.06	5.2
6	3	4.08	5.4
7	4	4.11	5.7
8	4	4.33	5.3
9	4	4.35	5.5
10	4	4.22	5.5
11	3	4.43	5.4
12	3	4.18	5.5
Total			~64

[a]liquid, organic phase after filtration

Table 6. Approximate composition of natural ethyl *trans*-2,*cis*-4-decadienoate from Stillingia oil after enzymatic transesterification.

Ethyl ester of	GC Analysis (DB Wax)	
	Retention Index	Area [%]
cis-3,*trans*-5-decadienoic acid	1835	≤ 5
trans-2,*cis*-4-decadienoic acid	1848	≥ 90
cis-2,*trans*-4-decadienoic acid	1860	≤ 0.5
trans-2,*cis*-4,*cis*-7-decatrienoic acid	1895	≤ 5
trans-2,*trans*-4-decadienoic acid	1918	≤ 1.0
cis-3,*trans*-5,*cis*-7-decatrienoic acid	2011	≤ 0.5

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