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CHARACTERIZATION AND CONTROL OF KETONIC RANCIDITY IN THE LAURIC ACID OILS

by

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Abstract

Characterization and Control of Ketonic Rancidity in the Lauric Acid Oils

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Ketonic rancidity is associated with the lauric acid oils and butterfat. It arises when short and intermediate carbon chain length fatty acids (C6 to C14) are converted into methyl ketones (C5 to C13) by certain fungi. Ketonic rancidity will not occur in fats and oils that do not contain these fatty acids.

Fermentation experiments with whole oils and simple triglycerides confirmed that only short and intermediate carbon chain length fatty acids were converted into methyl ketones. Methyl ketones produced contained one carbon atom less than the parent fatty acid. Tetradecanoic acid was the longest fatty acid to undergo conversion into its corresponding methyl ketone.

Experiments with free fatty acids established that short and intermediate carbon chain length fatty acids inhibited the growth of *Penicillium crustosum*. Evidence was presented to demonstrate that the mitochondrion was a site of antifungal activity. It was concluded that the conversion of fatty acids into methyl ketones was a detoxification mechanism for their removal from the environment.

Extrinsic factors (temperature, pH, a_w, preservatives and O_2 removal) were used to control fungal growth and ketonic rancidity. Fungal homeostatic mechanisms often enabled *P.crustosum* to grow under unfavourable environmental conditions. It was suggested that a combination of preservation measures should be used to prevent ketonic rancidity in the lauric acid oils and their products.

Advanced studies, courses and conferences undertaken in connection with the programme of research in partial fulfilment of the degree of Doctor of Philosophy

Studies and Courses

1. Attendance of lectures that were part of the taught MSc in Analytical Chemistry. Department of Chemistry, Sheffield City Polytechnic. September 5th to December 5th, 1985.

2. Visits to the natural products research laboratory to study analytical techniques. Bush Boake Allen Ltd, London. October 24th to 28th, 1985 and January 9th to 11th, 1986.

3. "Using MINITAB - Interactive statistics made easy." Department of Computer Services, Sheffield City Polytechnic. October 23rd to Novemeber 6th, 1986.

4. "Fungal Biotechnology." Course attended with financial support from Bush Boake Allen Ltd. University of Strathclyde. September 8th to 13th, 1986.

Conferences

1. "Metabolites of Higher Fungi." University of Liverpool. September 24th to 26th, 1985.

2. XIV International Congress of Microbiology. University of Manchester. September 2nd to 6th, 1986.

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HATTON, P.V. & KINDERLERER, J.L. 1986 The effect of substrate on production of aliphatic methyl ketones by *Penicillium cylopium* westling.p[115],Proceedings of the 14th International Congress of Microbiology, University of Manchester.

HATTON, P.V., HANSBRO, P. & KINDERLERER, J.L. 1988 The ultrastructure of *Penicillium cyclopium* utilising commercial oils or simple triglycerides as sole carbon sources. Poster presented at the summer conference of the Society for Applied Bacteriology, University of Surrey. Journal of Applied Bacteriology 65, xxiv.

HATTON, P.V. & KINDERLERER, J.L. 1988 Detoxification of sorbic acid by *Penicillium cyclopium*. Poster presented at the summer conference of the Society for Applied Bacteriology, University of Surrey.* Journal of Applied Bacteriology 65, xxiv.

KINDERLERER, J.L., HATTON, P.V. CHAPMAN A.J. & ROSE, M.E. 1988 Essential oil produced by *Chrysosporium xerophilum* in coconut. *Phytochemistry* 27, 2761-2763.

* Commended - third out of forty-two posters.

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Abbreviations used in the text

CMI	Commonwealth Mycological Institute
cm	Centimetre
Co	Company
°C	Degrees centigrade
DCM	Dichloromethane
DEGS	
h	Hour
IMI	Imperial Mycological Institute
l	Litre
M	Molar
mg	Milligram
min	Minute
ml	Millilitre
mm	Millimetre
mM	Millimolar
mmol	Millimole
ND	Not detected
nm	Nanometre
UK	United Kingdom
µg	Microgram
µm	Micrometre
c.f.u.	Colony forming unit
DEGS	Diethylene glycol succinate
FAME	Fatty acid methyl ester
µmol	Micromole

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This thesis is dedicated to my friends at Saint Thomas's Church for their unfailing love and support throughout this project.

Revelation 3, 8

1. Introduction

A form of rancidity due to ketones, caused by moulds and encouraged by moisture, sometimes occurs in butter fat and in coconut and palm kernel oils. (Pearson 1976)

Fats and oils are constituents of most foods. They are important in a food for a number of reasons. They give a characteristic mouth-feel as well as acting as a solvent for the fat-soluble vitamins and flavour compounds (Kinsella 1969, Gunstone and Norris 1983).

All fats and oils are prone to rancidity. Rancidity is characterised by an unpalatable odour following oxidation or hydrolysis of triglycerides in the fat or oil (Pearson 1976, Coultate 1984, Robards *et al* 1988). This process often takes place in foods that are subjected to lengthy periods of storage. Loss in quality may parallel a reduction in the shelf-life of a fat, oil or stored food which can lead to financial loss at any stages in the food chain. It is consequently important to investigate the causes of rancidity and the measures that can be taken for its control. Some knowledge of the chemical and physical properties of fats and oils is required in order to understand rancidity.

1.1 The chemical and physical properties of fats and oils

The major components (90-95% w/v) of all fats and oils are the triglycerides (Coultate 1984). Triglycerides are esters of fatty acids with glycerol and the general formula for these compounds is given in Figure 1.1. The fatty acids are named according to the Geneva convention where the name of the acid is related to the hydrocarbon which is formed if the carboxyl group (COOH) is replaced by a methyl group (CH_3). Numbers indicate the position of double bonds or substituted groups and the carboxyl group is designated C1. Some examples of fatty acids are given in Figure 1.2. If all three acids are the same the triacyglycerol is called a simple triglyceride. If one fatty acid is different it is called a complex triglyceride. Complex triglycerides are the major storage lipids of plants and animals. Vegetable oils generally contain a high proportion of long carbon chain length unsaturated fatty acids while animal fats contain a higher proportion of saturated fatty acids. The aliphatic fatty acids vary greatly in their chemical and physical characteristics depending on the length of the carbon chain and the degree of unsaturation. The fatty acid composition accounts for the different properties of individual fats and oils.

The melting points for an aliphatic series of fatty acids are shown in Figure 1.3. These values show alternation between odd and even numbered carbon chains.

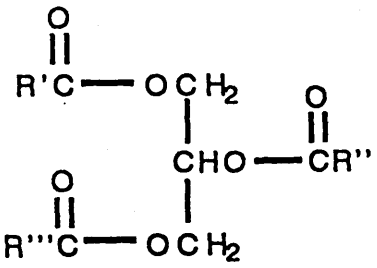


Figure 1.1 : General formula for a triglyceride where R', R'' and R''' may be saturated or unsaturated carbon chains.

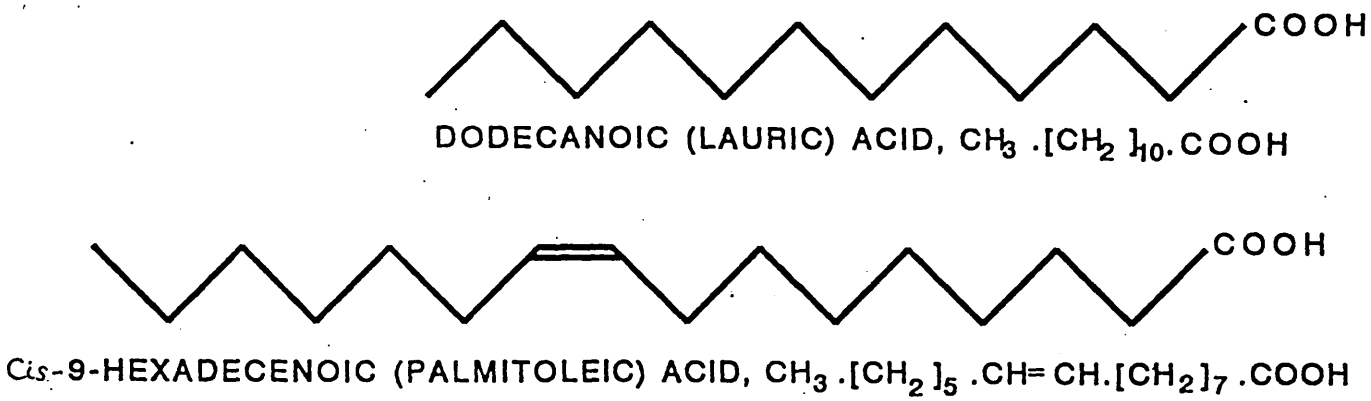


Figure 1.2 : Chemical structures of two common fatty acids.

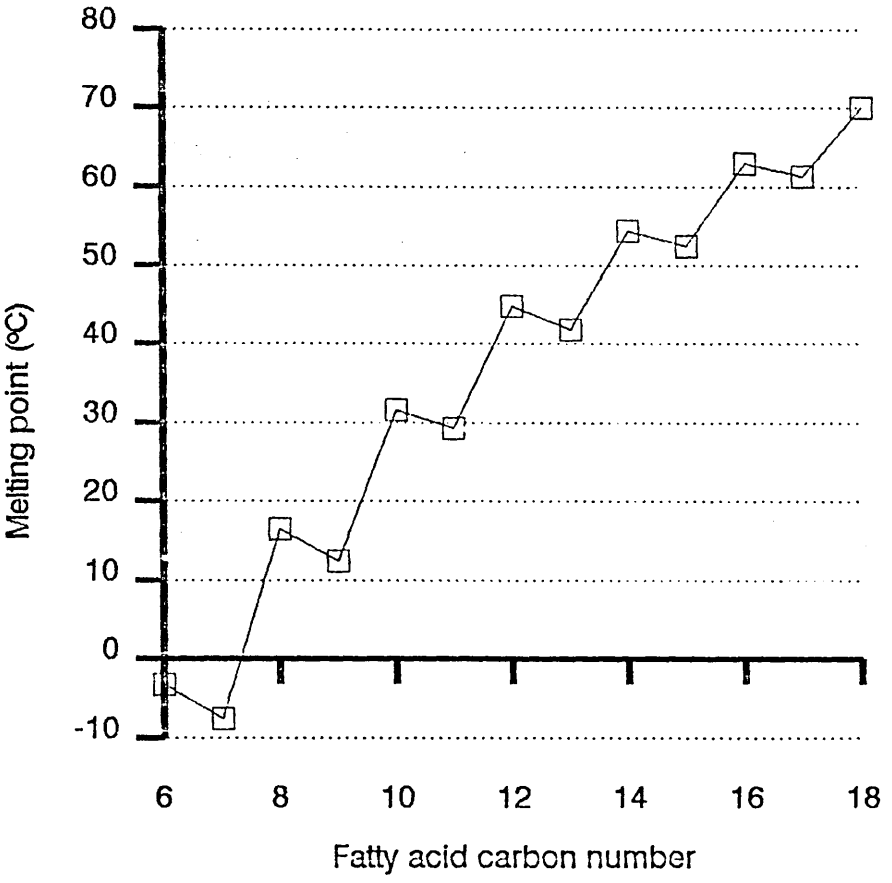


Figure 1.3 : Melting points of saturated fatty acids. Data derived from Handbook of Chemistry and Physics(1984).

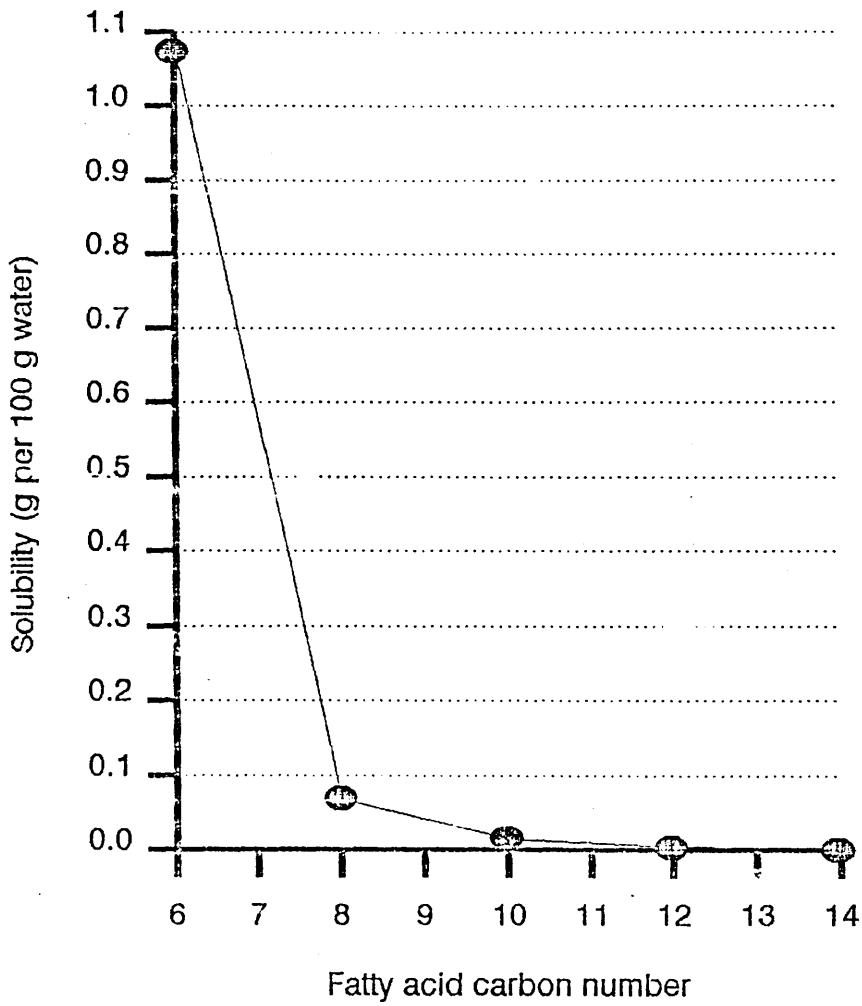


Figure 1.4 : Solubility in water of saturated fatty acids (Data recorded at 20°C).

Handbook of Chemistry and Physics (1984).

This is related to the arrangement of molecules within the crystal. The fatty acids may exist as solids in three forms (A, B and C). When heated A and B forms of even carbon chain acids are transformed into the C form. Therefore it is the melting point of the C form which is always observed. Odd numbered carbon chain acids usually exist in the B form and this accounts for the lower melting points of this series.

The solubility of fatty acids in both hydrophilic and hydrophobic areas such as membranes is important. Acids with more than six carbon atoms, though only slightly soluble in water, are more soluble than the corresponding hydrocarbons owing to the hydrophilic nature of the carboxyl group. Solubility in water decreases with increasing chain length (Figure 1.4). The solubility of saturated fatty acids in organic solvents indicates their solubility in membranes (lipophilicity). Here, too, solubility decreases with increasing chain length but odd and even acids show alternation; the acids with an odd number of carbon atoms are approximately as soluble as their even numbered homologues with one less carbon atom.

The major fatty acids in most fats and oils are those with eighteen carbon atoms (see Table 3.1). The lauric acid oils are unusual in that over 50% (w/w) of their constituent fatty acids are of short or intermediate carbon chain length (C6 to C14). Dodecanoic (lauric) acid is the predominant

fatty acid (Cornelius 1977, Young 1983, Hatton and Kinderlerer 1986).

Two main lauric acid oils are used industrially. These are coconut oil, obtained from *Cocos nucifera* L., and palm kernel oil, obtained from *Elaeis guineenis* L. The low slip points of these oils is due to the presence of short chain saturated fatty acids (Young 1983). This accounts for the fact that the lauric acid oils are widely used in the food and pharmaceutical industries. Lauric hard butters are used in the manufacture of margarine and synthetic creams as well as in the manufacture of soaps. The presence of low molecular weight fatty acids gives a soap that is free lathering and water soluble.

Considerable work has been undertaken to understand the causes and nature of rancidity in fats and oils. Three types of rancidity have been identified. These are hydrolytic, ketonic and oxidative.

1.2 Hydrolytic rancidity

Hydrolytic rancidity is due to the hydrolysis of triglycerides to give free fatty acids (FFA). Conversion of triglycerides into FFA and glycerol may be due to a chemical hydrolysis catalysed by acid or base catalysis (for example, the production of sodium or potassium palmitate and stearate in the manufacture of soap). Enzymatic hydrolysis due to

the action of the lipase group of enzymes (glycerol ester hydrolases; EC: 3.1.1.3) may also cause hydrolytic rancidity:

$$\text{TRIGLYCERIDES} + \text{WATER} \xrightarrow{\text{LIPASES}} \text{FFA} + \text{GLYCEROL}.$$

The increase in FFA may lead to soapy tastes in oils, fats and processed foods (Young 1983, Robards *et al* 1988). This is a particular problem in the lauric acid oils where the more volatile short carbon chain length fatty acids (C6 to C10) are easily hydrolysed and give a distinctive odour (Young 1983).

Lipases are widespread in nature where they are produced by animals, plants, bacteria and fungi. In fats, oils and foods the lipases may be present in the food itself or they may be of microbial origin. Production of lipolytic enzymes by fungi was recognised at the end of the nineteenth century (Biffen 1899). Fungal lipases have been studied extensively. *Aspergillus flavus* produced a lipase which attacks coconut oil to liberate free fatty acids (Hoover *et al* 1973). *Penicillium cyclopium* and *Penicillium crustosum* produce particularly active lipases (Oi *et al* 1967, Iwai *et al* 1975). This ability of moulds to hydrolyse triglycerides accounts for the increased concentrations of free fatty acids in oilseeds affected by fungal spoilage (Eggins and Coursey 1968).

1.3 Ketonic rancidity

Various workers have described a variation of hydrolytic rancidity whereby short carbon chain length fatty acids (C6 to C14) are converted into an homologous series of aliphatic methyl ketones by certain moulds (Starkle 1924, Stokoe 1928, Thaler and Eisenlohr 1941a, b). Ketonic or 'perfume' rancidity was first described by Biffen who suggested that amyl butyrate was responsible for the ester-like odour of rancid coconut (Biffen 1899). The compounds responsible for ketonic rancidity were later shown to be methyl ketones (Stokoe 1928, Starkle 1924). Working with *P. palitans*, Stokoe established that ketonic rancidity in coconut oil could be caused by this fungus. He identified 2-heptanone, 2-nonanone and 2-undecanone as well as secondary alcohols and their esters with free acids (Stokoe 1928). Further studies confirmed that the methyl ketones and secondary alcohols were produced by fungi from short carbon chain length (C6 to C14) fatty acids (Thaler and Eisenlohr 1941 a,b). This early work was reviewed by Foster (1949).

Many of the early studies of methyl ketone formation were undertaken because of the importance of these compounds as flavours in the mould-ripened cheeses. The formation of methyl ketones by fungi in dairy products was reviewed by Hawke (1966). He defined four enzymic mechanisms that operated in mould-ripened cheeses:

1. The liberation of free fatty acids from the triglycerides of milk by lipases.

2. Oxidation of the free fatty acids to β-ketoacids.

3. Decarboxylation of β-ketoacids to methyl ketones.

4. Reduction of methyl ketones to secondary alcohols.

This sequence is analogous to that which occurs in ketonic rancidity (Kellard *et al* 1985).

Ketonic rancidity was neglected until recently when Kinderlerer and her co-workers described ketonic rancidity as "an oxidative variation of the hydrolytic type of rancidity" (Kellard *et al* 1985). In common with hydrolytic rancidity, the initial step is the hydrolysis of triglycerides by lipases to release free fatty acids (Section 1.2). The next stage is oxidative and leads to the conversion of free fatty acids into methyl ketones. It is the accumulation of methyl ketones and their reduction products, the secondary alcohols, in a food or oil which leads to a rancid odour (Kellard *et al* 1985).

Three oils (coconut oil, palm kernel oil and butter fat) are susceptible to ketonic rancidity. The triglycerides of these oils contain appreciable concentrations of the short chain length fatty acids that can be converted into methyl ketones (Bezard *et al* 1970, Cornelius 1977, Young 1983). Ketonic rancidity has also been identified in dessiccated coconut and margarine (Kinderlerer 1984, Frisvad, personal

communication). The off-flavour notes and threshold values of the methyl ketones are given in Table 1.1.

Many but not all fungi are able to convert the short chain fatty acids into methyl ketones. This ability appears to be widespread among members of the Phycomycotina, Ascomycotina and Deuteromycotina (Foster 1949 , Franke and Heinen 1958, Franke *et al* 1961). The present situation is summarised in Table 1.2.

In early studies there was confusion as to whether spores or mycelia carried out the conversion of short carbon chain length fatty acids into methyl ketones. Several groups argued that spores alone were responsible (Gehrig and Knight 1958, 1961 and 1963, Lawrence 1966, 1967, Dartey and Kinsella 1973 a, b). It appears that both spores and mycelia are active in carrying out the conversion (Lewis and Darnall 1970, Lewis 1971, Hatton and Kinderlerer 1986). The failure to observe ketone production by mycelia was because conditions promoting ketogenesis were different for spores and mycelia. Glucose and some amino acids (proline, alanine and serine) stimulate the conversion of fatty acids into methyl ketones by spores (Dartey and Kinsella 1973 a, b, Lewis and Darnall 1970). However, these nutrients suppress this activity in vegetative mycelia (Lewis 1971).

Only fatty acids of short and intermediate chain length are converted into methyl ketones. Lauric (dodecanoic) acid was reported to be the longest fatty acid to undergo conversion into a methyl ketone (Stärkle 1924, Stokoe 1928).

Table 1.1 Flavour thresholds and off-flavour notes of aliphatic methyl ketones *

Compound	Flavour threshold in water (ppm)	Flavour note
2-Pentanone	2.3	Pear drops
2-Hexanone	0.9	Ethereal
2-Heptanone	0.7	Rancid almonds
2-Octanone	0.2	Weakly ethereal
2-Nonanone	0.2	Weakly turpentine
2-Undecanone	0.5	Weakly turpentine
2-Tridecanone	0.5	-

* Data adapted from Forss (1972) and Kellard *et al* (1985).

Table 1.2 Ketogenic and non-ketogenic fungal species

Ketogenic species	Non-ketogenic species
Penicillium palitans[1]	*Odium lactis*[2]
Penicillium glaucum [2,3]	*Absidia glauca* [4]
Penicillium roquefortii [7,9,11,12]	*Oospora lactis* [4]
Penicillium camembertii [6,14]	*Alternaria tenuis* [4]
Penicillium citrinum [13]	*Alternaria solani*
Penicillium crustosum [15]	*Fusarium sambucinum* [4]
Aspergillus niger [4,5,10]	*Candida albicans* [8]
Eurotium chevalieri [13]	
Eurotium herbariorum [13]	
Rhizopus spp. [4]	
Botrytis spp. [4]	

[1] Stärkle 1924
[2] Stokoe 1928
[3] Thaler and Eisenlohr 1941a
[4] Franke and Heinen 1958
[5] Frank *et al* 1961
[6] Schwartz and Parks 1963
[7] Gehrig and Knight 1963
[8] Adams *et al* 1963
[9] Lawrence and Hawke 1968
[10] Lewis and Darnall 1970
[11] Dartey and Kinsella 1973a,b
[12] King and Clegg 1980
[13] Kinderlerer and Kellard 1984
[14] Okumura and Kinsella 1985
[15] Hatton and Kinderlerer 1986

Subsequently myristic (tetradecanoic) acid was found to be converted into 2-tridecanone (Foster 1949). There is one report that spores of *Penicillium roquefortii* can convert palmitic (hexadecanoic) acid into 2-pentadecanone (Dartey and Kinsella 1973b). However, Dartey and Kinsella had used C^{14} labelled palmitic acid which may have given results that could not be obtained using a natural fat or oil as a substrate. The conversion of palmitic acid into 2-pentadecanone has not been observed in coconut or palm kernel oil.

Uptake of the short carbon chain length fatty acids into the mycelium is probably by diffusion, although group translocation has been demonstrated in mammalian systems (Noy *et al* 1986). On entering the cell the conversion of fatty acids into methyl ketones is thought to proceed via a β-oxidation mechanism. Evidence was first provided by Thaler and Eisenlohr, who demonstrated that spores of *P.glaucum* formed methyl ketones from β-hydroxy acids (1941a). Shortly afterwards a β-oxidation mechanism for the degradation of fatty acids in mamalian mitochondria was proposed (Lehninger 1945). However, the final step in the fungal pathway was not confirmed until β-ketoacyl decarboxylase activity was detected in spore preparations (Franke and Heinen 1958). This activity was demonstrated in both spores and mycelia of *P.roquefortii* (Hwang *et al* 1976). The pathway for the abnormal β-oxidation leading to methyl ketone and secondary alcohol production is given in Figure

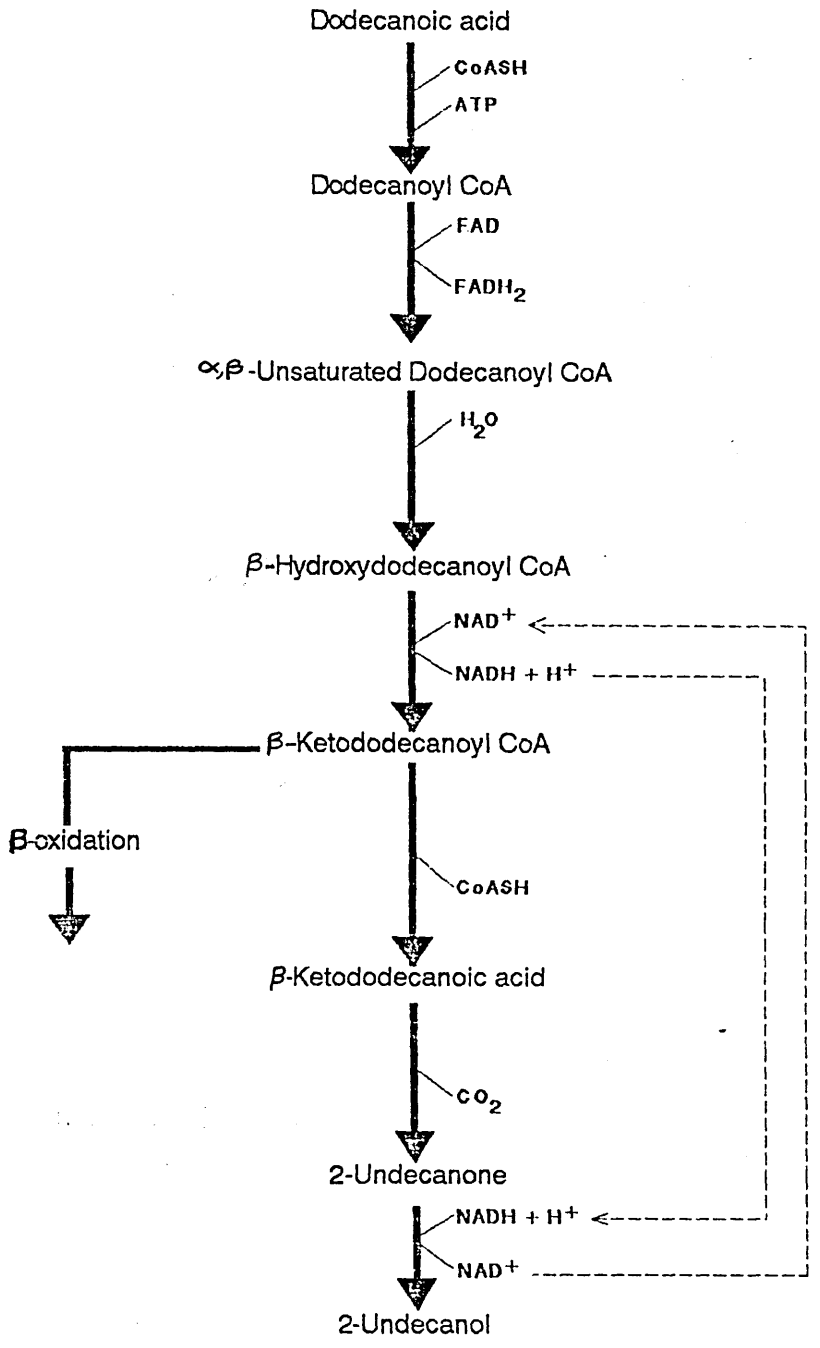


Figure 1.5 : Pathway leading to conversion of dodecanoic acid into 2-undecanone and 2-undecanol.

1.5. The site of this pathway is not known. Very little research has been undertaken on fungal β-oxidation due to the difficulty in obtaining a cell-free system (Smith and Berry 1975, Weete 1980).

The purpose of the conversion of short carbon chain length fatty acids into methyl ketones is not known. Several groups have established that these fatty acids can inhibit the growth and oxygen uptake of fungi (Wyss *et al* 1945, Rothman *et al* 1946, Rolinson 1954, Chattaway and Thompson 1956, Das and Bannerjee 1981). This has led some groups to suggest that methyl ketone formation is a detoxification mechanism for the removal of short carbon chain length fatty acids from the environment (Franke *et al* 1962, Lewis and Darnall 1970, Lewis 1971). Other workers have suggested that the production of methyl ketones is a means of recycling coenzyme A where the complete β-oxidation could not take place (Lawrence and Hawke 1968).

1.4 Oxidative rancidity

Oxidative rancidity arises from the autoxidation of unsaturated fatty acids by atmospheric oxygen. This reaction may be accelerated by heat, light or catalysts such as metal ions or metalloproteins. End products include aliphatic fatty acids, aldehydes, ketones, alcohols and esters (Galliard 1973, Frankel 1982 and 1984) Frankel has summarised the situation and

stated that "the oxidation of unsaturated lipids can produce an appallingly complex mixture of volatiles that can significantly affect the organoleptic properties of foods in extremely small quantities" (Frankel 1982). An indication of this complexity is given in Table 1.3 where the volatile decomposition products of triolein and trilinolein are listed. From this Table it can be seen that methyl ketones account for less than 1.0% of the total volatiles after oxidation of these unsaturated fatty acids.

Oxidation of free or esterified fatty acids and occurs in three stages. These are:

1. Initiation: initiation reactions give rise to highly reactive free radical species.

2. Propagation: free radicals react with oxygen to produce fatty acid hydroperoxides. These break down with the generation of free radicals which serve to maintain the chain reaction.

3. Termination: the reaction is terminated when the free radical species reach a sufficiently high concentration to combine with the formation of stable end products.

The mechanism of the initiation reactions is not fully understood. The suggested route is given in Figure 1.6.

Table 1.3 Volatile decomposition products after oxidation of triolein and trilinolein.

Volatile	Relative percent	
	Triolein[a]	Trilinolein[b]
Aldehydes		
Propanal		0.7
Butanal		0.4
Pentanal	0.9	3.6
Hexanal	1.9	17.2
Heptanal	5.1	8.2
Octanal	8.5	
Nonanal	22.4	
Decanal	2.8	
2-Hexenal		1.8
2-Heptenal	0.1	15.3
2-Octenal	0.5	4.4
2-Nonenal	2.1	0.2
2-Decenal	16.5	
2-Undecenal	11.1	
Acrolein		4.8
2,4-Decadienal		19.0
2,5-Epoxy-2-decenal		4.3
Other compounds		
Pentane		12.0
Hexane		0.7
Heptane	8.6	
Octane	9.7	
1-Pentanol		3.4
1-Heptanol	1.6	
1-Octanol	2.5	
1-Octen-3-ol		1.7
2-Pentylfuran		0.8

[a] other minor volatiles included methyl ketones (0,8%), acids (1,2%) and gamma lactones (0,8%)

[b] other minor volatiles included acids (1,8%), gamma lactones (0,5%), furan (0,4%) and methyl ketones (trace)

Adapted from Frankel (1982)

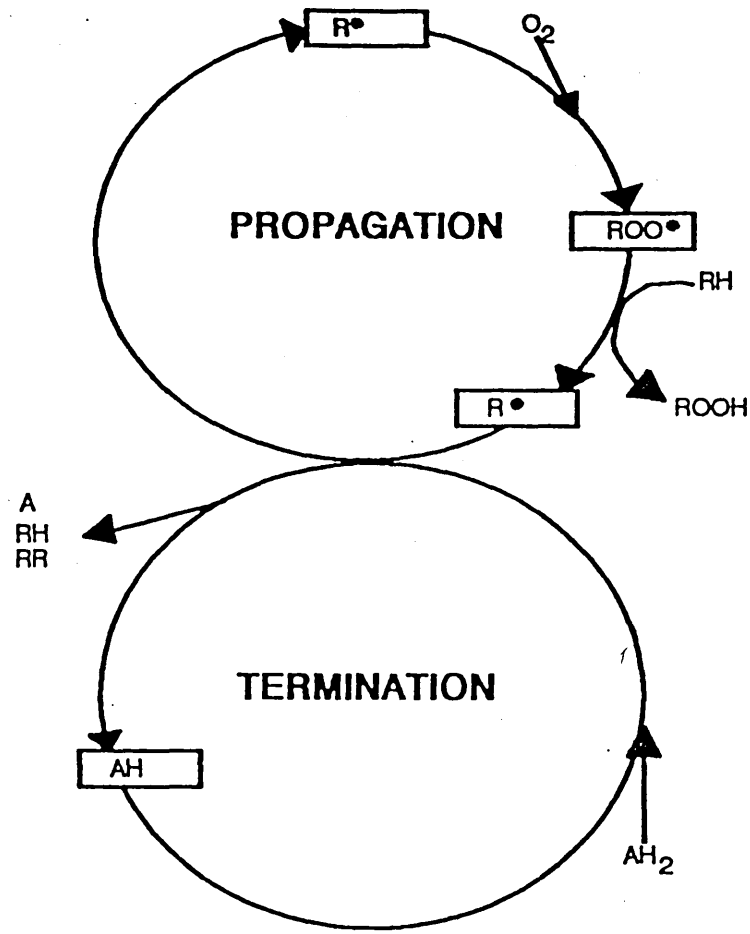


Figure 1.6,: Classical autoxidation route leading to hydroperoxide formation (ROOH) where RH = unsaturated lipid, R• = lipid radical, ROO• = lipid peroxy radical and AH_2= chain breaking antioxidant.*

* From Robards et al 1988.

Photo-oxidation is recognised as an alternative to the classical free radical mechanism for hydroperoxide formation (Sattar *et al* 1976). Unlike autoxidation photo-oxidation is not a free radical process as singlet oxygen reacts directly with the carbon-carbon double bonds of unsaturated fatty acids to form hydroperoxides.

A third route for hydroperoxide formation arises from the activity of lipoxygenase enzymes (EC: 1.13.11.12). The mechanism is fundamentally the same as for classical autoxidation but the enzymes are highly specific for a particular substrate. As a result, this mechanism is characterised by the formation of specific intermediates and breakdown products.

A wide variety of hydroperoxides are produced as the fat absorbs oxygen from the atmosphere. These compounds are relatively non-volatile and do not contribute to the odour or flavour of the rancid fat. Regardless of the mechanism of formation, decomposition of hydroperoxides will result in the production of the final volatile off-flavour compounds. Such termination reactions lead to formation of the range of compounds presented in Table 1.3.

There are two important differences between oxidative and ketonic rancidity. Fats and oils containing long chain unsaturated fatty acids are susceptible to oxidative rancidity whereas those containing saturated short chain fatty acids are susceptible to ketonic rancidity. Table 1.3 shows that under 1.0% (w/w) of the products of oxidative

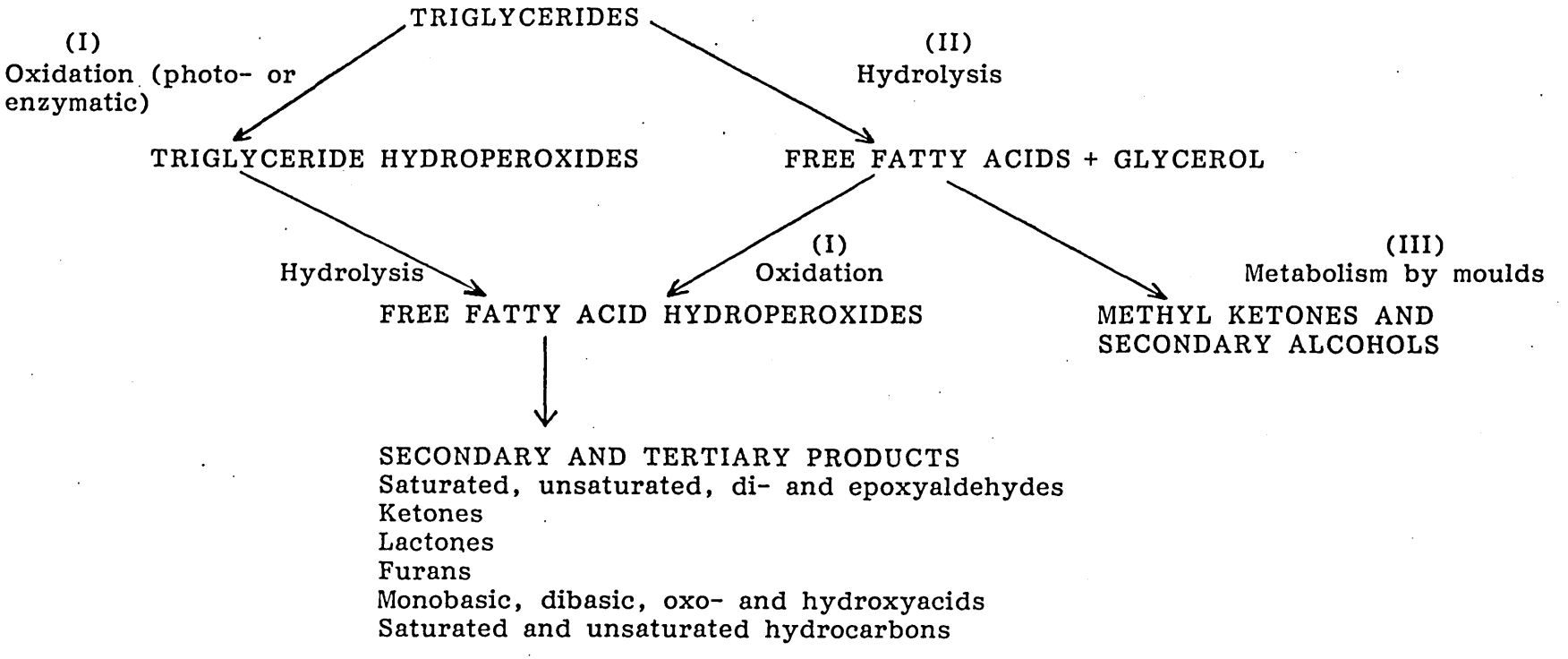


Figure 1.7 : General reaction scheme for (I) oxidative, (II) hydrolytic and (III) ketonic rancidity.*

* Adapted from Robards et al 1988.

rancidity were methyl ketones. Methyl ketones and secondary alcohols are the only compounds associated with ketonic rancidity.

The pathways for the development of rancidity in oils and fats are summarised in Figure 1.7. It is the chemical and physical characteristics of the oils and fats which, to a large extent, determine the type of rancidity that is likely to occur. Ketonic rancidity will only arise in fats containing short carbon chain length (C6 to C14) fatty acids after contamination by certain moulds. Chemical oxidation is only a problem in fats that contain a high proportion of unsaturated acids.

1.5 Selection of Fungus

Twenty-nine fungal species were isolated from good quality Sri Lankan desiccated coconut (Kinderlerer 1984). However, material which had been stored under poor conditions and had subsequently become rancid commonly contained only *Penicillium crustosum* (synonym *P. cyclopium*) and *Eurotium repens* (Kinderlerer and Clark 1986). Low numbers (6.1×10^2 c.f.u.'s per gram) of moulds were found even though the consumer returns were rancid and discoloured. It was suggested that the end products of spoilage (methyl ketones, secondary alcohols and free-fatty

acids) were toxic to the moulds and had a fungicidal effect (Kinderlerer and Clark 1986). Because of its resistance to the end products of spoilage *P. crustosum* was selected for further investigation.

Pitt describes *Pencillium crustosum* as a ubiquitous spoilage organism responsible for damage to stored corn, processed meats, biscuits and fruit juices (Pitt 1981). *Penicillium crustosum* has been isolated frequently from oilseeds (Samson *et al* 1976).

Where fungi grow in stored commodities there is the possibility that mycotoxins will accumulate (Schmidt and Esser 1985). The isolates used in this study were found to produce penitrem A, roquefortine C, terrestric acid and viridicatin (Frisvad 1985, Frisvad personal communication). Penitrem A is a tremorgen whilst both terestric acid and vindicatin are believed to have cardiotoxic properties (Moreau 1979).

1.6 Factors affecting fungal growth in foods

Deterioration in the quality of a food is often caused by only a small proportion of the micro-organisms originally present (Mossel and Ingram 1955). This was the case with the rancid desiccated coconut described in Section 1.5. The dominant species is that which determines the spoilage type; it is not necessarily the most numerous micro-organism.

Penicillium crustosum was a dominant species in rancid coconut (Kinderlerer and Clark 1986).

The conditions leading to dominance are determined by several factors. Four important parameters were defined (Mossel and Ingram 1955, Mossel 1983):

1. Intrinsic factors that depend upon the properties of the substrate.

2. Processing procedures that may often lead to the elimination of micro-organisms.

3. Extrinsic parameters which are the external selective influences.

4. Implicit factors that are properties of the dominant micro-organism.

While influencing the dominance of a particular micro-organism, these factors may also be used to control microbial growth in a food. The chemical and physical characteristics discussed in Section 1.1 represent some of the intrinsic properties of the substrate. These influence the susceptibility of a fat of oil to ketonic rancidity (Hatton and Kinderlerer 1986). Other intrinsic factors may be introduced into a food to control the growth of micro-organisms (ICMSF 1980). These include preservatives, solutes and certain acids.

The most important extrinsic factors are temperature, relative humidity and the composition of the gaseous phase (Mossel 1983). These factors can be used to control the growth of micro-organisms during production, distribution and storage of a food.

Implicit factors include the biological characteristics of *P. crustosum* that lead to its dominance in rancid coconut (Section 1.5). These factors may also influence the resistence of the fungus to the measures used to control growth and ketonic rancidity.

It is important to remember that the effect of a factor is influenced by the state of the other parameters. The combined influence of the many parameters results in three broad classes of foods (Mossel 1983). These are perishable, weakly preserved and shelf-stable commodities. In this order they represent foods with increasing resistance to microbial colonization and spoilage.

The aims of this research were to:

1. Characterise ketonic rancidity in the lauric acid oils.
2. Investigate the intrinsic and extrinsic factors that can prevent this reaction from taking place.

The system was studied using whole commercial oils and simple triglycerides. The influence of free fatty acids on fungal growth was also investigated.

2. Materials and Methods

2.1 Fungus

Pencillium crustosum (CMI 281919) was isolated from good quality Sri Lankan coconut (Kinderlerer 1984). A second isolate (CMI 300381) was obtained from Turkish hazelnuts (Dr Mary Phillips-Jones, unpublished). Dr Jens Frisvad identified both isolates by their ability to synthesize mycotoxins (see Section 1.5). *Penicillium crustosum* was distinguished from the closely related *P. cyclopium* by the ability to rot apples. Plate 2.1 shows the brown rot which was produced in Golden Delicious apples inoculated with *P. crustosum* compared to the brown rot produced by *P. expansum*. The two isolates were freeze-dried in vials and kept at 4°C until required for the production of stock cultures.

2.2 Maintenance of stock cultures

Slopes of malt extract agar (Oxoid CM59/60; 10 ml) in universal bottles (28 ml) were inoculated from a spore suspension using a sterile nicon wire (5 μm). A one point inoculation was made in the centre of the agar and the slopes were incubated at 25°C for 7 days to give fungal colonies which were covered in green spores. The cultures were stored at 4°C until required (no longer than 2 months).

(a)

(b)

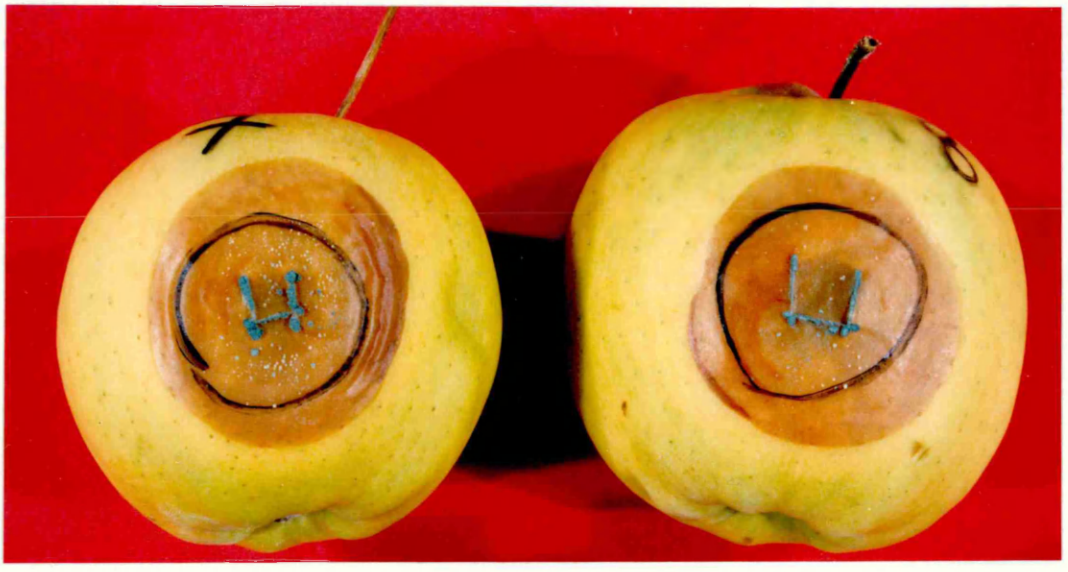


Plate 2.1 : Brown rot produced in Golden Delicious apples inoculated with (a) Penicillium crustosum and (b) Penicillium expansum.

2.3 Preparation of spore suspensions

Czapek medium contained (l^{-1}): $NaNO_3$ 2.0 g, KCl 0.5 g, KH_2PO_4 1.0 g, $MgSO_4.5H_2O$ 0.5 g, $FeSO_4.7H_2O$ 0.01 g, $CuSO_4.5H_2OO$ 0.005 g, $ZnSO_4.7H_2O$ 0.01 g, agar (Oxoid no. 1) 15 g, and sucrose 30 g (Johnson and Booth 1983). The salts, agar and sucrose were dissolved in single glass distilled water by heating to 100°C on a stirred magnetic hotplate. Medium(10 ml) was dispensed into universal bottles (28 ml) before sterilisation at 121°C (or 15 psi) for 15 min.

Each slope was inoculated using a spore suspension prepared from a stock culture (Section 2.2). The slopes were incubated at 25°C for seven days. Cultures showing heavy spore production were selected for preparation of starter cultures. Sterile distilled water (10 ml) containing five undrilled glass beads (4 mm diameter) was added to each slope and the spores were dislodged by gentle shaking. Clumps of spores were dispersed by mechanical shaking (5 minutes) using a Stuart flask shaker (Fisons Scientific Equipment, Loughborough, Leicestershire) at full speed. Mycelial debris was removed by filtration through two layers of sterile cheesecloth and a glass wool plug into a sterile Erlenmeyer flask (250 ml).

2.4 Determination of spore concentrations

A haemocytometer (improved Neubauer-type, Gallenkamp Ltd., Stockton-on-Tees) was used to determine the spore concentration. The spores lying in four large (0.04 mm^2) squares were counted in duplicate along a diagonal line across the grid. The number of spores per ml of suspension was calculated using the following equation:

$$\frac{A}{160} \times (4 \times 10^6) = B$$

A = number of spores counted in ten 0.04 mm^2 squares

B = number of spores per ml of suspension

The volume of inoculum containing 2.5 x 10^7 spores was calculated.

2.5 Conversion of triglycerides into methyl ketones by fermentation

2.5.1 The effect of time on the fermentation of palm kernel oil.

Czapek medium (Section 2.3) without sucrose and agar was prepared containing glycerol (20% v/v). The fermentation medium (50 ml) was dispensed with palm kernel oil (2 g) into each of eight foam-stoppered Erlenmeyer flasks (250 ml). These were sterilised at 121°C and 15 psi for 15 min. After autoclaving the flasks were allowed to cool before the addition of

spores (5.0 x 10^7)) with an automatic pipette (Gilson 'Pipetteman', Anachem Ltd., Luton, Beds).

Flasks were mounted on a rotary shaker (G2 Shaker, New Brunswick Scientific Co. Inc., New Jersey, U.S.A) at 200 rpm inside a refrigerated incubator (Vindon Scientific Ltd., Oldham, Lancs) at 25°C. Flasks were harvested in duplicate at intervals of 4, 8, 12, 16, 20, 24, 36, 48, 60, 72, 84, 96, 120 and 144 h. Internal standard (3-undecanone, 7.3 mg) was added to each flask prior to extraction (see Section 2.8).

2.5.2 The effect of pH on the conversion of triglycerides into methyl ketones

The basic fermentation medium described in Section 2.5.1 was modified by replacing KH_2PO_4 with 0.05 M phosphate buffer. Medium (25 ml) was dispensed into Erlenmeyer flasks (250 ml) with glyceryl trihexanoate or glyceryl trioctanoate (1 g) as a sole carbon source. The initial pH of these experiments was set at 4.5, 5.5, 6.5, 7.0 and 7.5. After sterilisation at 121°C and 15 psi the flasks were allowed to cool. Flasks were inoculated with spores (2.5 x 10^7) and incubated at 200 rpm and 25°C for 72 h. The experiment was set up in quadruplicate for each pH value. Internal standard (3-undecanone, 7.3mg) was added to each flask prior to extraction (see section 2.8).

Fermentations with glyceryl tridecanoate glyceryl tridodecanoate, palm kernel and coconut oil were set up at pH 7. These conditions were used throughout the remainder of the experiments. At this pH the phosphate buffer was most stable. (See Section 3.2.2.)

2.5.3 The effect of temperature on the conversion of the lauric acid oils and triglycerides into methyl ketones.

Medium (section 2.5.2) was used at pH 7.0. Palm kernel or coconut oils were used as sole carbon sources at temperatures of 4, 10, 20, 25, 30 and 37°C. The experiment at 4°C was carried out without shaking inside a refrigerator. The remaining experiments were carried out as described in section 2.5.2 and the temperature of the incubator adjusted as necessary.
The experiment was repeated using simple acid glycerides (glyceryl trihexanoate, glyceryl trioctanoate, glyceryl tridecanoate and glyceryl tridodecanoate) as sole carbon sources.

2.5.4 The effect of water activity on the conversion of the lauric acid oils into methyl ketones.

The effect of water activity was studied at 25°C and pH 7.0 using the media described in section 2.5.2. Experiments were performed in quadruplicate using

sodium chloride as an osmotic regulator. This was incorporated into the buffered Czapek medium at concentrations of 2.5, 5, 10 and 15% (w/v). Erythritol was also used as an osmotic regulator at concentrations of 10, 20, 30 and 40% (w/v). Figure 2.1 gives the water activities of these solutions in buffered Czapek media. Glycerol was not used as an osmotic regulator as it could be utilised as a carbon source.

2.5.5 The effect of preservatives on the conversion of the lauric acid oils into methyl ketones

Medium (25 ml) was dispensed into flasks as described in section 2.5.2. After sterilisation at 121°C and 15 psi for 15 min, sorbic acid (2,4-hexadienoic acid) was added as a solid to flasks over a range of concentrations from 1 to 20 mmol.l^{-1} (mmol sorbic acid per litre of medium). Experiments were carried out in quadruplicate using palm kernel or coconut oil as the sole carbon source. Internal standard (3-undecanone, 40.6 mg) was added after fermentation for 72 h at 25°C and 200 rpm.

The experiment was repeated using natamycin as a preservative at concentrations of 1.00, 0.50, 0.10, 0.05 and 0.01 mg.Kg^{-1} (mg natamycin per Kg media).

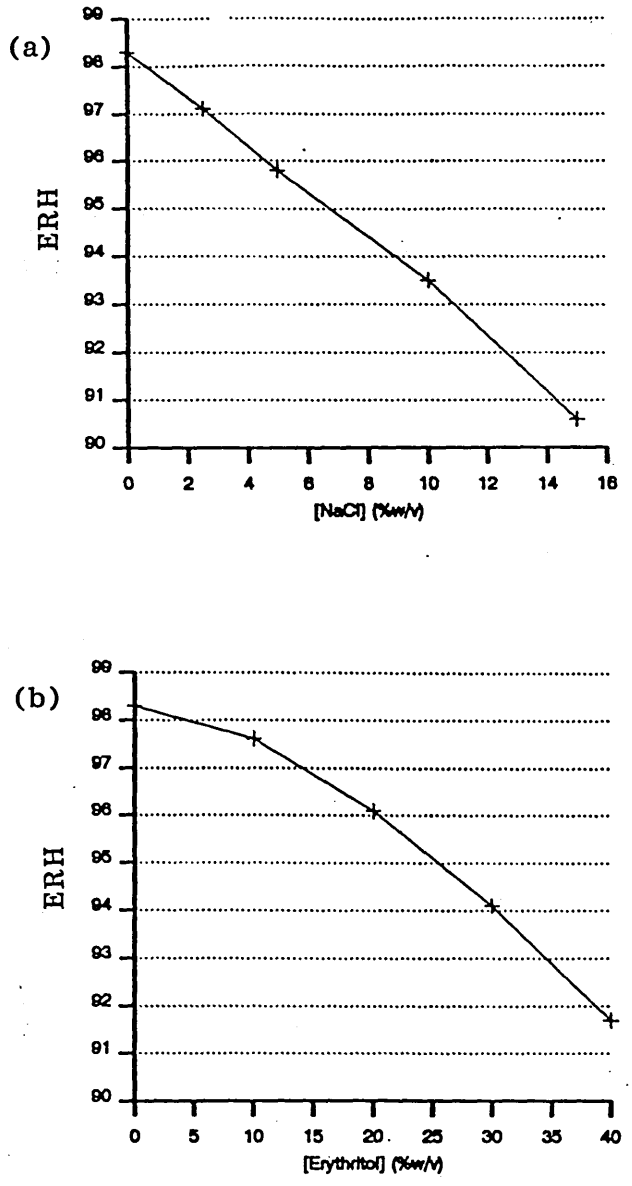


Figure 2.1 : Equilibrium relative humidities for solutions of (a) NaCl and (b) erythritol in buffered medium. Standard deviations did not exceed 0.1%

2.5.6 The effect of glucose on the conversion of coonut and palm kernel oil into methyl ketones

Glucose (3% w/v) was added to the pH buffered medium described in section 2.5.2. Medium (25 ml) was dispensed into flasks in quadruplicate with coconut or palm kernel oil (1 g). The flasks and media were sterilised at 121°C and 15 psi for 15 minutes and allowed to cool. Spores (2.5×10^7) were added and the flasks were incubated at 25°C and 200 rpm for 72 h.

2.5.7 The effect of light on the conversion of the lauric acid oils into methyl ketones

Flasks were set up as described in section 2.5.2 with palm kernel or coconut oil as a sole carbon source. Fermentations were exposed to black light (15 W, peak at 365 nm) for alternate 12 h periods throughout the experiment (Johnson and Booth, 1983).

2.5.8 The effect of the removal of oxygen on the growth of *Penicillium crustosum*

Czapek medium as described in section 2.3 was prepared with the sucrose content replaced with either palm kernel or coconut oil (30 g). After sterilisation at 121°C and 15 psi for 15 min the medium was shaken vigorously and poured into Petri dishes. Each of two Petri dishes were inoculated with a three-point

inoculation from a spore suspension using a nicon wire needle (5 μm). The plates were incubated in an anaerobic jar (Oxoid HP11) where a reducing atmosphere was generated with a gas generating kit (Oxoid BR38). Anaerobic conditions were confirmed with indicator paper (Oxoid BR55). After 14 days at 25°C the plates were compared with a duplicate series incubated under aerobic conditions. Following this the medium was removed and placed in a stomacher bag with glass distilled water (20 ml) and internal standard (3-undecanone; 7.3 mg). The contents were mixed in a stomacher (Colworth, Bury St Edmunds, Suffolk) for one minute prior to solvent extraction.

2.5.9 Fermentation of commercially important fats and oils

The following fats were used as substrates: hazelnut oil, olive oil, coconut oil, palm kernel oil, cocoa butter and edible beef tallow. Flasks were set up in quadruplicate using medium at pH 7 and the fermentation carried out as described in section 2.5.2. The internal standard 3-undecanone (7.3 mg) was added to each flask prior to solvent extraction.

2.6 Extraction of volatile methyl ketones

After addition of internal standard, dichloromethane (DCM, 10 ml) was added to each flask which was shaken vigorously for one minute. The contents of the flasks were transferred to glass centrifuge tubes (50 ml) and centrifuged at 4000 rpm for five min. (MSE 'Centaur 2', MSE Ltd., Crawley, Sussex) when the lower solvent phase was removed with a Pasteur pipette. The solvent extract was placed in a glass vial and stored at 4°C prior to analysis.

2.7 Determination of biomass

The fungal pellets were filtered through a sintered glass filter under vacuum and washed with glass distilled water. The residue was placed on a filter paper disc (Whatman no. 1, ashless) and dried to constant weight for 72 h at 80°C in a drying oven.

2.8 Selection of internal standard for gas chromatography

Four ketones (5-nonanone, 2-decanone, 6-undecanone and 3-undecanone) were evaluated for use as internal standards. Table 2.1 demonstrates that 3-undecanone was the most suitable compound.

The eight of internal standard was calculated by calibrating a positive displacement pipette (Gilson 'microman', Anachem

Ltd., Luton, Beds.) so that the weight of ketone dispensed was 7.3 mg.

Table 2.1 Comparison by gas chromatography of aliphatic methyl ketones for use as internal standards*

Compound tested	Comment on suitability
5-Nonanone	Retention time too close to 2-heptanol
2-Decanone	Possibly produced during fermentations
6-Undecanone	Used in early work but could not be resolved from 2-nonanol
3-Undecanone	Eluted in ideal position between 2-nonanol and 2-undecanone

* Results were obtained from a series of runs with a Carbonwax 20M column under different conditions.

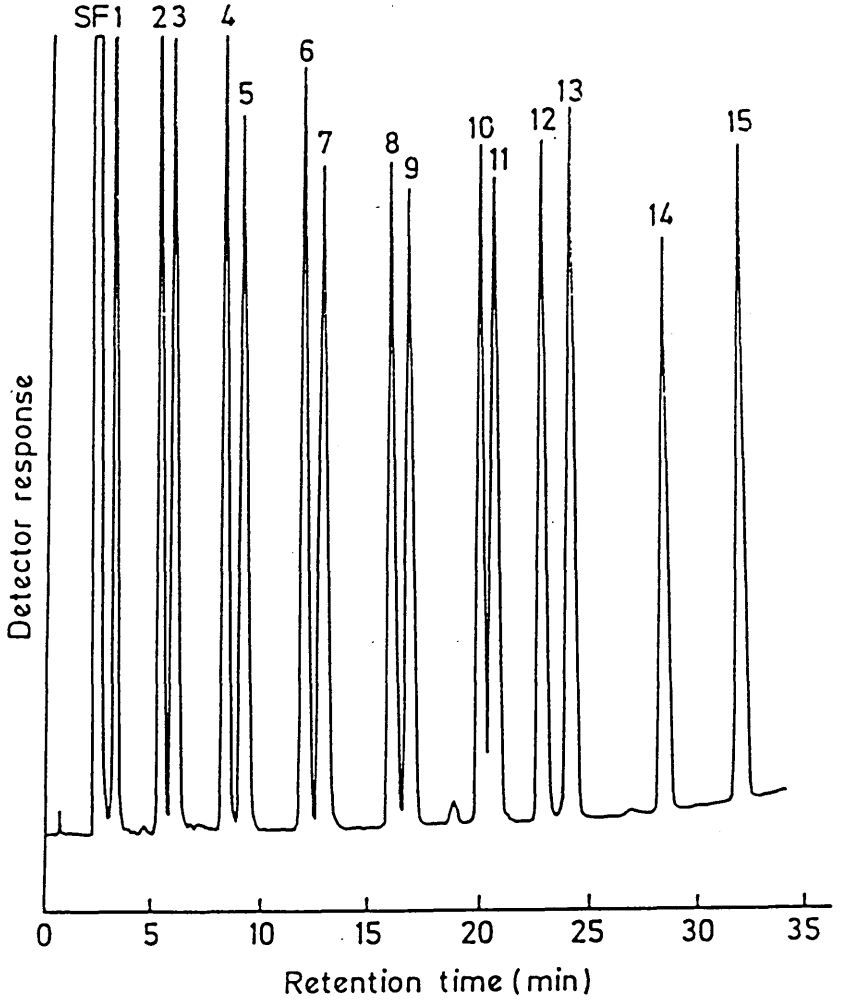
2.9 Analysis of methyl ketones and secondary alcohols by gas chromatography

Gas chromatography was carried out using a Varian 'Vista' series 6000 or a Varian 3400 gas chromatograph (Varian Associated Ltd., Walton-on-Thames, Surrey) with an SP 4100 computing integrator (Spectra-Physics, California, USA). Nitrogen was used as a carrier gas with the flow rate set at 40 $ml.min^{-1}$ with the injector block at 150°C and the flame ionisation detector (FID) at 200°C. Retention times relative to 3-undecanone were determined using standard mixtures of methyl ketones and secondary alcohols (see Figure 2.2). Compounds were identified by relative retention time and co-chromatography with known standards. Identifications were confirmed by gas chromatography/mass spectrometry.

2.10 Fatty acid composition of fats and single acid glycerides

2.10.1 Preparation of fatty acid methyl esters

Fatty acid compositions were determined for the following fats and single acid glycerides: hazelnut oil, olive oil, coconut oil, palm kernel oil, cocoa butter, edible beef tallow, glyceryl trihexanoate, glyceryl trioctanoate, glyceryl tridecanoate and glyceryl trioctadec-9-enoate. Fatty acid methyl esters (FAME) of these substrates were obtained using the

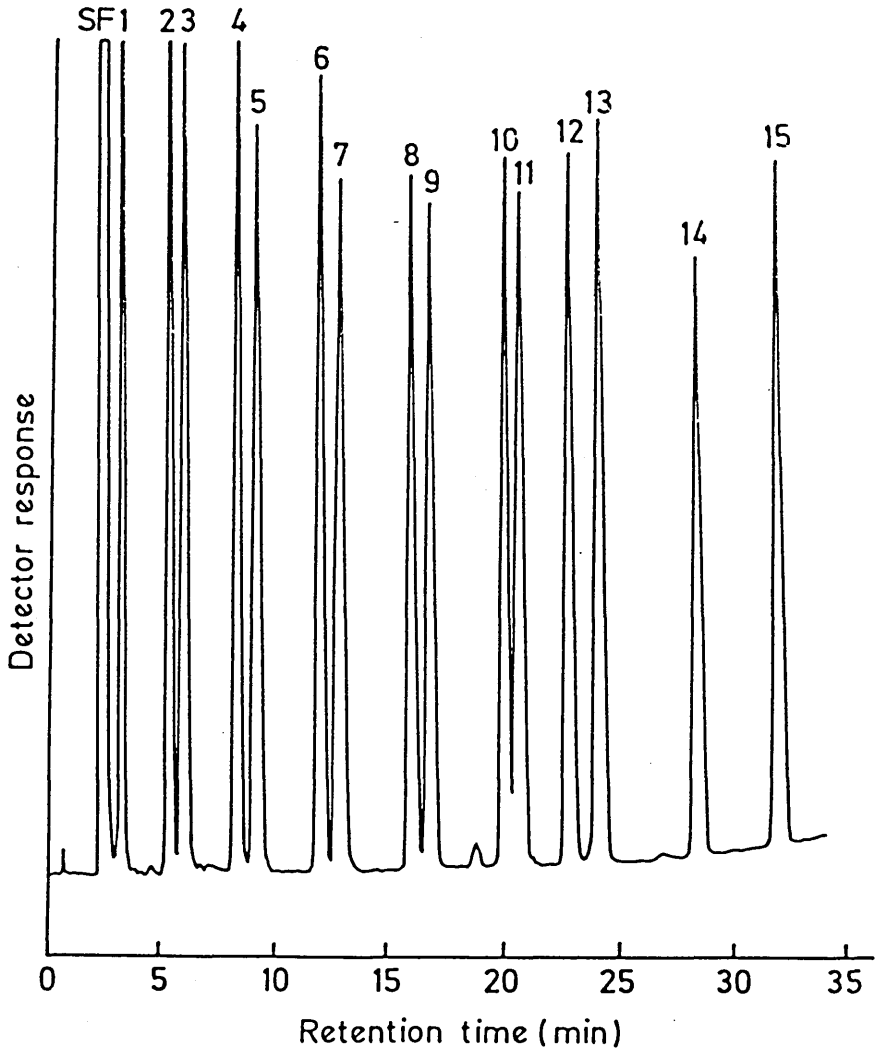


Conditions:

Column	2m x 2mm I.D. (steel).
Column packing	10%(w/w) Carbowax 20M on Chromosorb WAW, 80-100#.
Carrier gas	N_2(gas), 40 ml.min^{-1}.
Column oven program	70°C to 200°C at 3°C.min^{-1}.
Injection temp.	150°C.
Detector temp.	200°C.
Attenuation	x64
Range	10^{-10}.

Figure 2.2 : Methyl ketone and secondary alcohol standard run on gas chromatograph.*

* See Table 2.2 for peak identities



Conditions:

Column	2m x 2mm I.D. (steel).
Column packing	10%(w/w) Carbowax 20M on Chromosorb WAW, 80-100#.
Carrier gas	N_2(gas), 40 ml.min^{-1}.
Column oven program	70°C to 200°C at 3°C.min^{-1}.
Injection temp.	150°C.
Detector temp.	200°C.
Attenuation	x64
Range	10^{-10}.

Figure 2.2 : Methyl ketone and secondary alcohol standard run on gas chromatograph.*

* See Table 2.2 for peak identities.

Table 2.2 Response factors and relative retention times of aliphatic methyl ketone and secondary alcohol standard mixture.

Peak No.	Peak Identity	Relative Retention Time	Response Factor
1	2-Pentanone	0.147 ± 0.001	1.169 ± 0.011
2	2-Hexanone	0.238 ± 0.001	1.120 ± 0.013
3	2-Pentanol	0.265 ± 0.001	1.188 ± 0.019
4	2-Heptanone	0.366 ± 0.001	1.067 ± 0.005
5	2-Hexanol	0.402 ± 0.001	1.119 ± 0.007
6	2-Octanone	0.522 ± 0.001	1.021 ± 0.005
7	2-Heptanol	0.560 ± 0.001	1.065 ± 0.010
8	2-Nonanone	0.697 ± 0.001	1.095 ± 0.012
9	2-Octanol	0.731 ± 0.001	1.055 ± 0.017
10	2-Decanone	0.876 ± 0.001	1.035 ± 0.001
11	2-Nonanol	0.905 ± 0.001	1.019 ± 0.002
12	3-Undecanone (IS)	1.000	1.000
13	2-Undecanone	1.055 ± 0.000	0.968 ± 0.005
14	2-Undecanol	1.243 ± 0.001	0.983 ± 0.002
15	2-Tridecanone	1.397 ± 0.000	0.964 ± 0.001

* Results are the mean of one standard and four analyses (± S.D.).

Retention times and response factors determined relative to 3-undecanone.

complete esterification method of Hitchcock and Hammond (1980). The methylating reagent (5 ml) was prepared from sulphuric acid, methanol and toluene in a 1:10:20 (v/v) ratio respectively. Individual fats or glycerides (40 mg) were placed into each of three pear-shaped distillation flasks (100 ml) containing anti-bumping granules to which methylating reagent (5 ml) was added. Internal standard (undecanoic or heptadecanoic acid, 10 mg) was added and this mixture was refluxed for one hour. The reaction was stopped by the addition of glass distilled water (5 ml). Diethyl ether (5 ml) was added and the flasks were shaken to aid extraction of the FAME. The lower aqueous phase was removed by aspiration leaving the methyl esters in the ether layer. This was dried with anhydrous MgSO4 (2 g) for 24 h prior to analysis by gas chromatography.

2.10.2 Analysis of fatty acid methyl esters by gas chromatography

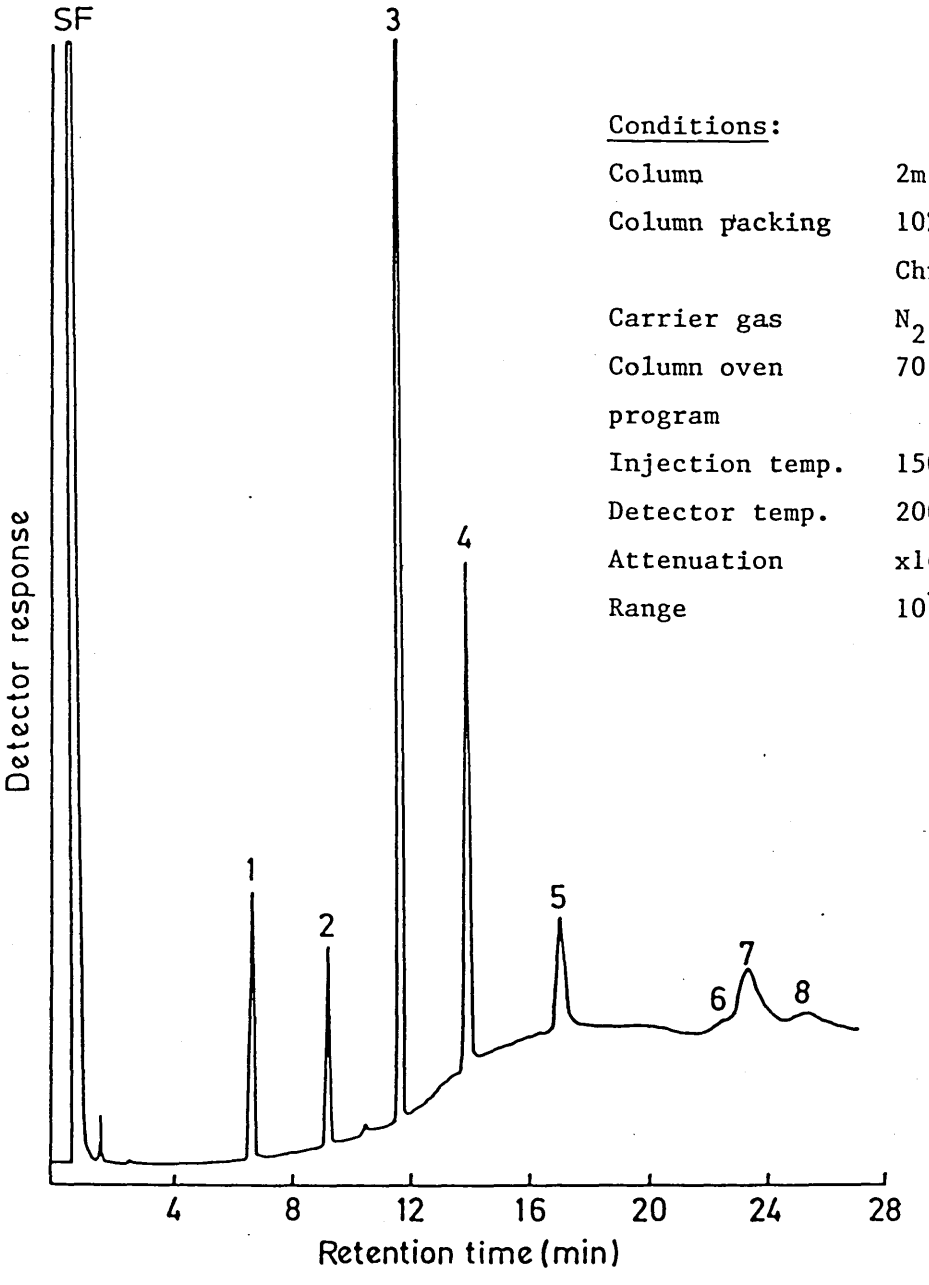
The analysis of short carbon chain fatty acid methyl esters (C6 to C12) was carried out using a Varian 'Vista' series 6000 gas chromatograph coupled to an SP 4100 computing integrator. Nitrogen was used as a carrier gas at a flow rate of 40 $ml.min^{-1}$. The column (2m x 4mm I.D.) was packed with 10% (w/w) Carbowax 20M on Chromosorb W-HP (mesh size 80/100). The oven was

programmed from 70 to 200°C at 10°C.min^{-1} with the injector block at 150°C and the detector at 220°C. For fatty acid methyl esters with a chain length greater than 14 carbon atoms the samples were analysed using a Varian 3400 gas chromatograph. Nitrogen was used as a carrier gas at a flow rate of 25 ml.min^{-1}. The column (2m x 4mm I.D.) was packed with 5% (w/w) DEGS (0.01% w/w H_3PO_4) on Chromosorb W-HP (mesh size 80/100). The oven was maintained at 165°C with the injector block at 200°C and the detector at 250°C. Individual fatty acids were identified by relative retention time to the internal standard and identifications were confirmed by co-chromatography with known standards. Figures 2.3 and 2.4 show the analysis of FAME for short and long chain fatty acids.

2.11 Physical properties of fats and single acid glycerides

2.11.1 Melting point determination

Melting points were determined for free fatty acids, single acid glycerides and whole oils using melting point apparatus (Gallenkamp, Fisons plc, Loughborough, Leics.). Samples were analysed in triplicate and the results compared with published values.



Conditions:

Column	2m x 2mm I.D. (steel).
Column packing	10%(w/w) Carbowax 20M on Chromosorb WAW, 80-100#.
Carrier gas	N_2(gas), 40 ml.min^{-1}.
Column oven program	70°C to 200°C at 10°C.min^{-1}.
Injection temp.	150°C.
Detector temp.	200°C.
Attenuation	x16.
Range	10^{-10}.

Figure 2.3 : Fatty acid methyl ester standard run on gas chromatograph.*

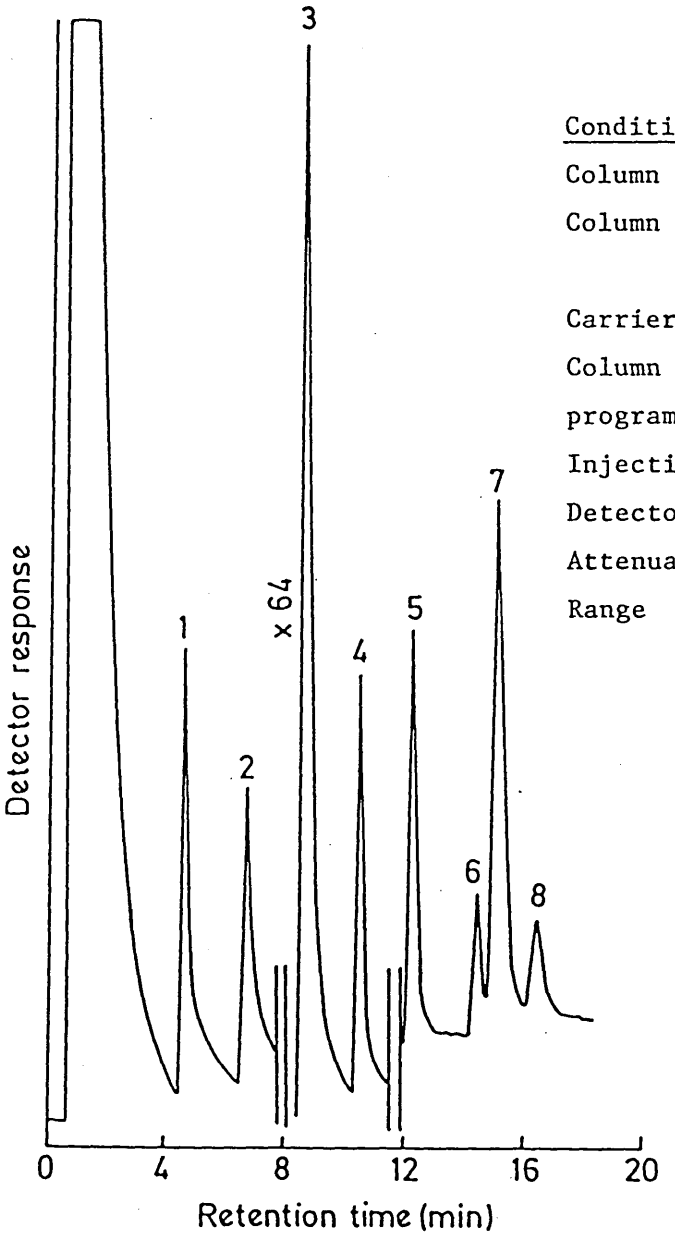
* See table 2.3 for peak identities.

Table 2.3: Response factors and relative retention times of fatty acid methyl ester standard mixture, carbowax 20 M column*.

Peak No.	Peak Identity	Relative Retention Time	Response Factor
1	Methyl octanoate	0.571 ± 0.001	1.033 ± 0.109
2	Methyl decanoate	0.793 ± 0.000	0.962 ± 0.111
3	Methyl dodecanoate	1.000	1.000
4	Methyl tetradecanoate	1.195 ± 0.001	0.884 ± 0.070
5	Methyl hexadecanoate	1.465 ± 0.003	0.866 ± 0.314
6	Methyl octadecanoate	1.948 ± 0.016	7.929 ± 5.974
7	Methyl octadec-9-eneoate	2.005 ± 0.002	2.153 ± 1.261
8	Methyl octadec-9,12-eneoate	2.169 ± 0.001	1.613 ± 0.219

* Results are the mean of one standard and four analyses (± S.D)

Retention times and response factors determined relative to methyl dodecanoate.



Conditions:

Column	2m x 2mm I.D. (steel).
Column packing	10%(w/w) D.E.G.S. on Chromosorb WAW, 80-100#.
Carrier gas	N_2(gas), 40 ml.min^{-1}.
Column oven program	80°C to 190°C at 10°C.min^{-1}.
Injection temp.	150°C.
Detector temp.	200°C.
Attenuation	x16.
Range	10^{-10}.

gure 2.4 : Fatty acid methyl ester standard run on gas chromatograph.*

See table 2.4 for peak identities.

Table 2.4: Response factors and relative retention times of fatty acid methyl ester standard mixture, DEGS Column*

Peak No.	Peak Identity	Relative Retention Time	Response Factor
1	Methyl octanoate	0.527 ± 0.002	1.752 ± 0.155
2	Methyl decanoate	0.771 ± 0.001	1.558 ± 0.272
3	Methyl dodecanoate	1.000	1.000
4	Methyl tetradecanoate	1.221 ± 0.001	1.076 ± 0.093
5	Methyl hexadecanoate	1.431 ± 0.002	1.086 ± 0.115
6	Methyl octadecanoate	1.690 ± 0.002	1.145 ± 0.143
7	Methyl octadec-9-eneoate	1.770 ± 0.001	0.919 ± 9.100
8	Methyl octadec-9,12-eneoate	1.928 ± 0.001	1.225 ± 0.190

* Results are the mean of one standard and four analyses (± S.D.)

Retention times and response factors determined relative to methyl dodecanoate.

2.11.2 Energy value determination

A ballistic bomb calorimeter (Gallenkamp, Fisons plc, Loughborough, Leics.) was used to determine the molar heat of combustion for whole oils and single acid triglycerides used as carbon sources. The instrument was calibrated using benzoic acid (Bureau of Analysed Samples, Ltd., Middlesborough). Six repeat tests were carried out to obtain a mean calibration constant (Y) for the instrument:

(i) calculation of calibration constant:

$$Y1 = \frac{26.4416 \times W1}{D2-D1} \text{ KJ. per division}$$

Where 26.4416 KJ.g = energy value of benzoic acid.

W1 = mass of benzoic acid (g).

D1 = galvanometer deflection without sample.

D2 = galvanometer deflection with benzoic acid.

The standardising test was repeated five more times and the average for Y calculated:

$$Y = \frac{Y1 + Y2 + Y3 + Y4 + Y5 + Y6}{6} \text{ KJ per division}$$

The calibration constant (Y) was determined before operating the instrument. It was then used to calculate energy values for unknown samples.

(ii) Calculation of energy value of unknown sample:

$$\text{Energy value} = \frac{(D3 - D1)}{Z} Y \quad KJ.g^{-1}$$

Z = Mass of sample (g)

D3 = Galvanometer deflection with sample

D3-D1 = Galvanometer deflection due to sample

Y = Calibration constant ($KJ.division^{-1}$)

Analyses of unknown samples were performed in triplicate.

2.12 The inhibition of fungal growth by fatty acids

Czapek medium was prepared and 20 ml was dispensed into Erlenmeyer flasks (125 ml) which were sterilized at 121°C/15 psi for 15 minutes. Caproic (hexanoic) acid was added to the flasks to give a range of concentrations from 0 to 50 $mmol.l^{-1}$. The flasks were inoculated with spores ($2.0x10^{7}$) from a spore suspension. Experiments were performed in triplicate at 25°C and 200 rpm on a rotary shaker mounted in an incubator (see section 2.5a).

The experiment was repeated using sorbic (2,4-hexadienoic), capric (decanoic), lauric (dodecanoic) and oleic (9-octadeceneoic) acids. Growth inhibition was determined relative to a control with no fatty acids present:

$$1 - \frac{W1}{W0} \times 100 = \% \text{ inhibition}$$

W1 = Dry weight (mg) of mould growing in presence of fatty acid

W0 = Dry weight (mg) of control

2.13 Detoxification of sorbic acid and the production of 1,3-pentadiene by *Penicillium crustosum*

Medium (400 ml) as described in section 2.3 was dispensed into each of four micro-carrier pots (500 ml; Techne, Duxford, Cambridgeshire). These were sterilised at 121°C and 15 psi for 15 min, cooled and inoculated with spore suspension (1 ml). Pots were incubated at 60 rpm and 25°C for 48 h when sorbic acid (4 mmol) was added to two pots. The incubation was continued for a further 24 h with the flasks containing sorbic acid in a separate incubator. Volatile 1,3-pentadiene was extracted into DCM (40ml) for gas chromatography or ^{1}H-chloroform (10 ml) for nuclear magnetic resonance spectroscopy (nmr). Analyses by gc/ms were performed at 70 EV using a Carlo Erba series 2150 gas chromatograph coupled to a Micromass 30 F mass spectrometer. Identification of 1,3-pentadiene was confirmed by nmr using a Brucker WP 80 SY at 80 MHz.

2.14 Examination of fungal ultrastructure by electron microscopy

Mycelial pellets were removed from shake culture and embedded in agar (1% w/v). These were trimmed with a scalpel to give blocks (1 mm^3) which were suspended in 15 ml of primary fixative (3% w/v glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2). After 1 h the buffer was replaced with fresh buffer for a further 30 min. This washing process was repeated once more.

Secondary fixation took place in a solution of osmium tetroxide (1% w/v) in sodium cacodylate buffer (0.1 M) for 1 h. The volume was sufficient to cover the sample. Osmium tetroxide is extremely hazardous and secondary fixation was carried out in a fume cupboard. Samples were dehydrated by passage through a series of acetone baths:

	Acetone conc. (% w/v in water)	Time (min)
1.	30	10
2.	50	10
3.	70	15
4.	90	15
5.	95	15
6.	100	20
7.	100	20

Samples were washed twice in propylene oxide for 15 min before infiltration with a 50/50 mixture of propylene oxide and resin. Samples were then placed in resin for 1 h at 37°C before finally being left overnight in fresh resin at room temperature. In the morning the samples were transfered to Beem capsules and polymerised with fresh resin for 16 h at 60°C.

Sections were cut on an ultramicrotome (Reichart OmU3,) and placed on copper grids. Sections were stained by immersion in a saturated solution of uranyl acetate in methanol for 15 min. After thorough washing in five methanol baths, the sections were stained with lead citrate. This took place in a Petri dish in the presence of sodium hydroxide to avoid the formation of lead carbonate. Sections were examined using a transmission electron microscope (JEOL 100 CX).

Materials

Except where otherwise indicated all chemicals were purchased from BDH Ltd (Poole, Dorset) and were of 'Analar' or equivalent grade. Solvents were obtained from Romil Chemicals (Romil Chemicals, Shepshed, Leics.) and were of HPLC grade. Synthetic triglycerides were obtained from Sigma Chemicals (Poole, Dorset). Commercial fats were a gift from Unilever PLC (Colworth House, Bedford, Beds.). The fatty acid methyl ester standard mixture was purchased

from Supelco, Inc., Bellefonte, Pennsylvania, USA). Materials for electron microscopy were purchased from Emscope Ltd. (Ashford, Kent).

3. Results and Discussion - Characterization of ketonic rancidity in the lauric acid oils.

3.1 The effect of time on the fermentation of palm kernel oil

Studies of the kinetics of fungal growth are helpful in understanding how growth is modified by changes in the environment. There have been two recent reviews of the growth kinetics of filamentous fungi (Righelato 1975, Trinci 1978). Several groups have investigated the influence of culture age on the conversion of fatty acids into methyl ketones and secondary alcohols by spores and fungal mycelium (Hawke 1966, Dwivedi and Kinsella 1974, Kinsella and Hwang 1976 a,b). Hawke demonstrated that the age of mycelium markedly affected its ability to convert octanoic acid into 2-heptanone (Hawke 1966). Mycelium was claimed to have maximum activity after 50 h (Kinsella and Hwang 1976 a). These results were contested by other groups who reported that fresh and aged (24 h) mycelium were equally capable of forming methyl ketones after an initial lag phase. The lag phase may be longer with fresh mycelium compared to aged mycelium (Dwivedi and Kinsella 1974).

Figure 3.1 shows that the greatest increase in biomass occurs between 60 and 72 h of fermentation. This has followed a period where the spores

have germinated and little increased biomass was recorded. The growth curve does not show the stationary or death phase usually described for fungi in liquid shake culture (Righelato 1975, Trinci 1978).

The lag phase represents the time taken for the fungal spores to germinate. Once the energy reserves of the spores were depleted the organism was forced to use the palm kernel oil as a sole carbon source. Fungi do not normally use lipids as a first choice of carbon source. During periods of nutrient deficiency reserves of lipid within the hyphae are metabolised (Gyllenberg and Raitio 1952). To utilise extracellular triglycerides the fungus must secrete lipases (Section 1.2). These enzymes hydrolyse lipids to produce free fatty acids. The fatty acids are taken up into their hyphae in the undissociated state (Lewis 1971). Methyl ketones were not detected during the lag phase. The absence of methyl ketones indicates that spores cannot convert palm kernel oil into methyl ketones. However, several groups have reported that spores can convert free short carbon chain fatty acids (C6 to C14) into the corresponding methyl ketone one carbon atom shorter than the parent acid (Lawrence 1966, Lawrence and Hawke 1968). Lawrence also reported that spores of *Penicillium roquefortii* could convert glyceryl trihexanoate into 2-pentanone and glyceryl trioctanoate into 2-heptanone (Lawrence 1967).

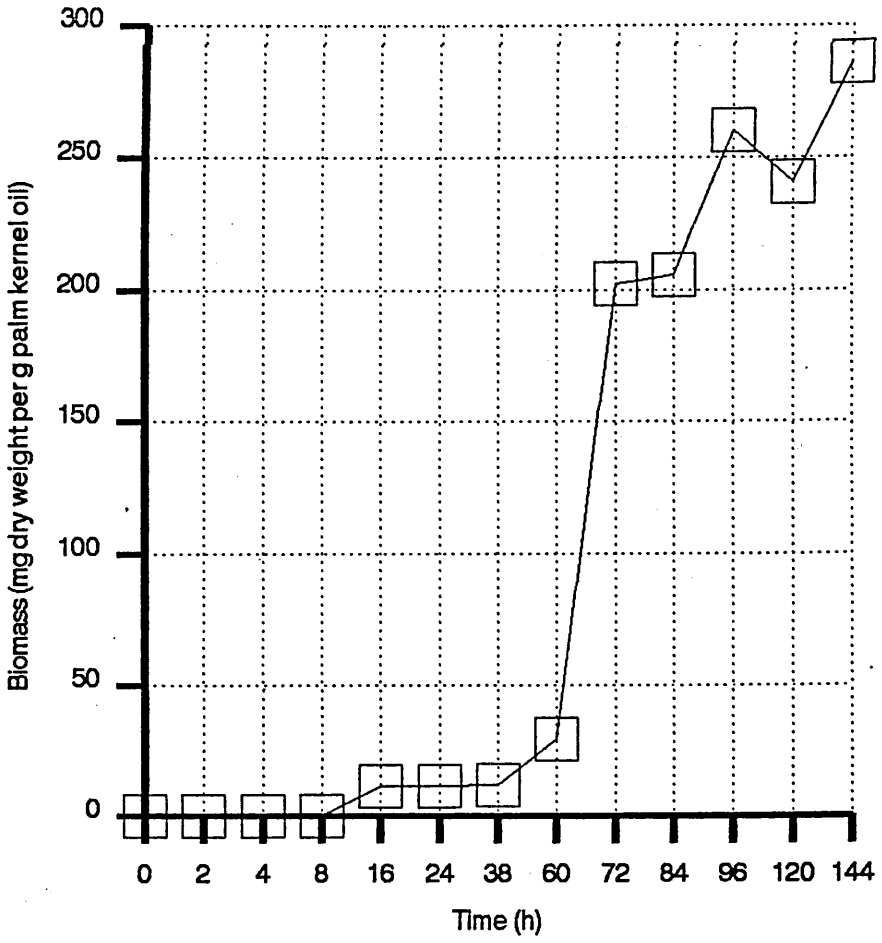


Figure 3.1 : Growth curve for Penicillium crustosum fermenting palm kernel oil (2 g) as a sole carbon source. Results are the mean of two flasks.

The increase in 2-undecanone concentration was similar to the increase in biomass for 84 h of the fermentation (Figure 3.2). The relationship between growth and ketone production provides further evidence that the production of these compounds is a result of the operation of a primary metabolic pathway as secondary metabolites are usually produced during the period of growth limitation (Bu'lock 1965). The effect of fermentation time on the detection of other methyl ketones is shown in Figure 3.3. The order of production of ketones was C7 , C8 and C9, C11, C13, with 2-heptanone appearing after only 16 h fermentation. This suggested that the reactivity of the fatty acids decreased with an increase in carbon chain length of the substrate. The decrease in mycelial growth rate at the end of the exponential growth phase was accompanied by a fall in the concentration of methyl ketones. Methyl ketones may undergo further metabolism or they may be lost due to their volatility. The further metabolism of methyl ketones has been recognised (Forney and Markovetz 1971, Kinderlerer 1987, Platen and Schink 1989).

From Figures 3.1 and 3.2 the maximum rate of ketone formation coincided with the exponential growth phase. This phase ended after 72 h growth in liquid shake culture. It was felt that 72 h was a suitable time to

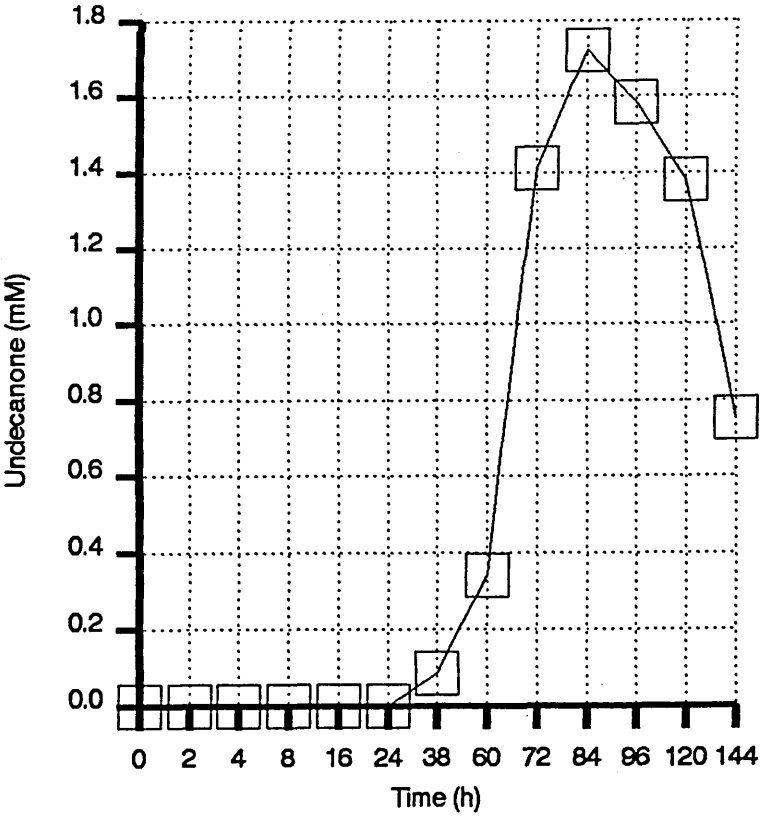


Figure 3.2 : Production of 2-undecanone by Penicillium crustosum during 144 h fermentation of palm kernel oil (2 g). Results are the mean of two flasks.

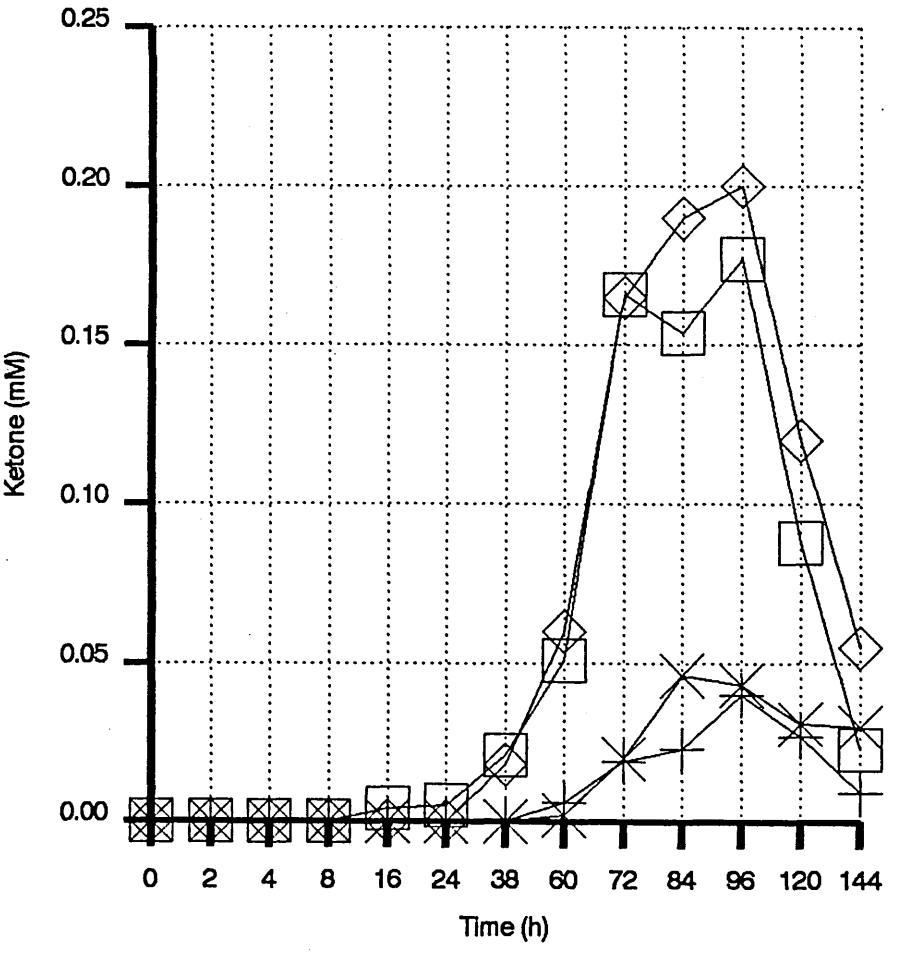


Figure 3.3 : Production of methyl ketones (C7,8,9 and 13) during 144 h fermentation of palm kernel oil (2 g). Results are the mean of two flasks.

harvest mycelium and extract methyl ketones in subsequent experiments.

3.2 Fermentation to demonstrate the effect of fatty acid composition of commercial oils on the production of methyl ketones

Spoilage of the lauric acid oils has not been studied since 1928 when Stokoe compared the spoilage of coconut oil with that of beef fat (Stokoe 1928). Methyl ketones were produced after growth of *P.palitans* on coconut oil but not on beef fat. A number of workers have established that fats and oils containing fatty acids with less than fourteen carbon atoms were prone to ketonic rancidity (Lawrence and Hawke 1968, Karahadian *et al* 1985, Hatton and Kinderlerer 1986).

In order to confirm which oils could serve as a substrate for methyl ketone formation the following oils: hazelnut oil, olive oil, beef tallow, cocoa butter, coconut oil and palm kernel oil were used as a sole carbon source. The fatty acid compositions of these oils are given in Table 3.1. This Table demonstrates that coconut and palm kernel oils contained short and intermediate carbon chain length fatty acids (C6 to C14) unlike the other oils and fats where the glycerides contain only long carbon chain length fatty acids (> C14).

Table 3.1: Fatty acid compositions for commercial oils used as sole carbon sources in fermentations with *Penicillium crustosum*.*

Fatty Acid (C no)	Fatty Acid Composition (g. 100 g oil)					
	Hazelnut oil	Olive oil	Beef tallow	Cocoa butter	Palm kernel oil	Coconut oil
C6:0	ND	ND	ND	ND	ND	0.5±0.0
C8:0	ND	ND	ND	ND	3.1±0.2	7.7±0.3
C10.0	ND	ND	ND	ND	3.2±0.2	5.9±0.4
C12:0	ND	ND	ND	ND	38.7±1.8	46.3±2.4
C14:0	ND	ND	6.5±0.8	ND	14.6±0.7	15.6±1.7
C16:0	4.9±0.1	10.3±0.5	29.0±1.3	21.0±1.8	7.7±0.5	8.3±0.7
C18:0	1.8±0.02	2.5±0.1	14.9±1.1	25.8±1.5	2.0±0.2	3.1±0.3
C18:1	67.5±1.0	62.1±2.8	27.4±2.1	30.2±2.4	12.3±1.1	5.7±0.4
C18:2	12.0±0.5	8.7±0.5	2.7±0.4	2.9±0.5	2.3±0.3	1.8±0.4

* Results are the arithmetic mean of three esterifications and nine analyses (±S.D.).

Methyl ketones were only detected after coconut or palm kernel oils were used as sole carbon sources. Table 3.2 shows the homologous series of methyl ketones (C5, 7, 9, 11 and 13) derived from the fermentation of coconut oil. A similar pattern for the conversion of palm kernel oil into methyl ketones is given in Table 3.3. In both cases the major product was 2-undecanone. This reflected the high proportion of dodecanoic acid in coconut and palm kernel oils (Table 3.1). 2-Tridecanone was the largest methyl ketone detected. This observation suggested that tetradecanoic acid was the cut-off point for production of methyl ketones. Table 3.1 shows that heptanoic and nonanoic acids were not detected. These acids would be the precursors for 2-hexanone and 2-octanone respectively. It is not known how these even numbered methyl ketones were produced. It is likely that these ketones are derived from different biosynthetic pathways to those involved in the production of the odd-numbered series (C5, C7, C9, C11 and C13).

Tables 3.2 and 3.3 show that greater concentrations of methyl ketones were produced from the fermentation of coconut oil than that of palm kernel oil. This reflected the differences in fatty acid composition between the two oils shown in Table 3.1. Coconut oil contained about two times the weight per gram of the

Table 3.2: The conversion of coconut oil into methyl ketones by two isolates of Penicillium crustosum.*

Substrate		Products				
			Isolate CMI 281919		Isolate CMI 300381	
Fatty acid (Carbon no)	Molar composition (mmol g^{-1} oil)	Methyl Ketone+	Concentration (mmol)	Molar Conversion (%)	Concentration (mmol)	Molar Conversion (%)
6:0	0.041	2-Pentanone	0.010±0.003	24.4±7.3	0.001±0.000	2.4±0.0
8:0	0.535	2-Heptanone	0.107±0.013	20.0±2.4	0.111±0.009	20.7±1.7
10:0	0.344	2-Nonanone	0.112±0.015	32.6±4.4	0.111±0.010	32.3±2.9
12:0	2.309	2-Undecanone	0.580±0.068	25.1±2.9	0.514±0.050	22.3±2.2
14:0	0.700	2-Tridecanone	0.018±0.004	2.6±0.6	0.012±0.002	1.7±0.3

* Results are the arithmetic mean of four fermentations and eight analyses (±S.D.). Fermentations were in aerobic liquid shake culture at 200 rpm, 25 °C and pH 7 with 1 g of coconut oil as a sole carbon source.

+ Secondary alcohols were less than 1% (w/w) of the total products and they were added to the corresponding methyl ketone. 2-Octanone accounted for less than 2% (w/w) of the total products. It was not included in the Table as it probably arises through a different pathway and is not directly produced from the short chain fatty acids of the coconut oil.

Table 3.3: The conversion of palm kernel oil into methyl ketones by two isolates of *Penicillium crustosum*.*

Substrate		Products				
			Isolate CMI 281919		Isolate CMI 300381	
Fatty acid (Carbon no)	Molar composition (mmol g^{-1} oil)	Methyl Ketone+	Concentration (mmol)	Molar Conversion (%)	Concentration (mmol)	Molar Conversion (%)
6:0	-	2-Pentanone	-	-	-	-
8:0	0.216	2-Heptanone	0.015±0.011	6.9±5.1	0.051±0.013	23.9±6.1
10:0	0.186	2-Nonanone	0.023±0.013	12.4±7.1	0.053±0.014	28.5±7.9
12:0	1.932	2-Undecanone	0.140±0.112	8.2±5.1	0.294±0.103	15.2±5.3
14:0	0.637	2-Tridecanone	0	0	0.006±0.000	0.9±0.0

* Results are the arithmetic mean of four fermentations and eight analyses (±S.D.). Fermentations were in aerobic liquid shake culture at 200 rpm, 25 °C and pH 7 with 1 g of palm kernel oil as a sole carbon source.

+ Secondary alcohols were less than 1% (w/w) of the total products and they were added to the corresponding methyl ketone. 2-Octanone accounted for less than 2% (w/w) of the total products. It was not included in the Table as it probably arises through a different pathway and is not directly produced from the short chain fatty acids of palm kernel oil.

shorter fatty acids (C6, C8 and C10) than palm kernel oil.

Tables 3.2 and 3.3 give conversion figures for fatty acids (C6 to C14) into methyl ketones. The molar conversion (%) was calculated from conversion of the fatty acid in the substrate into the corresponding methyl ketone (mmol product divided by mmol substrate x 100). Relatively high conversion rates have been achieved, unlike those reported for the two members of the genus *Eurotium* (Kinderlerer 1987). Unexpectedly, the highest conversion was that of decanoic acid into 2-nonanone where up to 33% of the substrate fatty acid was converted to methyl ketone. The order of conversion of the individual fatty acids was C10:0 > C12:0 > C8:0 & C6:0 for coconut oil (Table 3.2) and C10:0 > C12:0 & C8:0 for palm kernel oil (Table 3.3).

It is unlikely that the fungus would carry out such a metabolically wasteful reaction without a reason. Early work suggested that the production of methyl ketones was a means of recycling coenzyme A (Lawrence and Hawke 1968). On the other hand most workers have suggested that the production of these compounds is a means of removing fatty acids which may be fungicidal (Franke *et al* 1961, Lewis and Darnall 1970, Lewis 1971). Originally Stokoe had suggested that the short chain fatty acids of coconut oil were toxic. More recent evidence for the toxicity of the lauric acid

oils was provided by Moody and Weinhold (1972). These authors reported that *Armillaria mellea* was unable to grow in the presence of 5% (w/v) coconut oil (Moody and Weinhold 1972).

In order to investigate the importance of fatty acid composition fungal growth rates were compared using different commercial oils. Figure 3.4 shows the biomass produced after 72 h fermentation. It can be seen that the order of fungal growth was hazelnut oil > olive oil > coconut oil > beef tallow > cocoa butter > palm kernel oil. Figure 3.4 shows that the fungus grew best on oils that contained a high proportion of unsaturated fatty acids with eighteen carbon atoms. All the fermentations were set up with 1 g of fat. The energy values for these substrates were determined in order to show that the differences in growth rate were due to the fatty acid composition of the substrate. These values are given in Table 3.4. The energy values are approximately 45 $kJ.g^{-1}$ for all the oils and fats. The different growth rates given in Figure 3.5 were due to differences in the fatty acid composition of the substrate, not the energy value.

The physicochemical properties of fats and oils are determined by fatty acid composition. These properties are important in determining resistance to fungal spoilage. The substrates listed in Table 3.5 fall into two groups, those which were liquid at 25°C and those

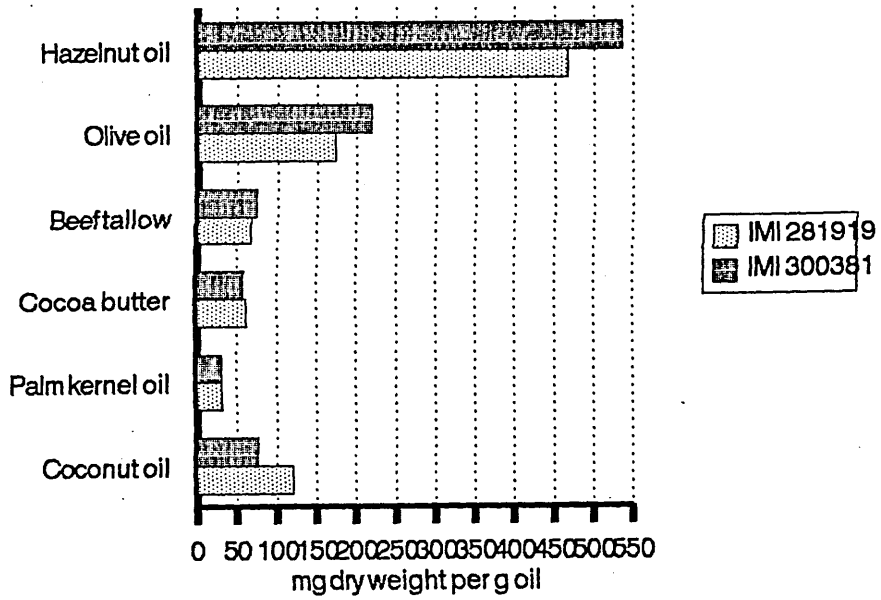


Figure 3.4 : Production of biomass by Penicillium crustosum using whole oils as sole carbon sources. Results are the arithmetic mean of four fermentations

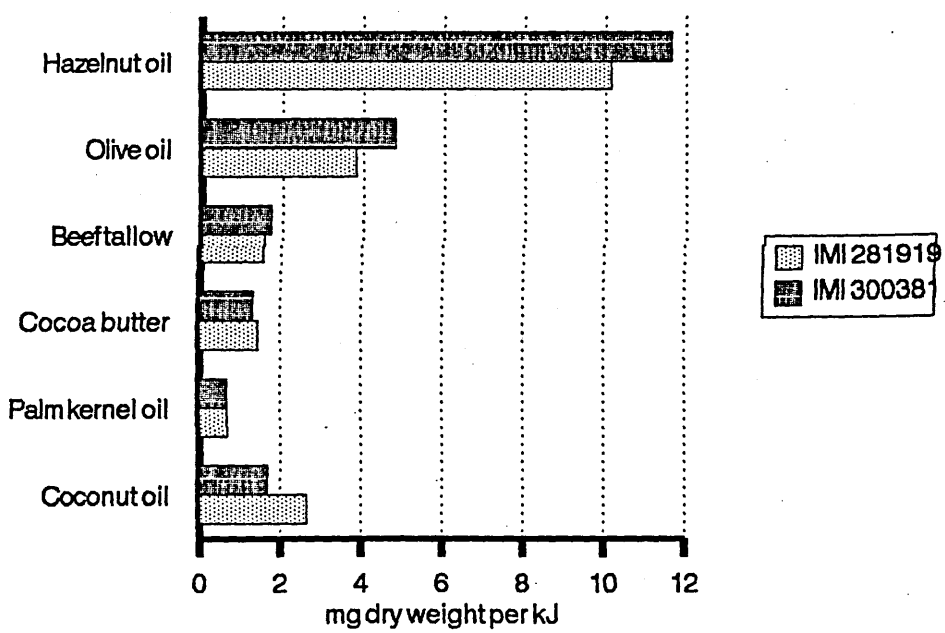


Figure 3.5 : Growth of Penicillium crustosum on whole oils as a function of substrate energy.

Table 3.4: Energy values of commercial oils.*

Commercial oil	Energy value ($KJ.g^{-1}$)
Hazelnut oil	46.5
Olive oil	45.7
Coconut oil	45.7
Palm kernel oil	45.7
Cocoa butter	43.5
Beef tallow	43.1

* Results are the arithmetic mean of one analysis and three samples. The standard deviation did not exceed 3.0 $KJ.g^{-1}$.

which were solid. Oils which were liquid at 25°C (hazelnut, olive and coconut) supported more growth than those which were solid (beef tallow, cocoa butter and palm kernel oil). However, coconut and palm kernel oils supported less growth than oils with corresponding slip-points but different fatty acid compositions (Figure 3.4). These two oils contained short and intermediate carbon chain length fatty acids (C6 to C14). The presence of these fatty acids appeared to inhibit the growth of the organism. This is further evidence that the production of methyl ketones from the lauric acid oils is a means of removing fatty acids that would otherwise further inhibit fungal growth.

Table 3.5 Slip-points of commercial oils *

Oil	Slip-point (°C)
Hazelnut oil	<20
Olive oil	<20
Coconut oil	23.7(±0.3) to 26.1(±0.2)
Palm kernel oil	24.8(±0.3) to 27.3(±0.4)
Cocoa butter	27.9(±0.2) to 31.0(±1.3)
Beef tallow	38.9(±3.1) to 44.2(±0.8)

* Results are the arithmetic mean of three samples and six analyses (±S.D.).

3.3 Fermentation to show the effect of fatty acid composition of simple triglycerides on the production of methyl ketones

Further experiments were required to confirm that glycerides containing short carbon chain length fatty acids inhibited the growth of *P. crustosum*. Simple triglycerides were used as model substrates to study inhibition of fungal growth and the conversion of short carbon chain length fatty acids into methyl ketones. The following simple triglycerides were used as a sole carbon source: glyceryl trihexanoate, glyceryl trioctanoate, glyceryl tridecanoate, glyceryl tridodecanoate, glyceryl tritetradecanoate, glyceryl trihexadecanoate, glyceryl trioctadecanoate and glyceryl trioctadec-9-eneoate. Figure 3.6 gives the biomass (mg dry weight) produced after fermentation of simple triglycerides (1 g) by *P. crustosum* in liquid shake culture at 200 rpm, 25°C and pH 7.0 for 72 h. The relative growth rates presented in Figure 3.6 suggest that the order of preference was C18:1 > C8:0 > C6:0 > C10:0 > C12:0. No growth took place on simple triglycerides of tetradecanoic (C14:0), hexadecanoic (C16:0) or octadecanoic (C18:0) acids.

The maximum growth rate achieved using glyceryl trioctadec-9-eneoate (triolein) accounted for the similar growth rates observed with hazelnut and olive oils (Figure 3.4). These oils contained a high

proportion of 9-octadeceneoic acid (Table 3.1). This confirms a previous report that long chain unsaturated fatty acids can stimulate fungal growth (Wardle & Schisler 1969). Oils that contained a high proportion of unsaturated fatty acids were liquid at 25°C (Table 3.5). The growth rates on hazelnut and olive oils were greater than those for fats containing predominantly saturated long carbon chain length fatty acids (beef tallow and cocoa butter). It is possible that the substrate specificity of the extracellular lipases may affect the organisms ability to utilise different oils, fats or simple trigylcerides as substrates. It has been reported that the lipases of *P.crustosum* preferentially hydrolyse glycerides containing octanoic or 9-octadeceneoic acids (Oi *et al* 1967, Iwai *et al* 1975).

There was a considerable difference between the molecular weights of the simple triglycerides due to the different lengths of the component fatty acids. This influenced the energy values of the substrates and these were determined by bomb calorimetry. Table 3.6 shows that the energy value of the triglyceride increased with increasing carbon chain length up to C18. These results were confirmed by Maggio (personal communication). However, the different energy values for the simple triglycerides did not alter the trend shown in Figure 3.6. Figure 3.7 shows that the

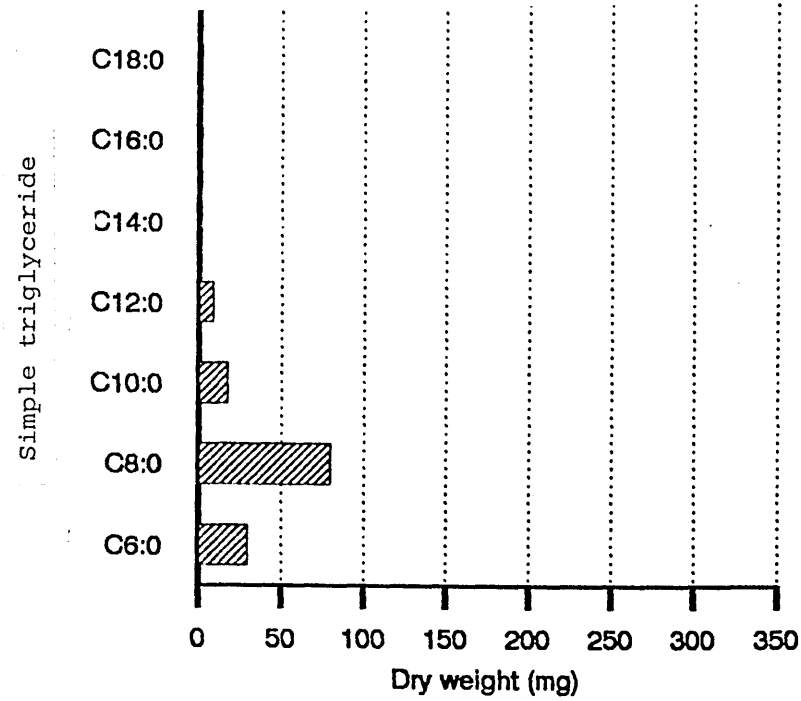


Figure 3.6 : Production of biomass by Penicillium crustosum using simple triglycerides as sole carbon sources (see Table 3.7).

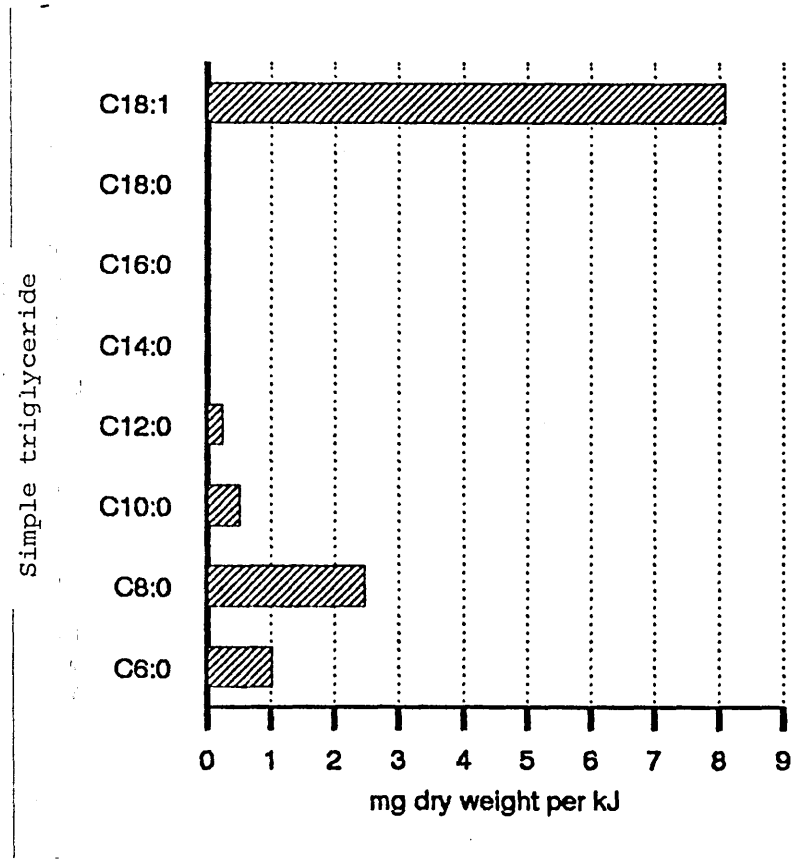


Figure 3.7 : Growth of Penicillium crustosum on simple triglycerides as a function of substrate energy. Results are the arithmetic mean of eight fermentations and two isolates.

Table 3.6: Energy values of simple triglycerides.

Triglyceride	Energy value ($KJ.g^{-1}$)	
	Determined*	Reference+
Glyceryl trihexanoate	29.6	31.5
Glyceryl trioctanoate	32.3	34.1
Glyceryl tridecanoate	34.2	-
Glyceryl tridodecanoate	34.8	-
Glyceryl tritetradecanoate	36.7	38.4
Glyceryl 9-octadeceneoate	40.2	39.1

* Results are the arithmetic mean of one analysis and three samples. The standard deviation did not exceed 2.0 $KJ.g^{-1}$.

+ Maggio, personal communication.

different growth rates were not a function of available energy but rather the fatty acid composition of the simple triglyceride.

Two physical properties of fats, oils, simple triglycerides or their component fatty acids may be important in determining their susceptibility to attack by moulds. These are the slip-point and solubility in water or fat (see Section 1.1). Triglycerides containing fatty acids which are liquid at 25°C (C6:0, C8:0 and C18:1) supported more growth than substrates that are solid at this temperature. The effect of temperature on this process is examined in more detail in Section 3.2.2. The short and intermediate carbon chain length fatty acids (C6:0 to C12:0) are slightly soluble in water (Figure 1.3). Triglycerides that were both solid at 25°C and insoluble in water (C14:0, C16:0 and C18:0) supported no fungal growth (Figure 3.6).

Methyl ketones were only detected after growth on simple triglycerides of hexanoic, octanoic, decanoic and dodecanoic acids. Table 3.7 gives the dry weight (mg), product (mmol) and molar conversion (%) after these simple triglycerides were used as a sole carbon source. Table 3.7 also gives the yield^{biomass} which was calculated by dividing product (μmol) by biomass (mg dry weight). The order of molar conversion was C10 > C6 > C8 > C12. This differed from the order found for coconut or palm kernel oils (Tables 3.2 and 3.3).

Table 3.7: Conversion of simple triglycerides into methyl ketones by two isolates of *Penicillium crustosum*.*

Simple Triglyceride (C-number)	Isolate CMI 281919				Isolate CMI 300381			
	Biomass (mg dry wt)	Product - Ketones (mmol)	Molar Conversion (%)	Yield ^{biomass} (μmol mg^{-1})	Biomass (mg dry wt)	Product - Ketones (mmol)	Molar Conversion (%)	Yield ^{biomass} (μmol mg^{-1})
C6	36.2±3.1	0.531±0.054	6.8±0.7	14.7±1.7	23.5±6.2	0.475±0.076	6.1±1.0	21.3±6.2
C8	86.7±8.2	0.322±0.035	5.1±0.6	3.8±0.7	72.4±18.4	0.278±0.054	4.4±0.9	4.1±1.5
C10	20.2±7.6	0.432±0.361	8.5±7.1	19.2±12.3	14.0±1.4	0.860±0.139	16.9±2.7	60.2±5.5
C12	10.3±5.4	0.022±0.012	0.5±0.3	2.4±1.0	5.9±2.4	0.040±0.009	0.9±0.2	7.6±1.1

* Results are the arithmetic mean of four fermentations and eight analyses (±S.D.). Fermentations were carried out in aerobic liquid shake culture at 200 rpm, 25 °C and pH 7 with 1 g of substrate as a sole carbon source. Secondary alcohols accounted for less than 1% (w/w) of the total products and they were added to the corresponding methyl ketone concentration.

However, the results in Table 3.7 confirm that more decanoic acid was converted into the corresponding methyl ketone than any other fatty acid. The yield biomass was greatest for decanoic acid and the implication was that this acid was the most inhibitory to fungal growth.

Under these conditions there was no evidence for the conversion of simple triglycerides to methyl ketones other than those one carbon atom shorter than the parent acid. This suggests that the fatty acyl CoA undergoes partial β-oxidation and contradicts the results presented by Dartey and Kinsella who suggested that dodecanoic and tetradecanoic acids were converted into an homologous series of methyl ketones (Dartey and Kinsella 1973a,b).

The results in this section suggest that the conversion of short chain fatty acids into methyl ketones is a detoxification mechanism. Further studies were required to confirm that short carbon chain length fatty acids which were components of the lauric acid oils were fungistatic or fungicidal.

3.4 The toxicity of short chain fatty acids

The antimicrobial properties of fatty acids have been studied since the beginning of the century (Clarke 1899, Kiesel 1913). The earliest significant

investigation of the antifungal properties of fatty acids was carried out in 1945 (Wyss *et al* 1945). These authors demonstrated that fatty acids had fungistatic and fungicidal activity towards *Aspergillus niger*, *Trichophyton interdigitale* and *Trichophyton purpureum*. These early studies prompted further research with a view to using fatty acids as preservatives or chemotherapeutic agents (Rothman *et al* 1946, Chattaway and Thompson 1956, Kabara *et al* 1972, 1977, Hunkova and Fencl 1977, 1978, Das and Banerjee 1981). These authors determined toxicity as inhibition of growth or as a decrease in oxygen uptake.

Early studies indicated that the carbon chain length of a fatty acid was an important determinant in toxicity. Table 3.8 gives the different carbon chain lengths reported to show optimum antifungal activity. Further work revealed that several factors determined the effectiveness of fatty acids as inhibitors of fungal growth and metabolism. These can be summarised as follows:

1. The length of the carbon chain (Kiesel 1913, Wyss *et al* 1945 Rolinson 1954, Chattaway and Thompson 1956).

2. The pH of the media (Wyss *et al* 1945, Rolinson 1954, Chattaway and Thompson 1956, Lawrence and Hawke 1968, Lewis and Darnall 1970).

3. The species of mould (Wyss *et al* 1945).

4. The composition of the media (Hunkova and Fencl 1977).

5. The concentration of fatty acid (Wyss *et al* 1945, Rolinson 1954, Chattaway and Thompson 1956, Hunkova and Fencl 1977, 1978, Das and Banerjee 1981).

In addition Hunkova and Fencl divided the magnitude of effect of a fatty acid into four categories.

1. Subthreshold concentrations that stimulate respiration and oxygen uptake.

2. Threshold concentrations that inhibit growth but not the rate of substrate consumption.

3. Above-threshold concentrations that inhibit growth and substrate utilisation.

4. High concentrations with a microbicidal effect.

Several mechanisms for the antifungal activity of fatty acids have been proposed. An early theory was that fatty acid anions could bind to proteins which subsequently led to inhibition of key enzymes and cell death (Sampson *et al* 1955). Several groups did not

Table 3.8: Optimum carbon chain length for antifungal activity of short chain fatty acids.

Mould species	Optimum chain length
Penicillium chrysogenum [1]	C10
Aspergillus niger [2]	C11
Unknown cellulolytic mould [3]	C12
Trichophyton interdigitale [2]	C13
Microsporium canis [4]	C14

1 Rolinson 1954

2 Wyss *et al* 1945

3 Tetsumoto 1933

4 Chattaway and Thompson 1956

agree and suggested that acidification of cell contents was the process that inhibited oxygen uptake and mycelial growth. This was later disproved by groups who showed that free fatty acids had little effect on intracellular pH (Lewis and Darnall 1970, Hunkova and Fencl 1978). Fungal cells are generally resistant to changes in pH (see Section 4.2).

The effect of free fatty acids (C6 to C12) on the growth of *Penicillium crustosum* was studied in liquid shake culture at 25°C and pH 7 with sucrose as a sole carbon source. Figure 3.8 shows growth inhibition (%) against concentration for a 72 h fermentation. The minimum inhibitory concentrations (MICs) are given in Table 3.9. The order of toxicity is C11 > C10 > C12 > C8 > C6. This order was predicted in Section 3.2 using the yields (ketone per mg biomass) for coconut and palm kernel oil. Tetradecanoic acid was not inhibitory to *P. crustosum*. Longer carbon chains than C14 do not exhibit antifungal activity due to their insolubility in water (Wyss *et al* 1945). It is of interest that undeceneoate is used for treatment of dermatomycoses. Maximum toxicity was found for undecanoic acid.

The toxicity of octanoic acid towards *P. crustosum* isolated from hazelnuts (CMI 300381) was investigated. The dose response curves for both isolates are compared in Figure 3.9. The hazelnut isolate was more sensitive to this acid than *P. crustosum* isolated from dessiccated

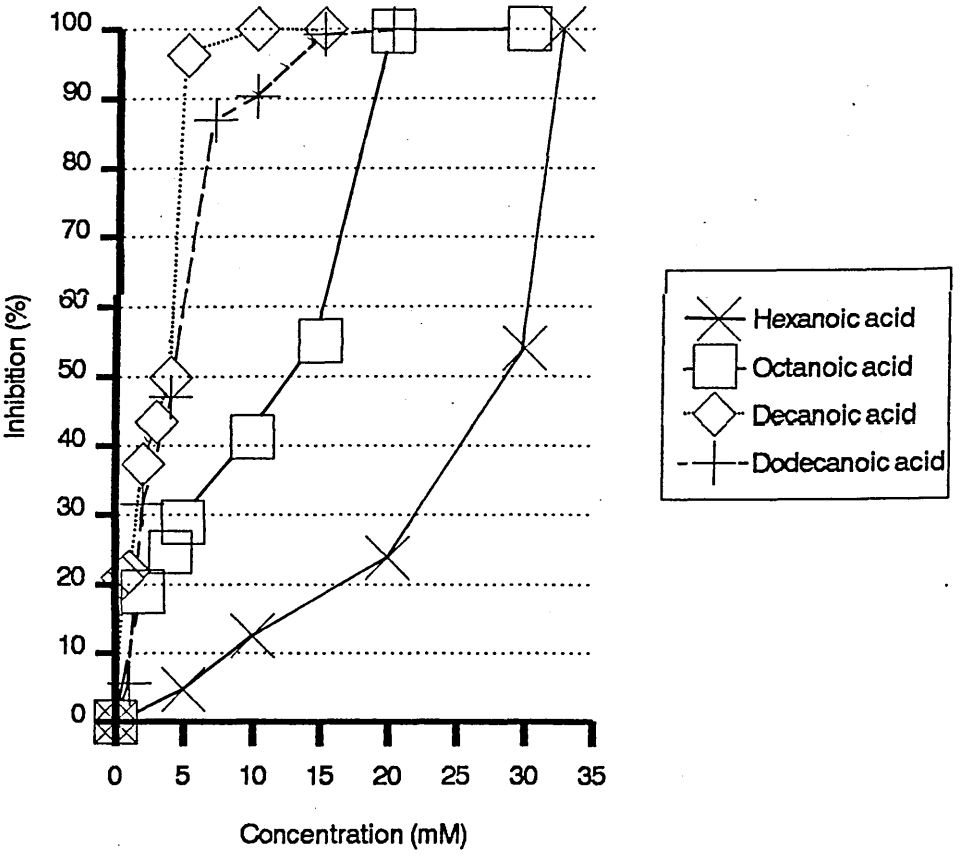


Figure 3.8 : Dose response curves for the inhibition of growth of Penicillium crustosum (CMI 281919) by short carbon chain length fatty acids. Growth inhibition was determined in aerobic liquid shake culture at 200 rpm, 25 °C and pH 7.0. Results are the arithmetic mean of three fermentations.

Table 3.9: Comparative sensitivity of Penicillium crustosum (CMI 281919) to free fatty acids.*

Free Fatty Acid	Minimum Inhibitory Concentration	
	($mmol.l^{-1}$)	($mg.Kg^{-1}$)
Hexanoic acid, C6:0	32.5	3776
Octanoic acid, C8:0	20.0	2884
Decanoic acid, C10:0	10.0	1723
Undecanoic acid, C11:0	4.7	880
Dodecanoic acid	15.3	3066

* Fermentations carried out in liquid shake culture at 200 rpm, 25 °C and pH 7 for 72 h with sucrose (3% w/v) as a sole carbon source.

coconut (CMI 281919). The MIC of octanoic acid towards the hazelnut isolate was 10.5 mmol per litre. The MIC was only 50% of that determined for the mould isolated from dessiccated coconut. Resistance to short chain fatty acids had developed in this isolate. The mould may have adapted to survive in coconut where the oil contains glycerides composed of short carbon chain length fatty acids.

Further evidence for the toxicity of short carbon chain length fatty acids was obtained from a study of the ultrastructure of *P. crustosum*. There are no reports of the effect of fatty acids on the ultrastructure of filamentous fungi. *Penicillium crustosum* was grown using sucrose, cocoa butter, palm kernel oil and single acid triglycerides as sole carbon sources. Plates 3.1 and 3.2 show typical hyphal cells after growth on sucrose. Normal organelles were also observed after growth on cocoa butter (Plates 3.3 and 3.4). These were not seen after growth on palm kernel oil. There was a loss of mitochondrial cristae and an increase in the electron density of the cytoplasm (Plate 3.5). Cocoa butter contains glycerides of long carbon chain fatty acids (> C16) whilst palm kernel oil contains short chain fatty acids (C8 to C14) (Table 3.1). *Penicillium crustosum* was grown on a range of simple triglycerides (C6:0, C8:0, C10:0, C12:0) to confirm that the short chain fatty acids were

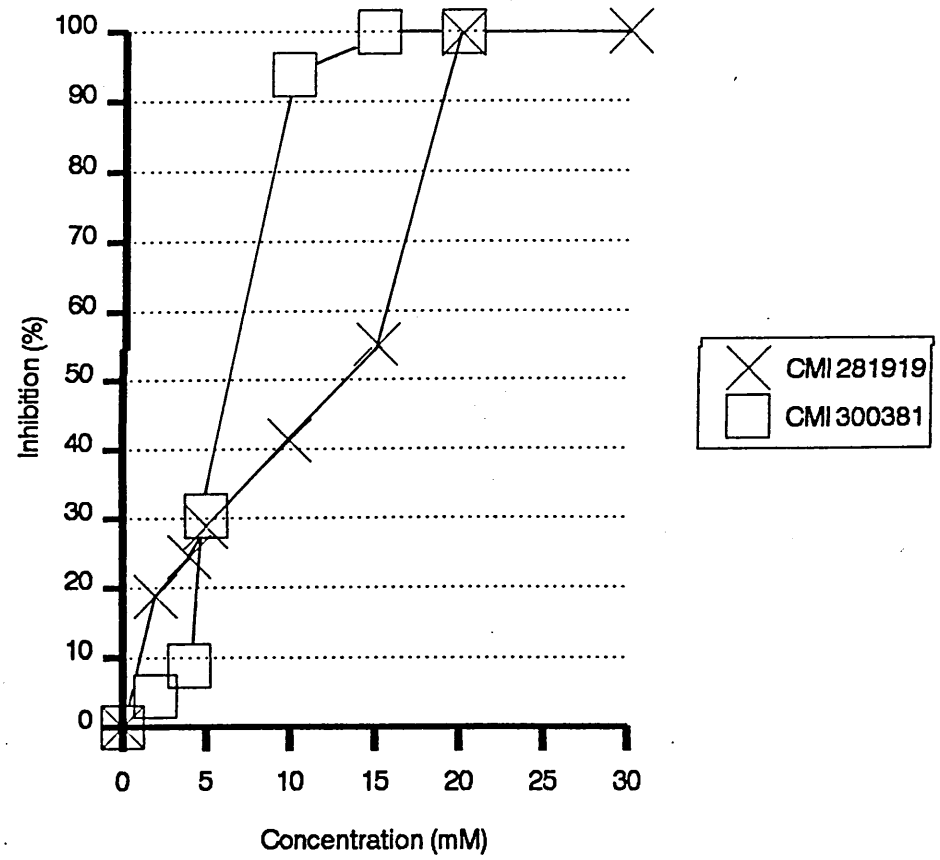


Figure 3.9 : Toxicity of octanoic acid towards two isolates of Penicillium crustosum. Growth inhibition was determined in aerobic liquid shake culture at 200 rpm, 25 °C and pH 7.0 with sucrose as a sole carbon source. Results are the arithmetic mean of three fermentations.

Key to Plates 3.1 to 3.6

C Cytoplasm

cm Cytoplasmic membrane

er Endoplasmic reticulum

M Mitochondrion

mb Microbody

N Nucleus

V Vacuole

vs Vesicle

W Cell wall

X Mitochondrion without cristae

(Beckett et al 1974.)

All scale bars represent 1.0 µm except where indicated.

Micrographs were taken by P. Hansbro as part of an undergraduate project.

Plate 3.I : Penicillium crustosum grown on sucrose as a sole carbon source.

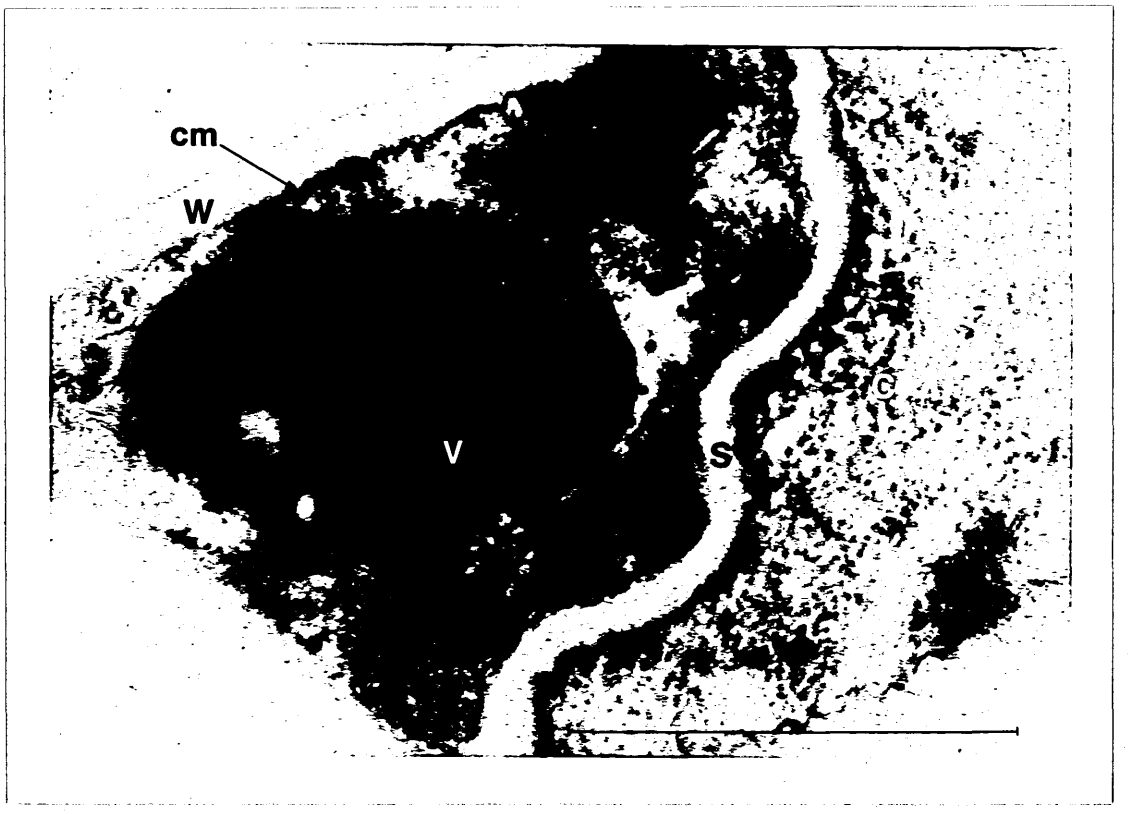


Plate 3.2 : Penicillium crustosum grown on sucrose as a sole carbon source.

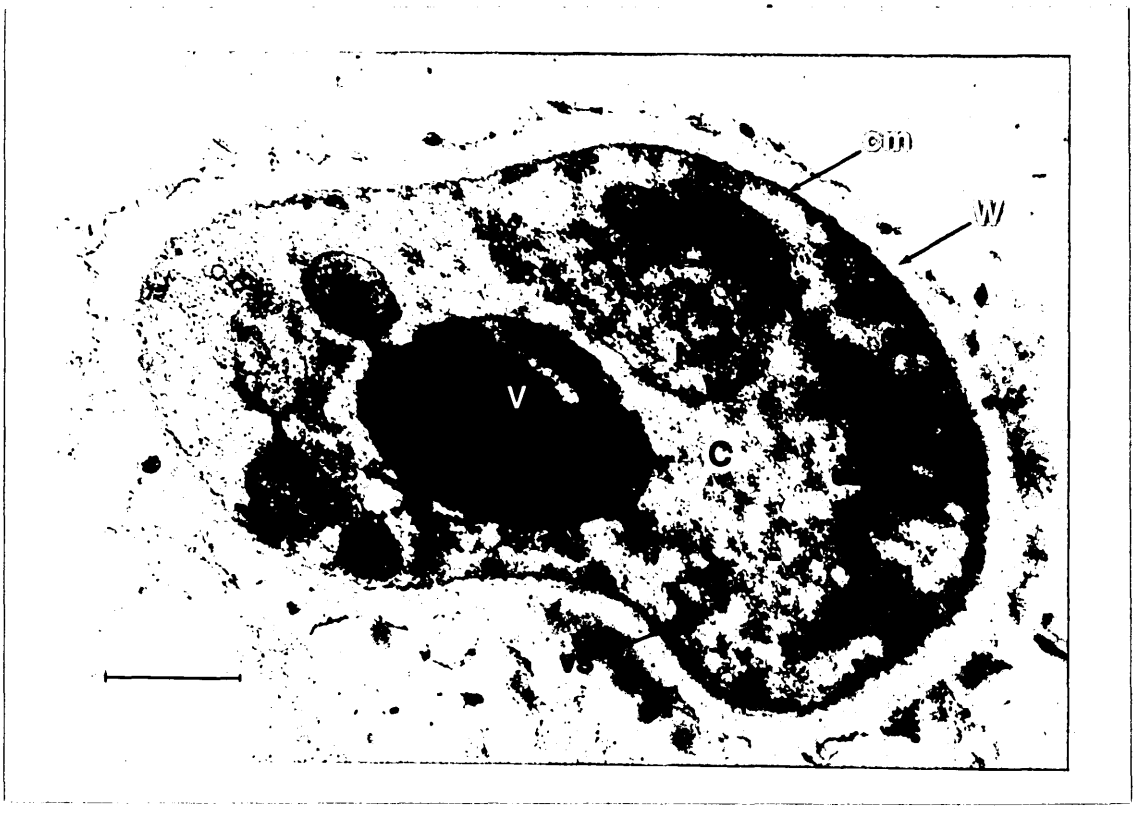


Plate 3.3 : Penicillium crustosum grown on cocoa butter as a sole carbon source.

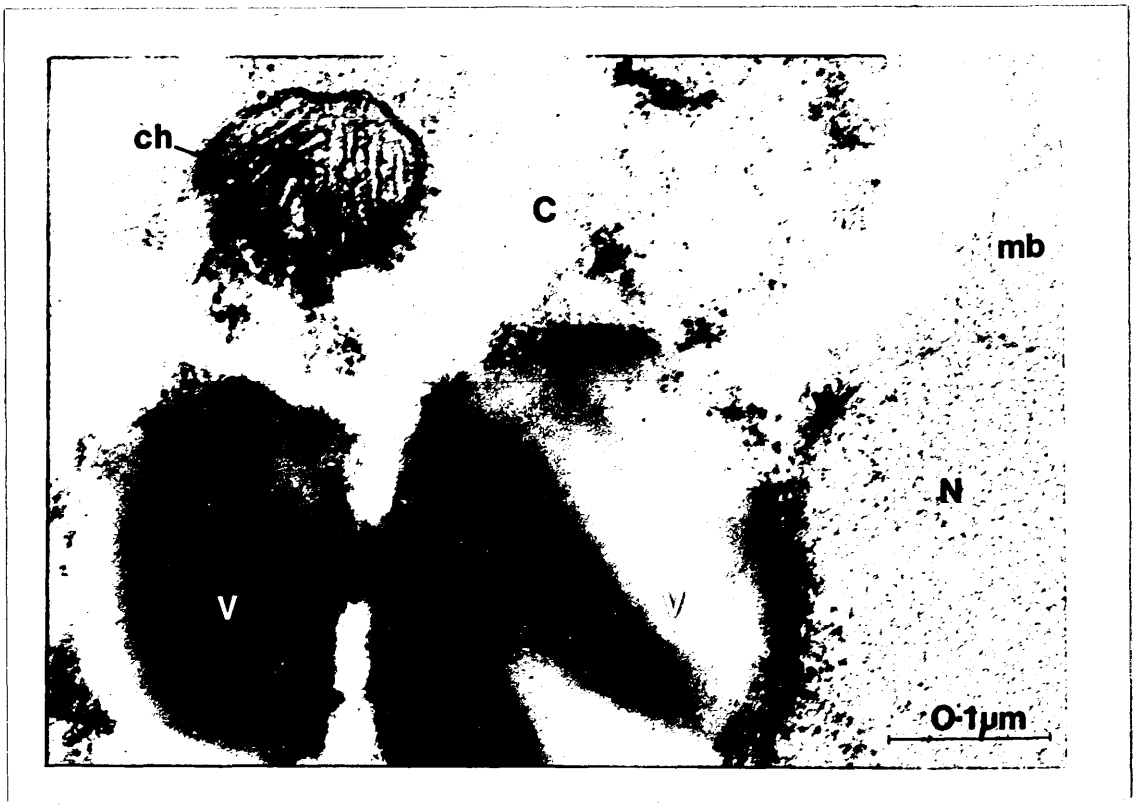


Plate 3.4 : Penicillium crustosum grown on cocoa butter as a sole carbon source.

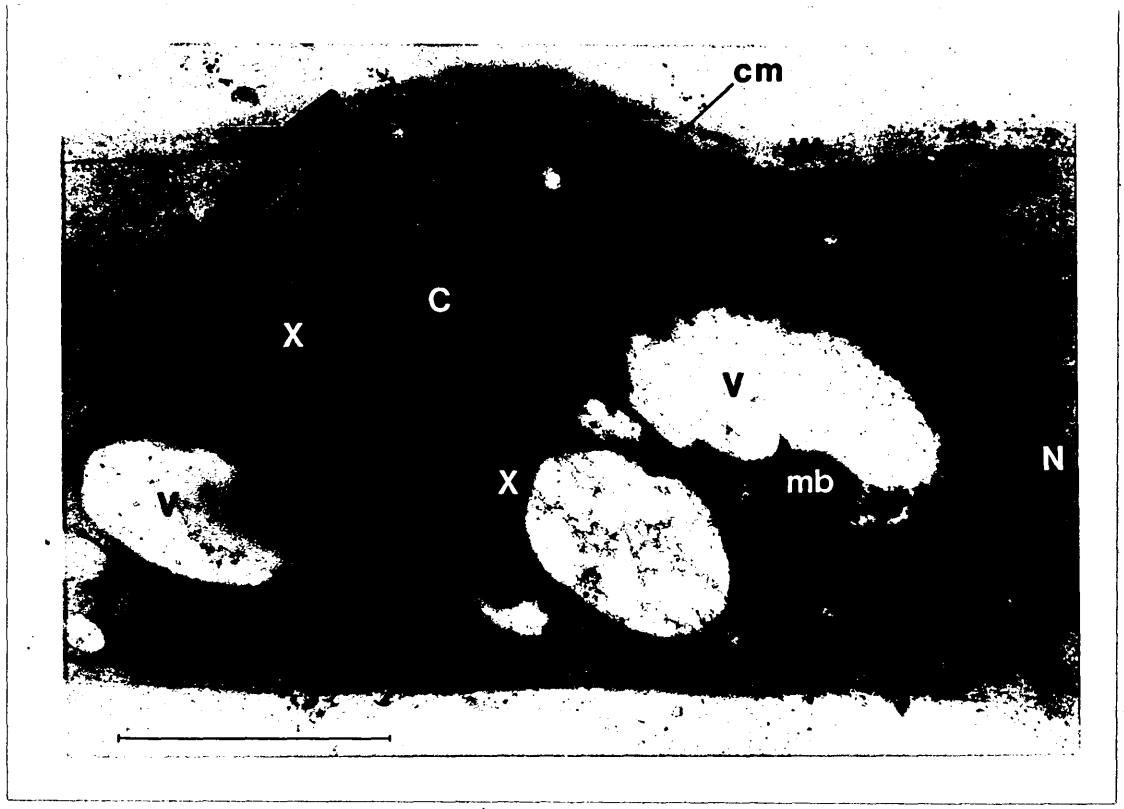


Plate 3.5 : Penicillium crustosum grown on palm kernel oil as a sole carbon source.

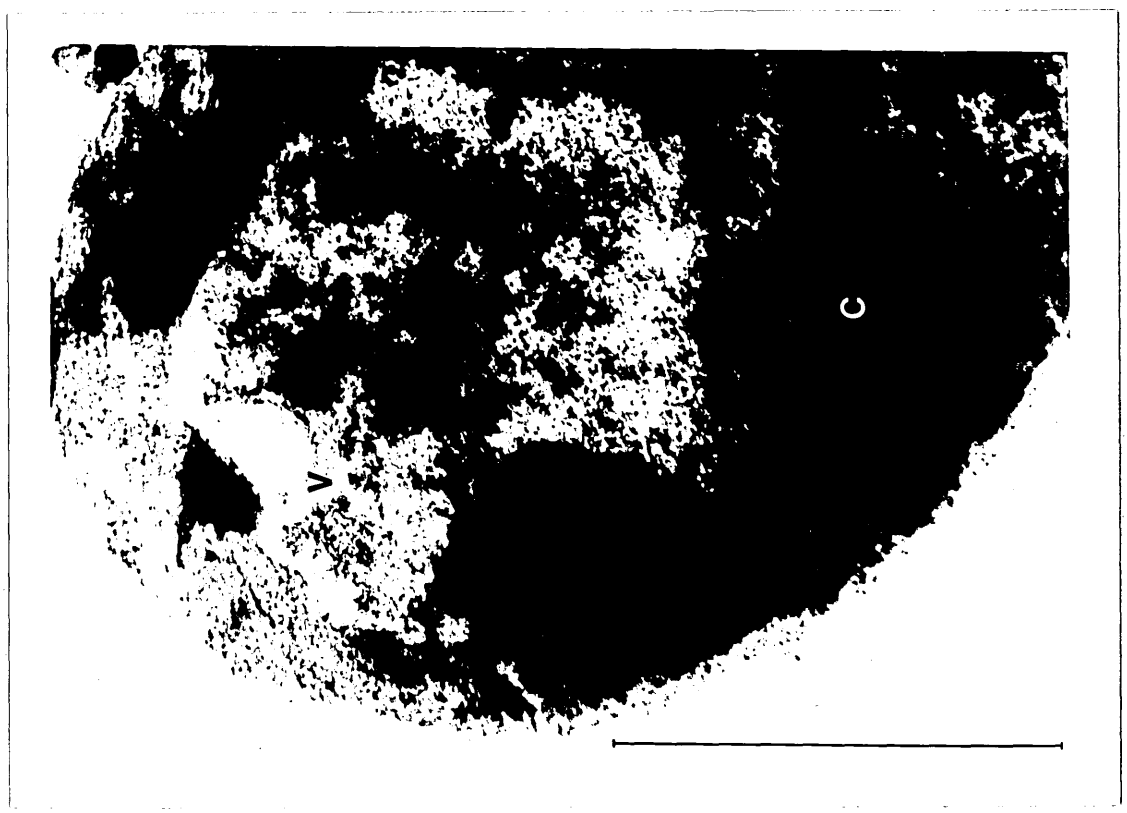


Plate 3.6 : Penicillium crustosum grown on glyceryl tridecanoate as a sole carbon source.

responsible for the changes in ultrastructure. Mycelial cells grown on these triglycerides contained a mass of electron dense cytoplasm and unidentifiable organelles within the cell wall (Plate 3.6). In the yeast *Candida albicans* short chain fatty acids have the same effect upon ultrastructure (Adams *et al* 1963). Only electron dense cytoplasm was observed after treatment of yeast cells with a sodium salt of hexanoic acid.

This study suggests that a key site of action for short chain fatty acids is the fungal mitochondrion. A recent theory suggests that short chain fatty acids act as uncouplers of oxidative phosphorylation in the mitochondria. Similar evidence for the uncoupling of oxidative phosphorylation was provided by groups studying isolated mammalian mitochondria (Scholefield 1956, Hird and Weidemann 1966). The mechanism of uncoupling was unknown. Later the development of the chemiosmotic theory helped to explain this activity (Mitchell 1972).

4. Results and Discussion - Control of ketonic rancidity in the lauric acid oils

4.1 The effect of temperature on fungal growth and ketonic rancidity

Extremes of temperature have been used for the preservation of foods for hundreds of years. Temperature control has become a critical factor in ensuring a safe food supply. It is the most important environmental factor affecting the growth and survival of micro-organisms (ICMSF 1980). Poor control of temperature during storage can allow bacteria or fungi to grow. Fungi differ in their temperature optima. Some fungi can survive in extremely cold environments, although growth at low temperatures is minimal. Most penicillia are regarded as psychrotrophs with an optimum growth temperature of between 25 and 30°C. *P. crustosum* has an optimum temperature for growth of 23°C and is able to grow at 4°C (Ayerst 1969).

The effect of temperature on ketonic rancidity has not been studied. However, the effect of temperature on the maturation of blue cheeses by *P. roquefortii* has been investigated (Dartey and Kinsella 1973a,b). The optimum temperature for the conversion of free octanoic

acid into 2-heptanone was 25°C (Lawrence 1966). The low temperatures (10°C) used for cheese ripening may limit the rate of ketone formation and affect the proportions of ketones produced (Hawke 1966).

4.1.1 The effect of temperature on production of biomass after fermentation of the lauric acid oils

Penicillium crustosum was grown in liquid shake culture at 4, 10, 20, 25, 30 and 37°C (Section 2.5.3). Table 4.1 gives the results obtained when coconut oil was used as a sole carbon source while Table 4.2 gives the results obtained from palm kernel oil.

The effect of temperature on biomass production is summarised in Figure 4.1. The optimum temperature for growth on both substrates lay between 20 and 25°C. Growth was detected within 72 h over a temperature range of 10 to 30°C. Considerably more growth has taken place where coconut oil is the substrate. This phenomenon was discussed in Section 3.2. The resistance of palm kernel oil to fungal attack may partly account for its being produced in greater quantities than coconut oil (Ashby, personal communication).

Table 4.1: The effect of temperature on the conversion of coconut oil into methyl ketones by Penicillium crustosum in aerobic shake culture at 200 rpm and pH 7 for 72 h.*

Temp (°C)	Isolate CMI 281919				Isolate CMI 300381			
	Biomass (mg dry wt)	Product - Total Ketones (mmol)	Molar Conversion (%)	Yield $\frac{\text{biomass}}{(\mu\text{mol mg}^{-1})}$	Biomass (mg dry wt)	Product - Total Ketones (mmol)	Molar Conversion (%)	Yield $\frac{\text{biomass}}{(\mu\text{mol mg}^{-1})}$
4	0	0	-	-	0	0	-	-
10	0.6±0.2	0.057±0.055	1.5±1.4	90.5±87.3	2.6±0.7	0.008±0.000	0.2±0.0	3.1±0.0
20	30.6±2.7	0.106±0.012	2.7±0.3	3.5±0.4	28.0±1.3	0.198±0.015	5.0±0.4	7.1±0.5
25	120.7±49.6	0.839±0.110	21.4±2.8	6.9±0.9	76.2±40.5	0.758±0.074	19.3±1.9	9.9±1.0
30	12.7±3.2	0.354±0.038	9.0±1.0	27.8±3.0	29.5±3.4	0.504±0.034	12.8±0.9	17.1±1.2
37	0	0	-	-	0	0	-	-

* Results are the arithmetic mean of four fermentations and eight analyses (±S.D.). Fermentations were carried out with 1 g of coconut oil as a sole carbon source. Secondary alcohols accounted for less than 1% (w/w) of the total products and they were added to the corresponding methyl ketone concentration.

Table 4.2: The effect of temperature on the conversion of palm kernel oil into methyl ketones by Penicillium crustosum in aerobic shake culture at 200 rpm and pH 7 for 72 h.*

Temp (°C)	Isolate CMI 281919				Isolate CMI 300381			
	Biomass (mg dry wt)	Product - Total Ketones (mmol)	Molar Conversion (%)	Yield ^{biomass} (μmol mg^{-1})	Biomass (mg dry wt)	Product - Total Ketones (mmol)	Molar Conversion (%)	Yield ^{biomass} (μmol mg^{-1})
4	0	0	-	-	0	0	-	-
10	1.0±1.0	0.005±0.001	0.2±0.0	5.0±1.0	2.2±0.2	0.001±0.000	0.0±0.0	0.5±0.0
20	32.3±7.5	0.076±0.027	2.6±0.9	2.4±0.8	39.2±6.7	0.146±0.030	5.0±1.0	3.7±0.8
25	30.5±7.7	0.180±0.136	6.2±4.7	5.9±4.5	30.9±3.5	0.405±0.130	13.9±4.5	13.1±4.2
30	10.8±2.6	0.195±0.027	6.7±0.9	18.1±2.5	10.9±0.9	0.320±0.014	11.0±0.5	29.4±1.3
37	0	0	-	-	0	0	-	-

* Results are the arithmetic mean of four fermentations and eight analyses (±S.D.). Fermentations were carried out with 1 g of palm kernel oil as a sole carbon source. Secondary alcohols accounted for less than 1% (w/w) of the total products and they were added to the corresponding methyl ketone concentration.

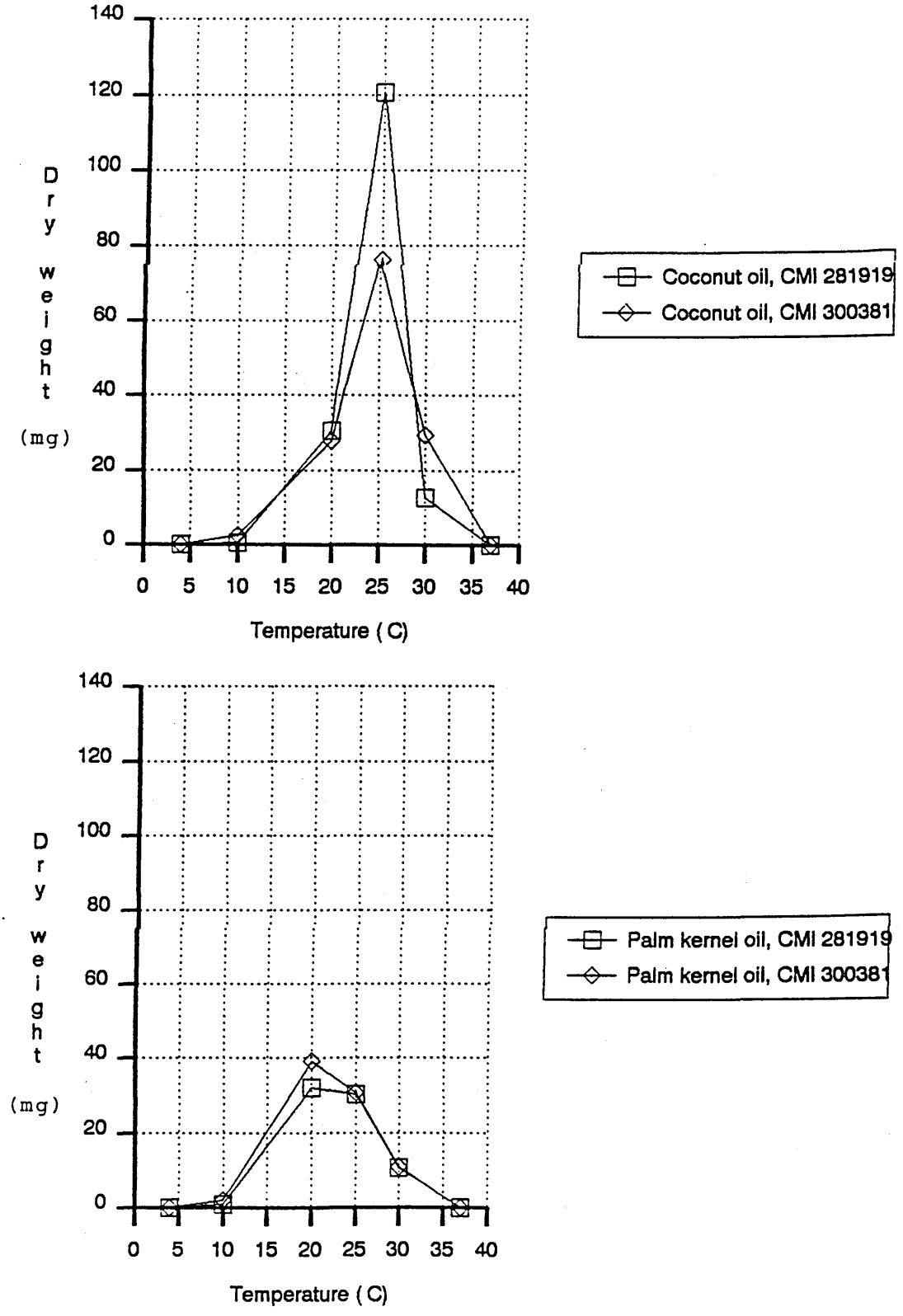
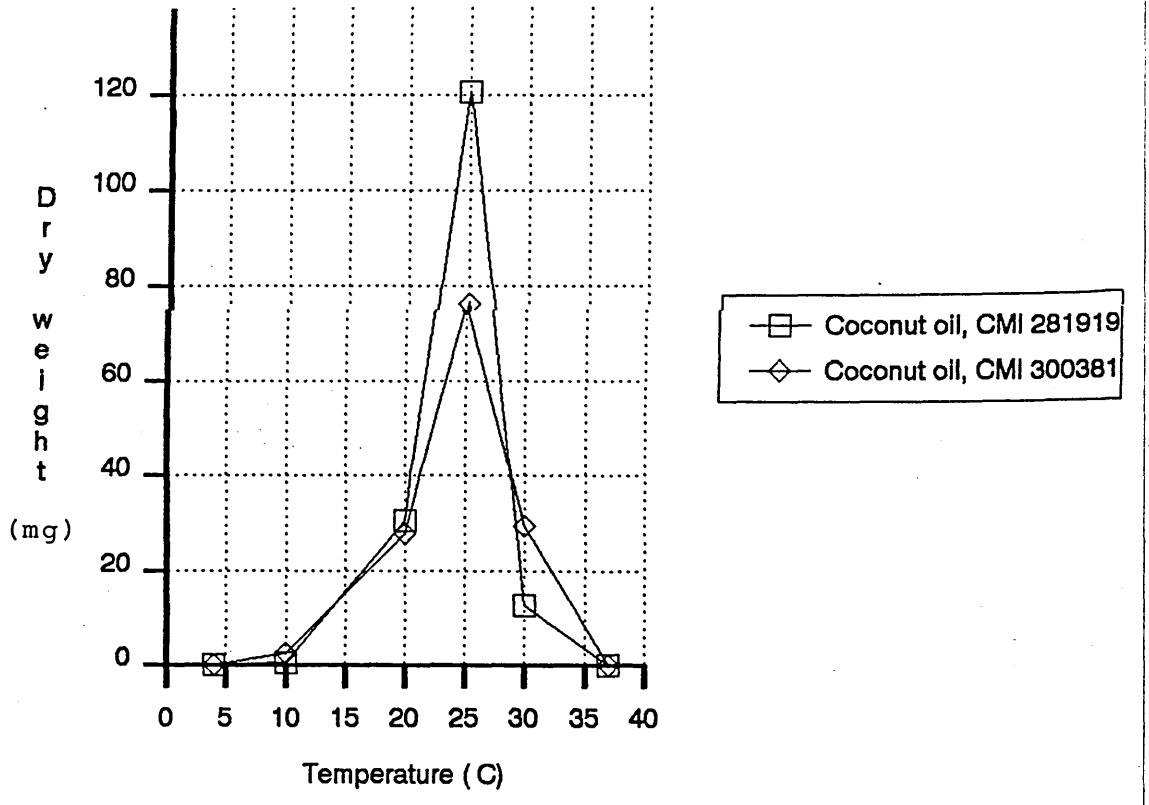


Figure 4.1 : The effect of temperature on production of biomass by Penicillium crustosum using coconut or palm kernel oils as sole carbon sources. Fermentations carried out in aerobic shake culture at 200 rpm and pH 7.0. Results are the arithmetic mean of four flasks (see Tables 4.1 and 4.2 for S.D.'s).



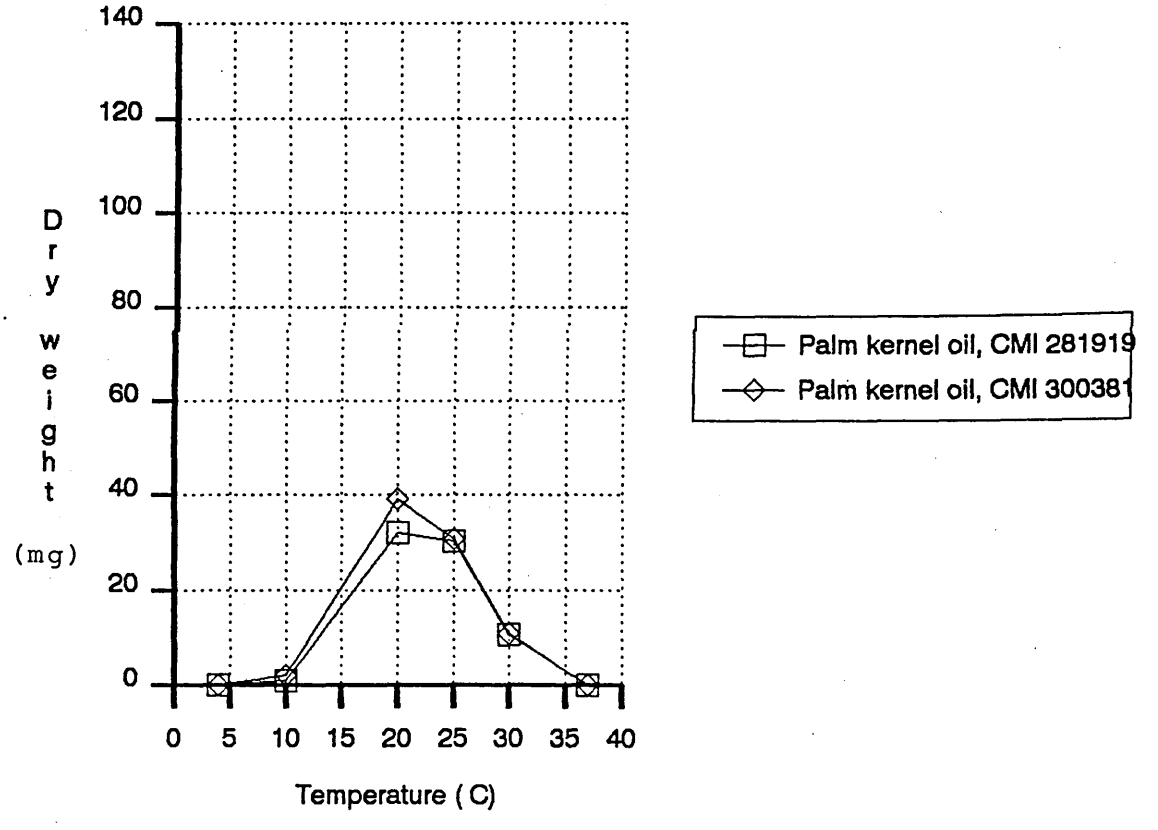


Figure 4.1 : The effect of temperature on production of biomass by Penicillium crustosum using coconut or palm kernel oils as sole carbon sources. Fermentations carried out in aerobic shake culture at 200 rpm and pH 7.0. Results are the arithmetic mean of four flasks (see Tables 4.1 and 4.2 for S.D.'s).

No growth occurred below 4°C and this appears to be a suitable temperature at which to store the lauric acid oils to avoid spoilage.

4.1.2 The effect of temperature on the conversion of the lauric acid oils into methyl ketones

The effect of temperature on the production of methyl ketones is given by Figure 4.2. Methyl ketones were not detected at temperatures where no growth occurred (4 and 37°C). This provided further evidence that it was mycelia and not spores that were responsible for the conversion of the lauric acid oils into methyl ketones.

The optimum temperature for production of 2-heptanone, 2-nonanone and 2-undecanone was 25°C. However, the optimum temperature for the production of 2-pentanone from coconut oil was 10° C as 2-pentanone was the most volatile product and could be lost by evaporation at higher temperatures. 2-Pentanone was not produced from palm kernel oil as hexanoic acid was absent from the substrate (Section 3.2).

The major product detected at each temperature after a 72 h fermentation was 2-undecanone. This reflected the fatty acid composition of the lauric acid oils where dodecanoic acid represented over 50%

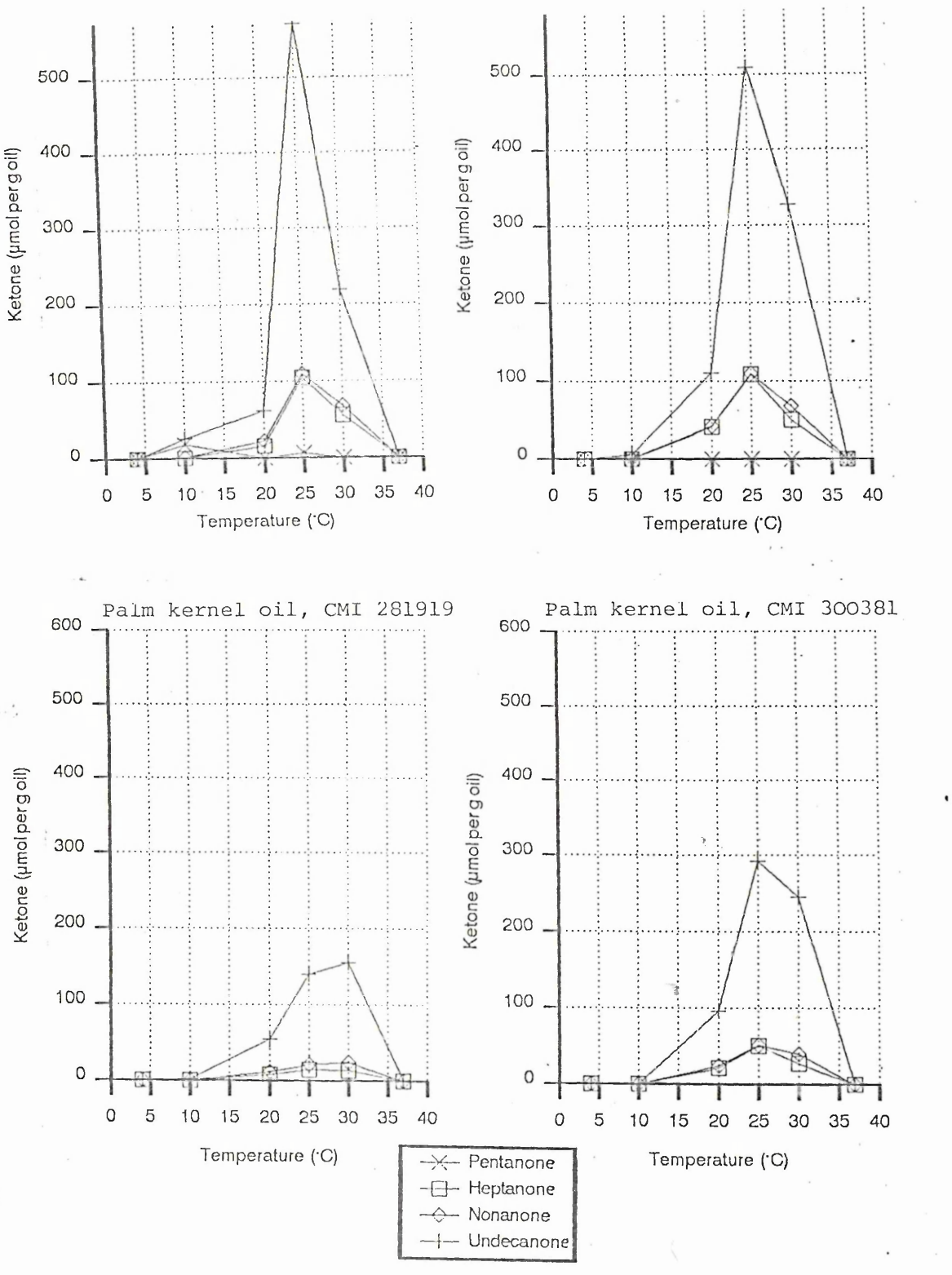


Figure 4.2 : The effect of temperature on the conversion of the lauric acid oils into methyl ketones by Penicillium crustosum. Fermentations carried out in aerobic shake culture at 200 rpm and pH 7.0 for 72 h with 1 g of oil as a sole carbon source. See Tables 3.2 and 3.3 for S.D.'s. Results are the arithmetic mean of eight analyses.

(w/w) of the acids (Table 3.1). It is of interest that the optimum temperatures for biomass and ketone production for both oils is the same, notably 25°C. More ketones were produced from coconut oil than palm kernel oil (Tables 4.1 and 4.2), by the fatty acid composition of the substrates. Coconut oil contained the greater proportion of short and intermediate carbon chain length fatty acids (C6 to C14).

4.1.3 Model fermentation experiments with simple triglycerides

Sections 3.1 to 3.2 cover fermentations utilizing a mixed substrate. From Section 3.3 the energy value of the simple glycerides increases with an increase in the hydrocarbon moiety of the fatty acid. In order to determine if ketone production depended on chain length of the fatty acid or solubility of the substrate in fat or water, fermentations with a range of simple triglycerides were undertaken.

The first reaction in the conversion of triglyceride into methyl ketone involves release of the free fatty acids. Figure 1.4 demonstrates that there is a marked decrease in the solubility in water as the chain length of the fatty acid increases. The decrease in water solubility is accompanied by an

increase in the slip point. Whilst glyceryl trihexanoate, glyceryl trioctanoate and glyceryl tridecanoate are liquid at 25°C, glyceryl tridodecanoate is a solid at this temperature.

The results of experiments using simple triglycerides are given in Tables 4.3 to 4.6. The effect of temperature on biomass production by *P. crustosum* using these carbon sources is shown in Figure 4.3. Table 4.7 summarises the optimum growth temperatures on each triglyceride. Optimum temperature for growth increases with increasing carbon chain of the component fatty acid. No growth was detected at 4 or 37°C on any simple triglyceride.

The effect of temperature on the conversion of simple triglycerides into methyl ketones is shown in Figure 4.4. The optimum temperature for conversion of glyceryl trihexanoate and glyceryl trioctanoate was either 20 or 25°C. Glyceryl tridecanoate was converted into 2-nonanone at an optimum temperature of 25°C. No 2-nonanone was detected at 4 and 10°C, while at 20°C only 0.1 to 0.2% of the total decanoic acid was converted into 2-nonanone (Table 4.5). The optimum temperature for the conversion of glyceryl tridodecanoate into 2-undecanone was from 25 to 30°C. Less 2-undecanone (< 0.5 mmol) was produced using glyceryl tridodecanoate as a substrate than

Table 4.3: The effect of temperature on the conversion of glyceryl trihexanoate into 2-pentanone by *Penicillium crustosum*.*

	Isolate CMI 281919				Isolate CMI 300381			
Temp (°C)	Biomass (mg dry wt)	Product - 2-Pentanone (mmol)	Molar Conversion (%)	Yield biomass (μmol mg^{-1})	Biomass (mg dry wt)	Product - 2-Pentanone (mmol)	Molar Conversion (%)	Yield biomass (μmol mg^{-1})
4	0	ND	0	0	0	ND	0	0
10	3.0±0.1	0.003±0.001	0.0±0.0	0.8±0.6	0.5±0.0	ND	0	0
20	44.8±8.5	0.489±0.074	6.3±1.0	11.4±3.7	23.5±1.0	0.364±0.099	4.7±1.3	15.6±4.4
25	36.2±3.1	0.531±0.054	6.8±0.7	14.7±1.7	23.5±6.2	0.475±0.076	6.1±1.0	21.3±6.2
30	7.1±0.9	0.038±0.008	0.5±0.1	5.3±0.8	9.4±1.8	0.058±0.016	0.7±0.2	6.1±1.4
37	0	ND	0	0	0	ND	0	0

* Fermentations carried out in aerobic liquid shake culture at 200 rpm and pH 7 for 72 h. Results are the arithmetic mean of four fermentations and eight analyses (±S.D.).

Table 4.4: The effect of temperature on the conversion of glyceryl trioctanoate into 2-heptanone by Penicillium crustosum.*

Temp (°C)	Isolate CMI 281919				Isolate CMI 300381			
	Biomass (mg dry wt)	Product - 2-Heptanone (mmol)	Molar Conversion (%)	Yield ^{biomass} (μmol mg^{-1})	Biomass (mg dry wt)	Product - 2-Heptanone (mmol)	Molar Conversion (%)	Yield ^{biomass} (μmol mg^{-1})
4	0	ND	0	0	0	ND	0	0
10	4.1±0.5	0.011±0.001	0.2±0.0	2.6±0.3	2.8±0.6	0.005±0.003	0.1±0.1	2.0±1.2
20	30.2±3.6	0.468±0.039	7.5±0.6	15.9±2.8	17.2±3.5	0.327±0.028	5.2±0.4	21.4±6.9
25	86.7±8.2	0.322±0.035	5.1±0.6	3.8±0.7	72.4±18.4	0.278±0.054	4.4±0.9	4.1±1.5
30	19.1±7.7	0.086±0.039	1.4±0.6	5.5±0.4	17.0±1.8	0.078±0.015	1.2±0.2	4.6±0.7
37	0	ND	0	0	0	ND	0	0

* Fermentations carried out in aerobic liquid shake culture at 200 rpm and pH 7 for 72 h. Results are the arithmetic mean of four fermentations and eight analyses (±S.D.).

Table 4.5: The effect of temperature on the conversion of glyceryl tridecanoate into 2-nonanone by Penicillium crustosum.*

Temp (°C)	Isolate CMI 281919				Isolate CMI 300381			
	Biomass (mg dry wt)	Product - 2-Nonanone (mmol)	Molar Conversion (%)	Yield ^{biomass} (μmol mg^{-1})	Biomass (mg dry wt)	Product - 2-Nonanone (mmol)	Molar Conversion (%)	Yield ^{biomass} (μmol mg^{-1})
4	0	ND	0	0	0	ND	0	0
10	0	ND	0	0	0	ND	0	0
20	7.9±4.3	0.003±0.000	0.1±0.0	0.5±0.3	7.5±3.0	0.010±0.000	0.2±0.0	1.7±1.0
25	20.2±7.6	0.432±0.361	8.5±7.1	19.2±12.3	14.0±1.4	0.860±0.139	16.9±2.7	60.2±5.5
30	3.2±2.2	0.031±0.014	0.6±0.3	11.1±4.3	16.6±4.7	0.200±0.106	3.9±2.1	12.0±3.9
37	0	ND	0	0	0	ND	0	0

* Fermentations carried out in aerobic liquid shake culture at 200 rpm and pH 7 for 72 h. Results are the arithmetic mean of four fermentations and eight analyses (±S.D.).

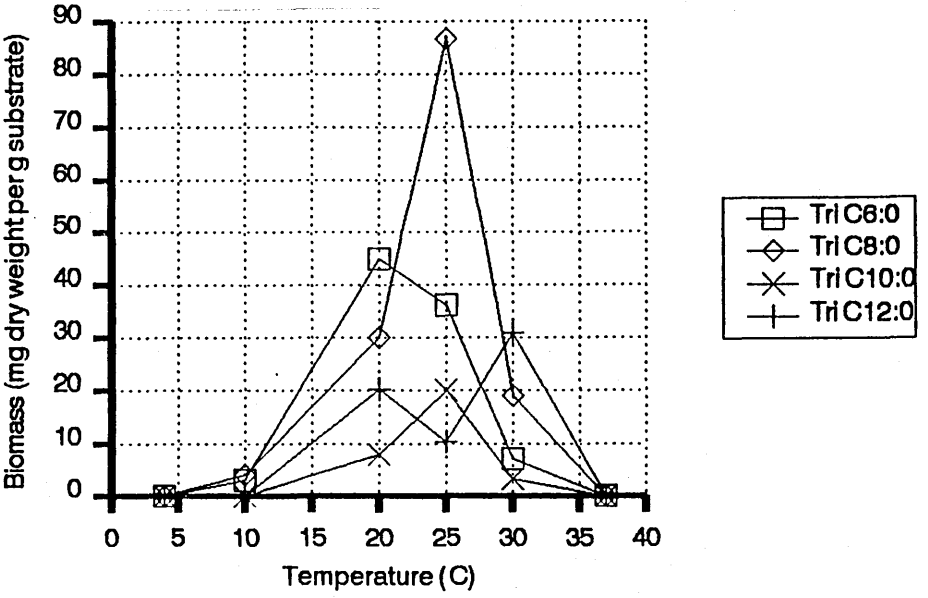
Table 4.6: The effect of temperature on the conversion of glyceryl tridodecanoate into 2-undecananone by Penicillium crustosum.*

Temp (°C)	Isolate CMI 281919				Isolate CMI 300381			
	Biomass (mg dry wt)	Product - 2-Undecanone (mmol)	Molar Conversion (%)	Yield ^{biomass} (μmol mg^{-1})	Biomass (mg dry wt)	Product - 2-Undecanone (mmol)	Molar Conversion (%)	Yield ^{biomass} (μmol mg^{-1})
4	0	ND	0	0	0	ND	0	0
10	0	ND	0	0	0	ND	0	0
20	20.0±3.3	0.005±0.004	0.0±0.0	0.3±0.2	12.2±6.6	0.030±0.015	0.7±0.4	2.5±0.5
25	10.3±5.4	0.022±0.012	0.5±0.3	2.4±1.0	5.9±2.4	0.040±0.009	0.9±0.2	7.6±1.1
30	30.9±18.1	0.035±0.013	0.8±0.3	1.5±1.5	41.7±4.8	0.015±0.004	0.3±0.1	0.4±0.1
37	0	ND	0	0	0	ND	0	0

* Fermentations carried out in aerobic liquid shake culture at 200 rpm and pH 7 for 72 h. Results are the arithmetic mean of four fermentations and eight analyses (±S.D.).

Table 4.7: Optimum temperatures for growth of Penicillium crustosum on simple triglycerides.

Sole carbon source	Growth optima (°C)	
	CMI 281919	CMI 300381
Glyceryl trihexanoate	20	20
Glyceryl trioctanoate	25	25
Glyceryl tridecanoate	25	30
Glyceryl tridodecanoate	30	30



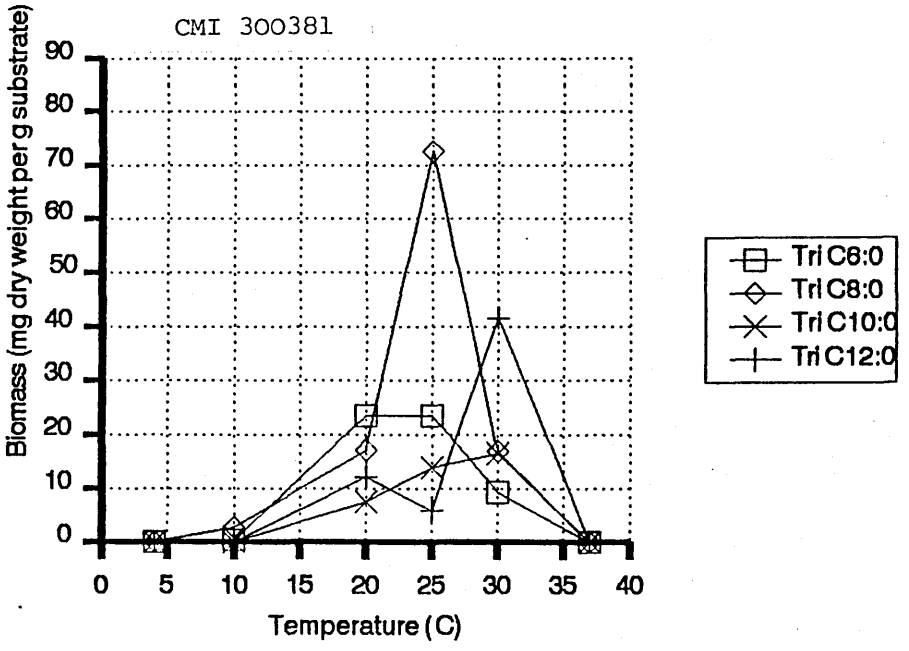
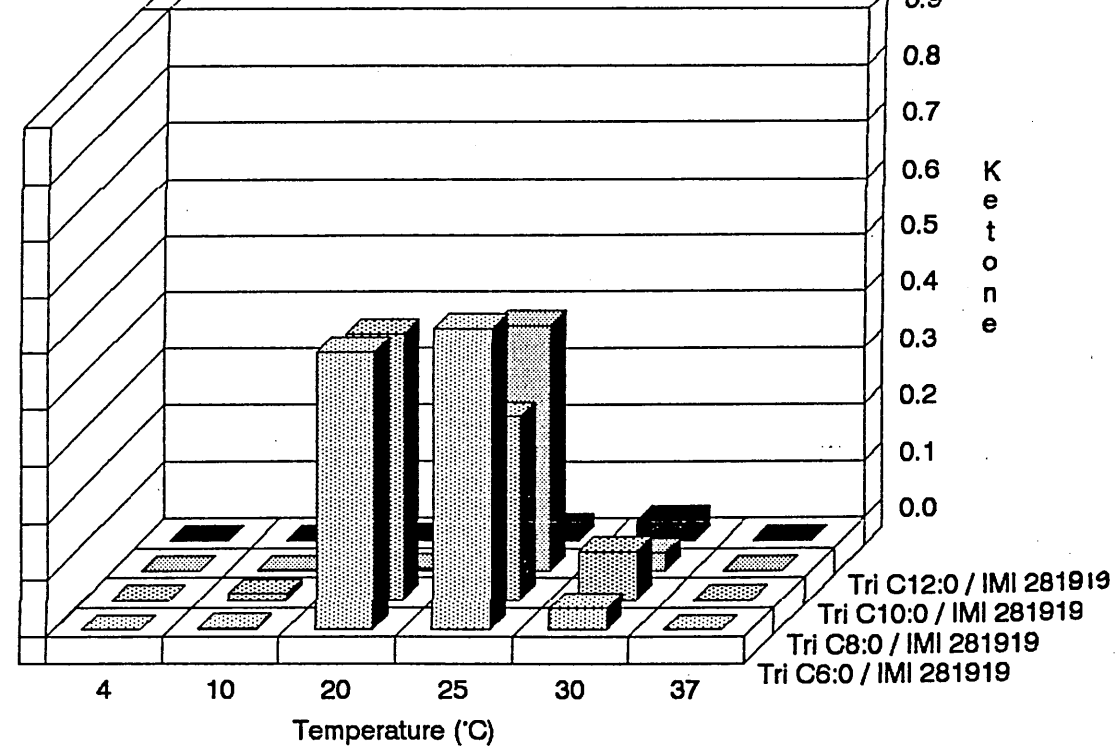


Figure 4.3 : The effect of temperature on production of biomass from simple triglycerides by Penicillium crustosum in aerobic shake culture at 200 rpm, 25 C and pH 7.0. Results are the arithmetic mean of four fermentations (see Tables 4.3 to 4.6 for S.D.'s).



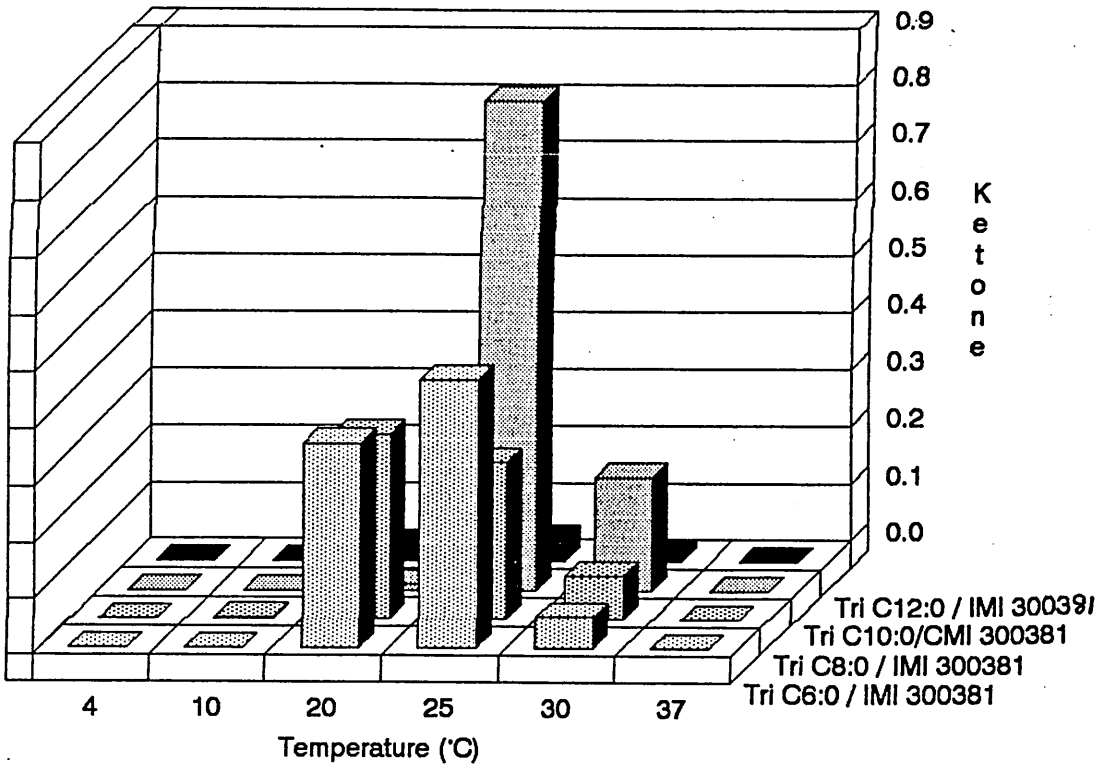


Figure 4.4 : The effect of temperature on the conversion of simple triglycerides into methyl ketones by Penicillium crustosum. Results are the arithmetic mean of eight analyses (see Tables 4.3 to 4.6 for S.D.

when the lauric acid oils were fermented. It appears that dodecanoic acid is more reactive when it is esterified in a mixed glyceride system. It is possible that the insolubility of glyceryl tridodecanoate in water and its high melting point (44.2°C) could explain the low conversion as well as the slow growth rate on this substrate.

It can be seen that the slip points of the simple triglycerides differ. The results given in Tables 4.3 to 4.6 confirm that molecular weight is an important factor in determining the susceptibility of a simple triglyceride to ketonic rancidity. Molecular weight of the simple triglyceride determines if the substrate is solid or liquid at the fermentation temperature. Growth rates were greater on substrates that were liquid at the fermentation temperature. No growth was detected at 4 or 10°C on glyceryl tridecanoate or glyceryl tridodecanoate after a 72 h fermentation. Only at 30°C did the growth rate on glyceryl tridodecanoate exceed that on the other substrates.

Methyl ketones were not detected when there was no fungal growth. This is further evidence that fungal spores are unable to convert triglycerides into methyl ketones and it is the vegetative mycelium that is active in this system. The greatest yield biomass (μmol product per mg dry weight) was

achieved at 25°C for all substrates except glyceryl trioctanoate (Tables 4.3 to 4.6). The greatest yield biomass for glyceryl trioctanoate (15.9 and 21.4 μmol product per mg dry weight) was found at 20°C. This suggests that the substrates other than glyceryl trioctanoate are at their most toxic at the optimum growth temperature for this fungus.

4.2 The effect of pH on the conversion of synthetic triglycerides into methyl ketones

The pH of a solution is a measure of the concentation of protons. The pH is defined as:

$$pH = -\log_{10}[H^+] \text{ or } pH = \log_{10}\frac{1}{[H^+]}$$

The ionisation equilibrium of a weak acid is given by:

$$HA \rightleftharpoons H^+ + A^-$$

The apparent equilibrium constant for this ionisation is:

$$K = \frac{[H^+][A^-]}{[HA]}$$

The pK of an acid is defined as:

$$pK = -\log K \text{ or } pK = \frac{1}{K}$$

The pK of an acid is the pH at which it is half-dissociated and the concentration of protons is equal to the acid concentration.

The pH of a medium or food is one of several factors which affect the growth of an organism in that

environment. By increasing the acidity of a food it is possible to enhance its microbiological stability (ICMSF 1980). This principle has been employed in the preservation of foods such as yogurt and pickles.

Simple triglycerides were used as model substrates to study the effect of initial pH on fungal growth and the conversion of short carbon chain length fatty acids into methyl ketones. The change in pH after fermentation of glyceryl trihexanoate and glyceryl trioctanoate at 25°C and 200 rpm in aerobic liquid shake culture is shown in Figure 4.5. It is known that fungi can alter the pH of their environment during growth (Smith and Berry 1975). This homeostatic mechanism has undoubtedly contributed to ΔpH and growth. Ketone production took place between pH 4.0 and 7.7. Buffered Czapek medium was most stable at pH 7.0 (Figure 4.5). This buffer system was used in the growth medium in all subsequent experiments.

The production of fungal biomass and methyl ketones after fermentation of glyceryl trihexanoate and glyceryl trioctanoate is given in Table 4.8. Rapid growth caused a considerable pH shift so that it was difficult to establish an optimum pH for the conversion of simple triglycerides into methyl ketones.

Hexanoic acid was converted into 2-pentanone and octanoic acid into 2-heptanone. There was no evidence

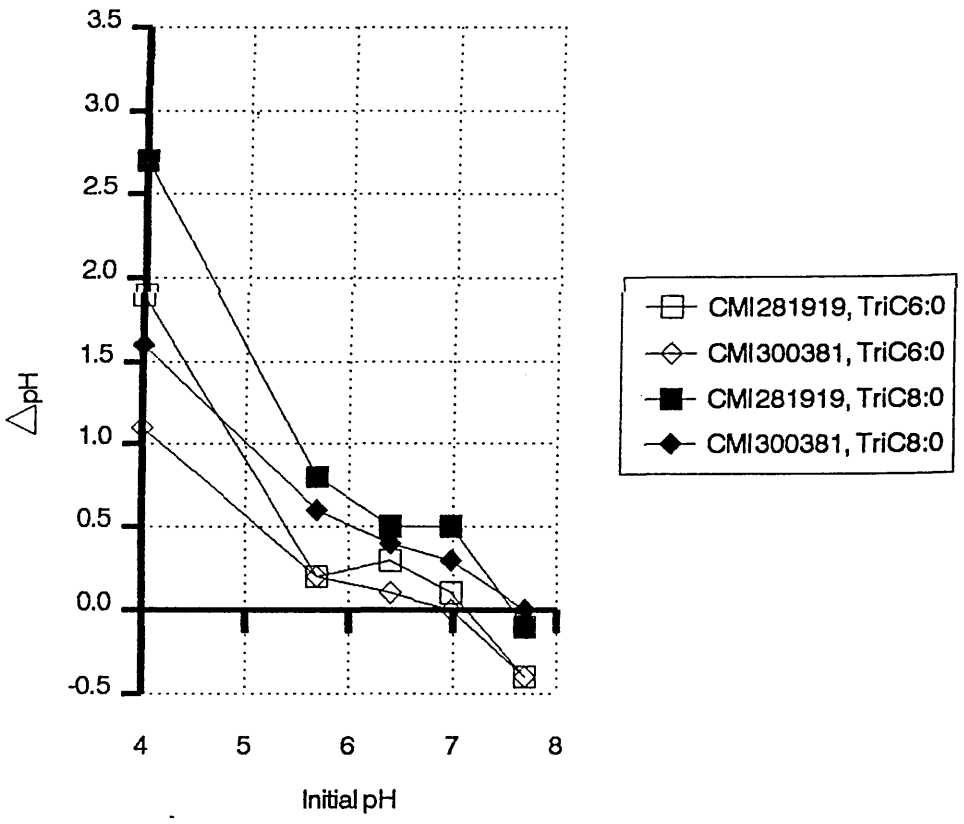


Figure 4.5 : Change in pH after growth of Penicillium crustosum in liquid shake culture with a 0.05 M phosphate buffer. Fermentations were conducted with 1 g of substrate at 25 C for 72 h. Results are the arithmetic mean of four fermentations.

Table 4.8: The effect of initial pH on the conversion of glyceryl trihexanoate into 2-pentanone and glyceryl trioctanoate into 2-heptanone by two isolates of Penicillium crustosum.*

Glyceryl trihexanoate

Isolate CMI 281919			Isolate CMI 300381		
Initial pH	Mycelial dry weight (mg)	2-pentanone (mmoles)	Initial pH	Mycelial dry weight (mg)	2-pentanone (mmoles)
4.0	28.3±9.8	0.396±0.069	3.7	2.9±1.0	0.098±0.016
5.7	19.0±4.7	0.621±0.088	5.7	18.4±2.9	0.383±0.109
6.4	52.3±11.6	0.705±0.093	6.4	17.6±5.4	0.485±0.095
6.9	36.2±3.1	0.531±0.054	6.9	23.5±6.2	0.475±0.076
7.7	17.1±2.1	0.157±0.054	7.7	8.3±1.7	0.064±0.022

Glyceryl trioctanoate

Isolate CMI 281919			Isolate CMI 300381		
Initial pH	Mycelial dry weight (mg)	2-pentanone (mmoles)	Initial pH	Mycelial dry weight (mg)	2-pentanone (mmoles)
4.0	117.5±8.6	0.323±0.216	4.0	68.5±8.2	0.277±0.012
5.8	82.0±23.8	0.409±0.137	5.8	52.3±7.4	0.302±0.099
6.3	65.3±28.1	0.433±0.101	6.3	44.6±13.9	0.377±0.088
6.9	86.7±8.2	0.322±0.035	6.9	72.4±18.4	0.278±0.054
7.7	0.4±0.2	0.029±0.002	7.7	23.7±4.6	0.371±0.047

* Results are the arithmetic mean of four fermentations and eight analyses (±S.D.). Fermentations carried out at 200 rpm and 25 °C with 1 g of triglyceride as a sole carbon source.

Table 4.8: The effect of initial pH on the conversion of glyceryl trihexanoate into 2-pentanone and glyceryl trioctanoate into 2-heptanone by two isolates of Penicillium crustosum.*

Glyceryl trihexanoate

Isolate CMI 281919			Isolate CMI 300381		
Initial pH	Mycelial dry weight (mg)	2-pentanone (mmoles)	Initial pH	Mycelial dry weight (mg)	2-pentanone (mmoles)
4.0	28.3±9.8	0.396±0.069	3.7	2.9±1.0	0.098±0.016
5.7	19.0±4.7	0.621±0.088	5.7	18.4±2.9	0.383±0.109
6.4	52.3±11.6	0.705±0.093	6.4	17.6±5.4	0.485±0.095
6.9	36.2±3.1	0.531±0.054	6.9	23.5±6.2	0.475±0.076
7.7	17.1±2.1	0.157±0.054	7.7	8.3±1.7	0.064±0.022

Glyceryl trioctanoate

Isolate CMI 281919			Isolate CMI 300381		
Initial pH	Mycelial dry weight (mg)	2-pentanone (mmoles)	Initial pH	Mycelial dry weight (mg)	2-pentanone (mmoles)
4.0	117.5±8.6	0.323±0.216	4.0	68.5±8.2	0.277±0.012
5.8	82.0±23.8	0.409±0.137	5.8	52.3±7.4	0.302±0.099
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7.7	0.4±0.2	0.029±0.002	7.7	23.7±4.6	0.371±0.047

* Results are the arithmetic mean of four fermentations and eight analyses (±S.D.). Fermentations carried out at 200 rpm and 25 °C with 1 g of triglyceride as a sole carbon source.

for the conversion of simple triglycerides into methyl ketones other than those with one carbon atom less than the parent fatty acid.

4.3 The effect of water activity on fungal growth and ketonic rancidity

Water is required for the growth of fungi as it is for other organisms. In addition, fungi require a film of water around the cells through which nutrients and enzymes can diffuse. Fungal growth can be prevented by removing water from the substrate. In the food industry water available for microbial growth is determined as water activity (a_w). This is defined as the ratio of the vapour pressure of water in the substrate to that of pure water at the same temperature and pressure.

$$a_w = \frac{p}{po}$$

a_w = water activity

p = water vapour pressure of substrate

po = water vapour pressure of pure water

It may be expressed as a percentage and it is then called equilibrium relative humidity (ERH). Available water may also be measured as osmotic potential. Osmotic potential is related to a_w by the expression

$$\Psi, \text{ Osmotic potential} = \frac{-RT \log_e a_w}{V}$$

where R is the universal gas constant, T the absolute temperature and V the partial molar volume of water. The food industry prefers to use a_w as a measure of available water and it has been retained in this section (Caurie 1983)..

The a_w of a food may be reduced by increasing the concentration of solutes in the aqueous phase. This is achieved by either removing free water (dehydration) or adding solutes. Both techniques are used to prevent the growth of micro-organisms in food commodities. Foods are not susceptible to microbial degradation if the a_w is below 0.6 (ICMSF 1980). The fungi that grow in the range 0.65 to 0.85 are termed xerophiles (Pitt 1975). Xerophilic moulds accumulate polyols inside the hyphae which maintain an osmotic balance with the external environment. These fungi are a serious problem in the food industry because of their ability to grow at low a_w. A recent example of this problem is the growth of *Chrysosporium farinocola* on bars of Fry's *Turkish Delight*. This industrial problem cost the Cadbury company in excess of £2.0 million (Kinderlerer, unpublished).

The minimum a_w at which conidia and ascospores germinate and grow has been widely researched (Ayerst 1969, Brown 1974, 1976, Pitt 1981). However, there are relatively few references in the literature to the

chemistry of spoilage due to growth of xerophilic fungi. The effect of a_w on ketonic rancidity has not been studied.

The effect of water activity on the conversion of the lauric acid oils into methyl ketones was studied in liquid shake culture at 25°C and pH 7.0. The water activity of the medium was adjusted by adding solutes (sodium chloride or erythritol). Sodium chloride is used in foods as a flavouring and preservative. Salting processes are applied predominantly to meat, fish and some vegetables (ICMSF 1980). Erythritol was a polyol which accumulated when *Chrysosporium xerophilum* was grown at reduced water activity (Phillips-Jones, unpublished). Erythritol was not used as a carbon source by *P. crustosum*. For this reason it was selected as an osmotic regulator.

The effect of sodium chloride on the growth of *P. crustosum* on coconut oil as a sole carbon source is given in Table 4.9. The growth rate of the mould was reduced by increasing the concentration of sodium chloride in the medium. The conversion of coconut oil into methyl ketones was also reduced. However, the amount of ketone produced per unit biomass ($yield^{biomass}$) was greatest at a salt concentration of 10% (w/v). The dose response curve for sodium chloride is shown in Figure 4.6. Sodium chloride completely inhibited mould growth when added to medium at a

Table 4.9: The effect of sodium chloride on the conversion of coconut oil into methyl ketones by *Penicillium crustosum*.*

[Sodium chloride] (% w/v)	Isolate CMI 281919				Isolate CMI 300381			
	Biomass (mg dry wt)	Product - Total Ketones (mmol)	Molar Conversion (%)	Yield ^{biomass} (μmol mg^{-1})	Biomass (mg dry wt)	Product - Total Ketones (mmol)	Molar Conversion (%)	Yield ^{biomass} (μmol mg^{-1})
0	120.7±49.6	0.839±0.110	21.4±2.8	6.9±0.9	76.2±40.5	0.758±0.074	19.3±1.9	9.9±1.0
2.5	58.7±5.7	0.503±0.203	12.8±5.2	8.6±3.5	69.9±10.1	0.541±0.043	13.7±1.1	7.7±0.6
5	49.0±6.1	0.343±0.070	8.7±1.8	7.0±1.4	47.3±3.3	0.508±0.049	12.9±1.2	10.7±1.0
10	4.4±2.3	0.072±0.014	1.8±0.4	16.4±3.2	13.7±3.0	0.303±0.042	7.7±1.1	22.1±3.1
15	0	ND	0	0	0	ND	0	0

* Fermentations carried out in liquid shake culture at 200 rpm and pH 7 for 72 h. Results are the arithmetic mean of four fermentations and eight analyses (±S.D.).

Table 4.10: The effect of sodium chloride on the conversion of palm kernel oil into methyl ketones by *Penicillium crustosum*.*

[Sodium chloride] (% w/v)	Isolate CMI 281919				Isolate CMI 300381			
	Biomass (mg dry wt)	Product - Total Ketones (mmol)	Molar Conversion (%)	Yield biomass (μmol mg^{-1})	Biomass (mg dry wt)	Product - Total Ketones (mmol)	Molar Conversion (%)	Yield biomass (μmol mg^{-1})
0	30.5±7.7	0.180±0.136	6.2±4.7	5.9±4.5	30.9±3.5	0.405±0.130	13.9±4.5	13.1±4.2
2.5	31.0±8.5	0.219±0.211	7.5±7.2	7.1±6.8	28.8±2.4	0.259±0.026	8.9±0.9	9.0±0.9
5	15.0±4.0	0.035±0.018	1.2±0.6	2.3±1.2	22.2±1.4	0.106±0.049	3.6±1.7	4.8±2.2
10	2.7±1.3	0.050±0.011	1.7±0.4	18.5±4.1	5.0±1.4	0.078±0.035	2.7±1.2	15.6±7.0
15	0	ND	0	0	0	ND	0	0

* Fermentations carried out in liquid shake culture at 200 rpm and pH 7 for 72 h. Results are the arithmetic mean of four fermentations and eight analyses (±S.D.).

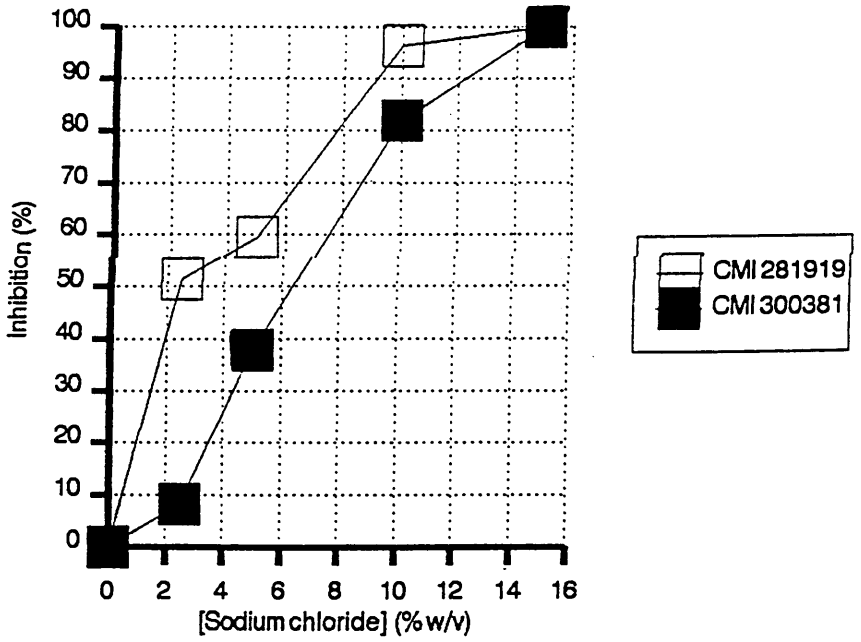


Figure 4.6 : Dose response curve for the inhibition of growth of Pénicillium crustosum by NaCl with coconut oil as a sole carbon source. Results are the arithmetic mean of four fermentations.

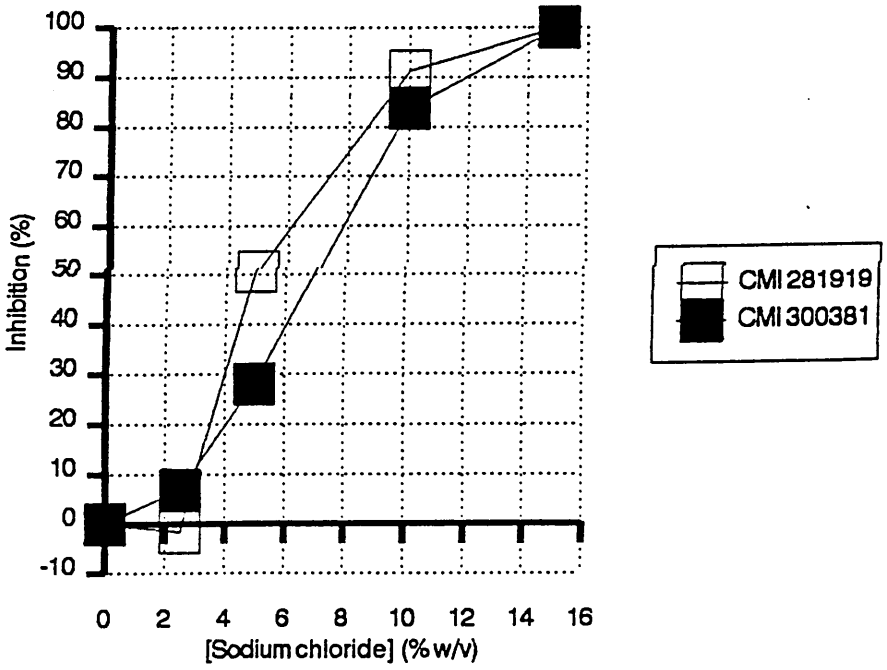


Figure 4.7 : Dose response curve for the inhibition of growth of Penicillium crustosum by NaCl with palm kernel oil as a sole carbon source. Results are the arithmetic mean of four fermentations.

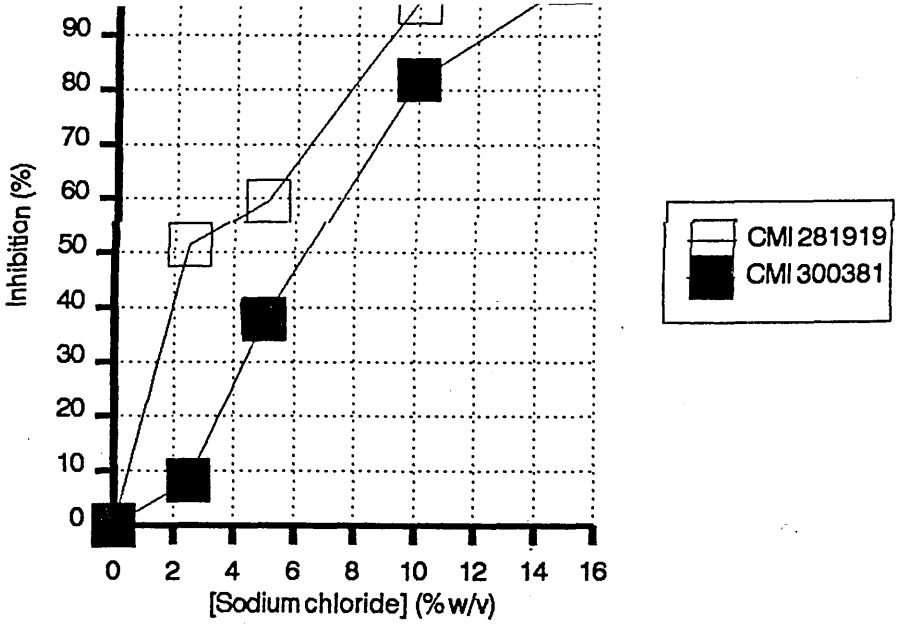


Figure 4.6 : Dose response curve for the inhibition of growth of Pénicillium crustosum by NaCl with coconut oil as a sole carbon source. Results are the arithmetic mean of four fermentations.

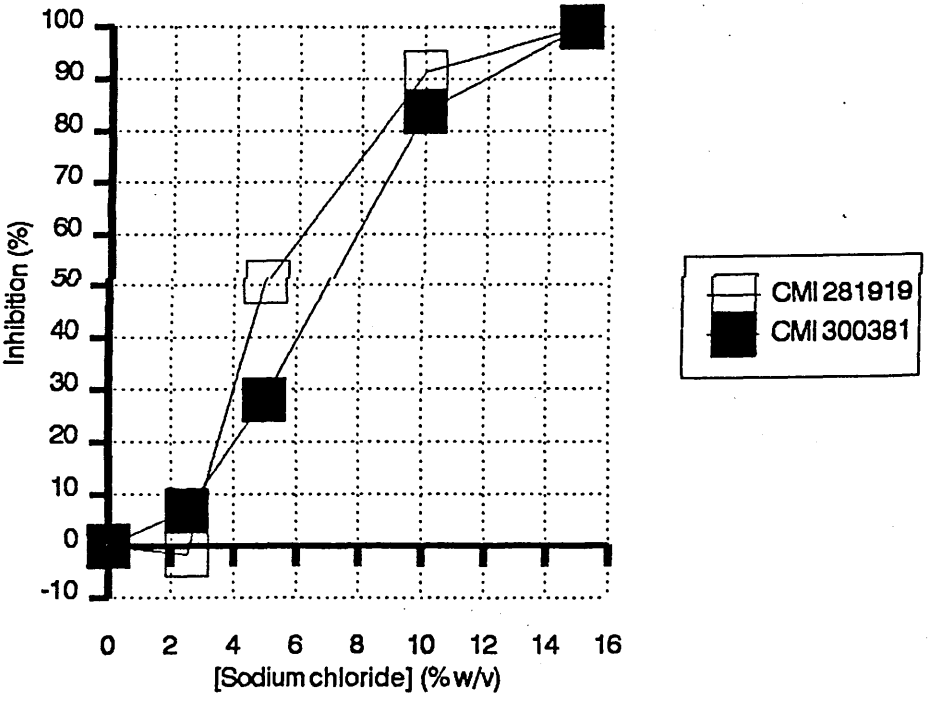


Figure 4.7 : Dose response curve for the inhibition of growth of Penicillium crustosum by NaCl with palm kernel oil as a sole carbon source. Results are the arithmetic mean of four fermentations.

concentration of 15% (w/v). At this concentration the medium had an a_w of 0.91.

In an experiment using palm kernel oil as a sole carbon source a similar pattern was found (Table 4.10). Production of biomass and methyl ketones were inhibited by increasing the concentration of solute. Maximum yield ketone biomass was found at 10% sodium choride concentration. When the mould was stressed by low a_w (0.94) it converted short carbon chain length fatty acids into methyl ketones rather than biomass. The dose response curves in Figure 4.7 show that growth was completely inhibited by a sodium chloride concentration of 15% (w/v). In this respect there was no difference between the two oils.

Table 4.11 gives the effect of erythritol on the growth of *P. crustosum*. The results showed increasing the concentration of erythritol produced a fall in growth rate and ketone production. When palm kernel oil was used as a carbon source the results were similar but maximum growth took place in the presence of 10% (w/v) erythritol. The dose response curves for erythritol are given in Figures 4.8 and 4.9. No growth was detected on either oil at an a_w of 0.92 (erythritol = 40% w/v).

The effect of water activity on the production of biomass by *P. crustosum* is summarised in Figure 4.10. Where the a_w was between 0.90 and 0.95 there was little difference between growth on the two oils. Here growth

Table 4.11: The effect of erythritol on the conversion of coconut oil into methyl ketones by *Penicillium crustosum*.*

[Erythritol] (% w/v)	Isolate CMI 281919				Isolate CMI 300381			
	Biomass (mg dry wt)	Product - Total Ketones (mmol)	Molar Conversion (%)	Yield $\frac{\text{product}}{\text{biomass}}$ (μmol mg^{-1})	Biomass (mg dry wt)	Product - Total Ketones (mmol)	Molar Conversion (%)	Yield $\frac{\text{product}}{\text{biomass}}$ (μmol mg^{-1})
0	120.7±49.6	0.839±0.110	21.4±2.8	6.9±0.9	76.2±40.5	0.758±0.074	19.3±1.9	9.9±1.0
10	98.3±7.5	0.456±0.041	11.6±1.0	4.6±0.4	87.2±9.3	0.409±0.031	10.4±0.8	4.7±0.4
20	58.3±22.1	0.502±0.090	12.8±2.3	8.6±1.5	2.6±1.3	0.059±0.033	2.3±0.8	22.7±12.7
30	14.7±0.5	0.170±0.006	4.3±0.2	11.6±0.4	2.9±1.3	0.015±0.007	0.4±0.2	5.2±2.4
40	0	ND	0	0	0	ND	0	0

* Results are the arithmetic mean of four fermentations and eight analyses (±S.D.). Fermentations carried out in aerobic liquid shake culture at 200 rpm and pH 7 for 72 h.

Table 4.12: The effect of erythritol on the conversion of palm kernel oil into methyl ketones by Penicillium crustosum.*

[Erythritol] (% w/v)	Isolate CMI 281919				Isolate CMI 300381			
	Biomass (mg dry wt)	Product - Total Ketones (mmol)	Molar Conversion (%)	Yield ^{biomass} (μmol mg^{-1})	Biomass (mg dry wt)	Product - Total Ketones (mmol)	Molar Conversion (%)	Yield ^{biomass} (μmol mg^{-1})
0	30.5±7.7	0.180±0.136	6.2±4.7	5.9±4.5	30.9±3.5	0.405±0.130	13.9±4.5	13.1±4.2
10	53.2±23.0	0.402±0.207	13.8±7.1	7.6±3.9	51.4±29.3	0.505±0.126	17.3±4.3	9.8±2.5
20	16.5±10.9	0.203±0.126	7.0±4.3	12.3±7.6	20.2±8.1	0.249±0.111	8.6±3.8	12.3±5.5
30	0.7±0.2	0.018±0.003	0.6±0.0	25.7±4.3	2.6±0.5	0.051±0.015	1.8±0.5	19.6±5.8
40	0	ND	0	0	0	ND	0	0

* Results are the arithmetic mean of four fermentations and eight analyses (±S.D.). Fermentations carried out in aerobic liquid shake culture at 200 rpm and pH 7 for 72 h.

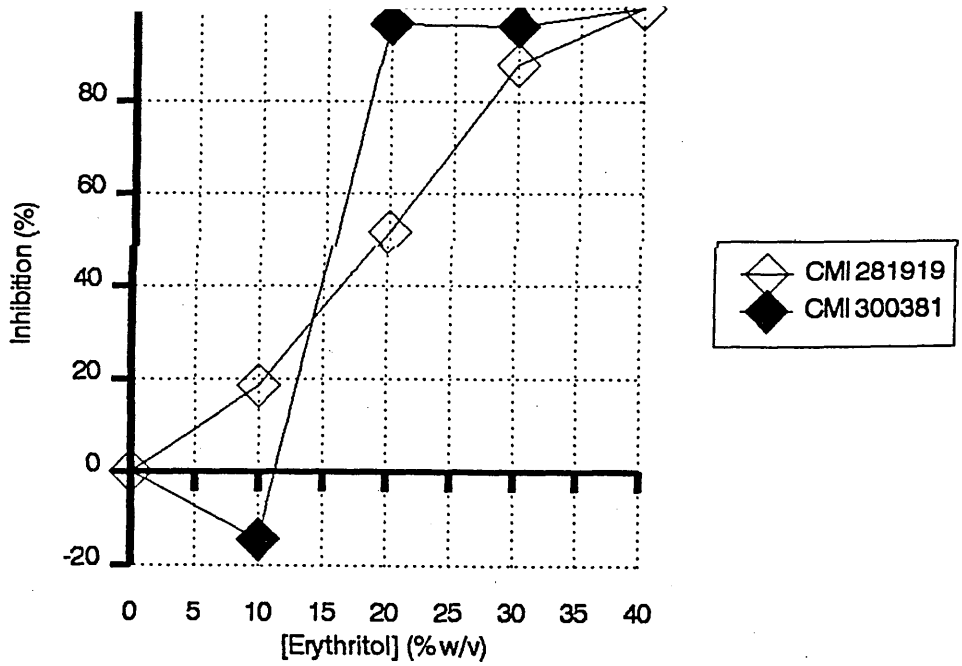


Figure 4.8 : Dose response curve for the inhibition of growth of Penicillium crustosum by erythritol with coconut oil as a sole carbon source. Results are the arithmetic mean of four fermentations.

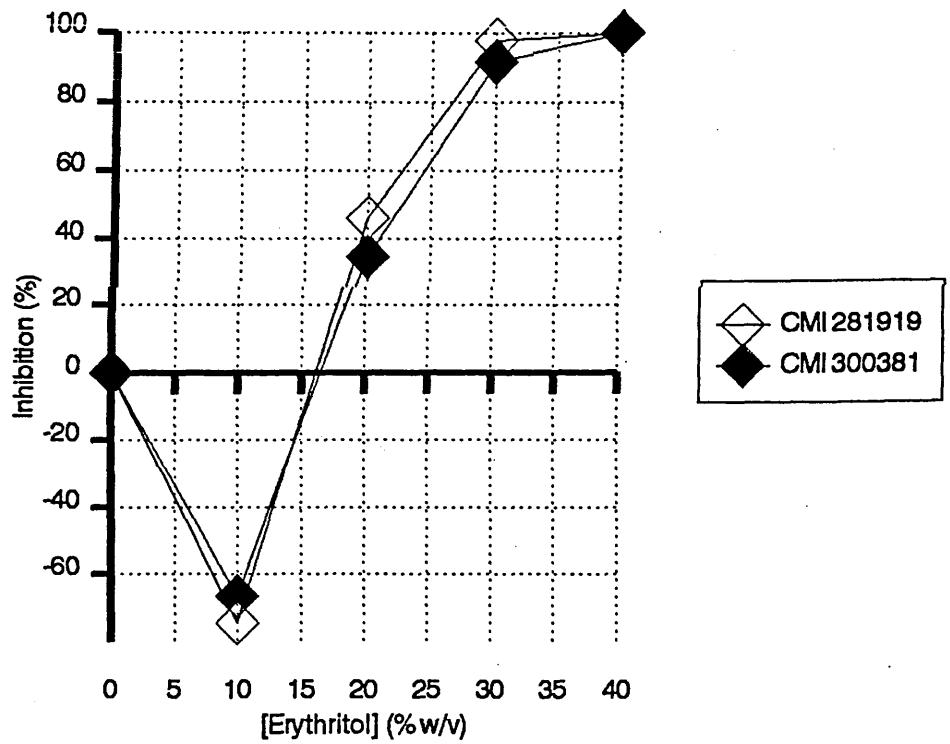
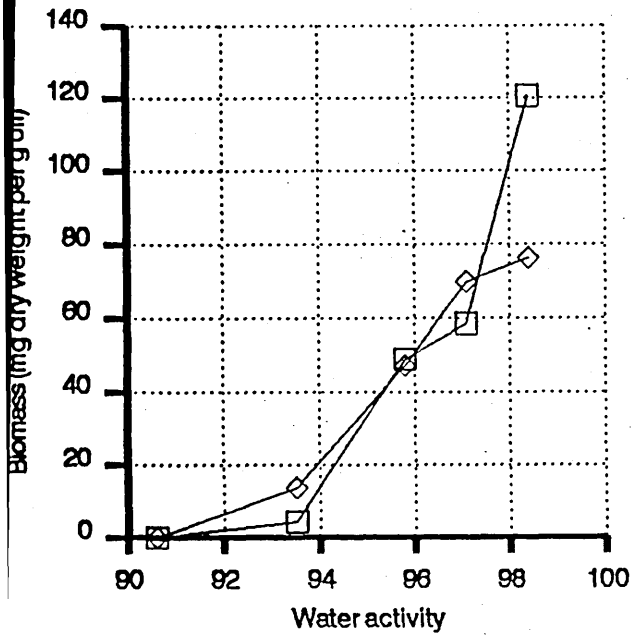
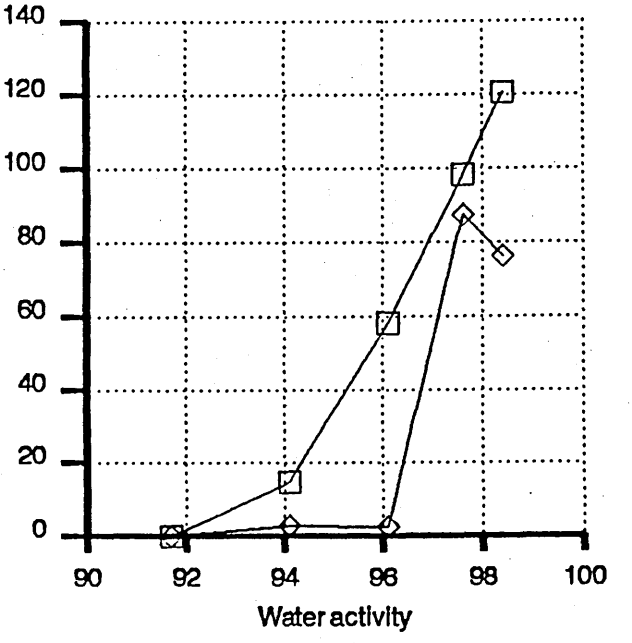


Figure 4.9 : Dose response curve for the inhibition of growth of Penicillium crustosum by erythritol with palm kernel oil as a sole carbon source. Results are the arithmetic mean of four fermentations.

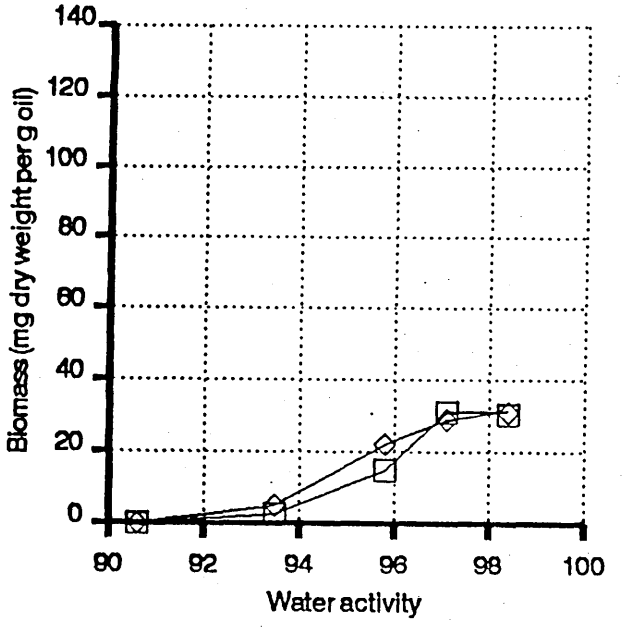
(a) Coconut oil/NaCl



(b) Coconut oil/Erythritol



(c) Palm kernel oil/NaCl



(d) Palm kernel oil/Erythritol

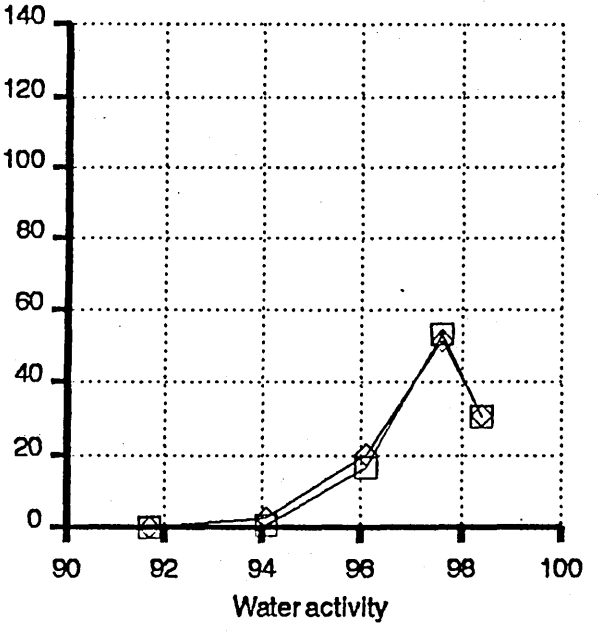
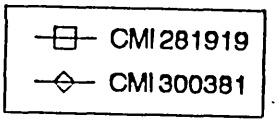
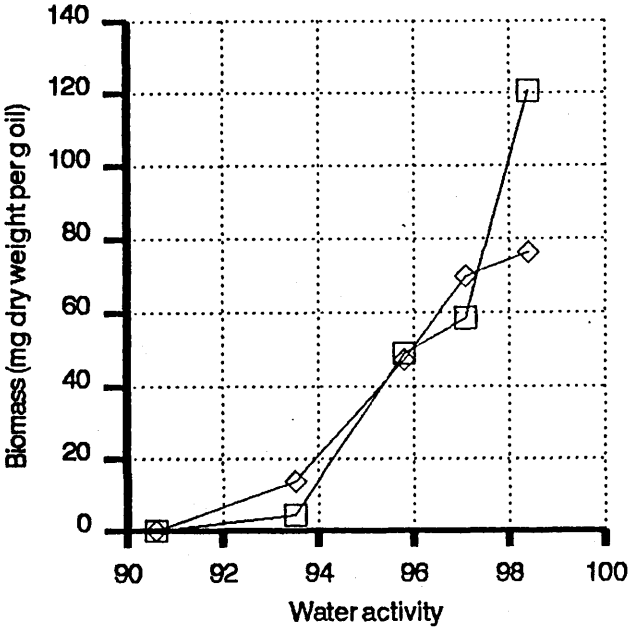
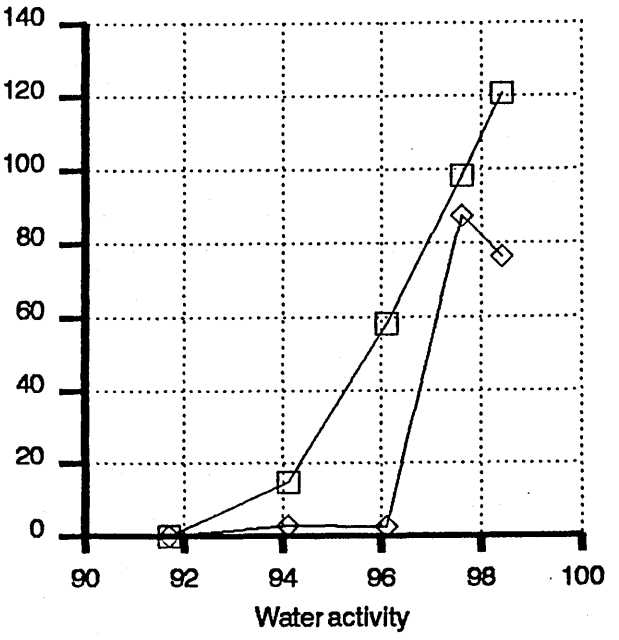


Figure 4.10 : The effect of water activity (expressed as ERH) on production of biomass from the lauric acid oils. Results are the mean of four fermentations (see Tables 4.9 to 4.12 for S.D.'s)

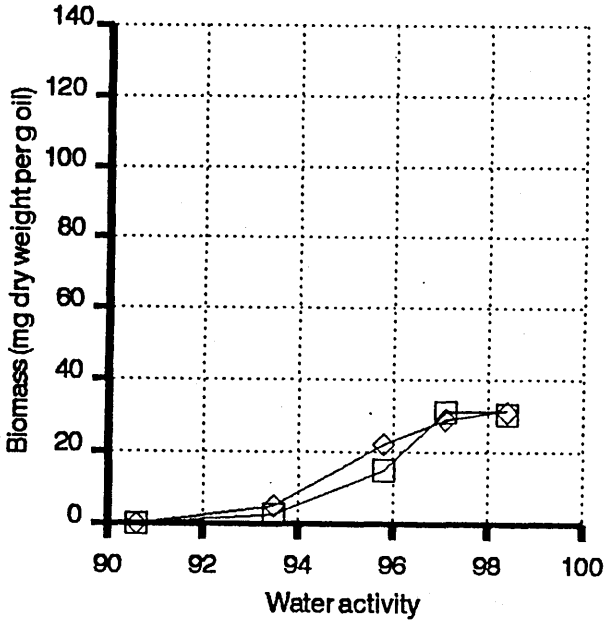




(c) Palm kernel oil/NaCl



(d) Palm kernel oil/Erythritol



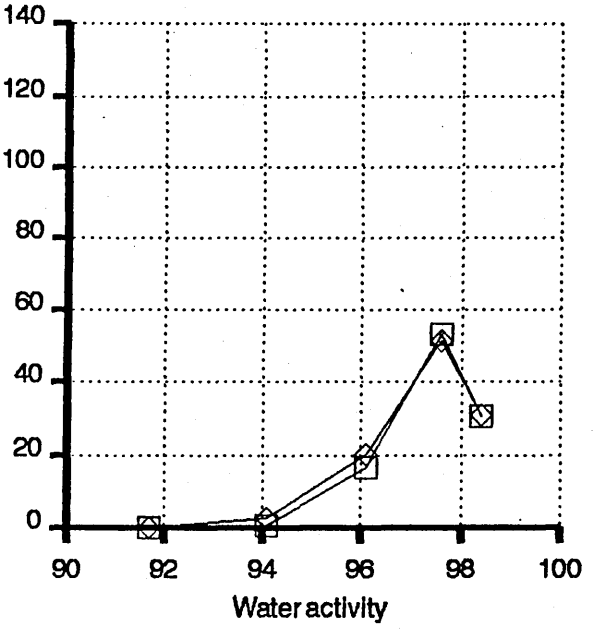
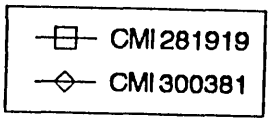


Figure 4.10 : The effect of water activity (expressed as ERH) on production of biomass from the lauric acid oils. Results are the mean of four fermentations (see Tables 4.9 to 4.12 for S.D.'s)



was probably controlled exclusively by availability of water. Above 0.95 there was a significant difference between coconut and palm kernel oil. The different properties of the oils were a more important factor than a_w over this range of solute concentrations. The two solutes had similar effects on growth and ketone production. It was likely that they functioned exclusively by reducing a_w.

4.4 The effect of preservatives on fungal growth and ketonic rancidity

Preservatives have an important role in preventing food spoilage and the growth of foodborne pathogens. A wide range of antimicrobial chemicals have been used as preservatives. This subject has been frequently reviewed (ICMSF 1980, Lueck 1980, Gould *et al* 1983, Branen and Davidson 1984). The mechanism of action of preservatives is based on one or a combination of three factors:

1. Destruction of the cell envelope (wall o cell membrane)
2. Inhibition of key enzymes or interference with energy transducing processes
3. Destruction of the genetic structure of the protoplast (adapted from de Boer 1988)

There are important limitations which restrict the use of preservatives. The microbicidal effects of preservatives are lost in foods which contain a large population of micro-organisms. They cannot return putrefying foods to a fresh state. Neither do preservatives have a complete spectrum of activity against moulds, yeasts and bacteria.

The use of preservatives to prevent ketonic rancidity has not been investigated. Sorbic acid and natamycin were used to study the effect of mould inhibitors on the growth of *P. crustosum* and the conversion of the lauric acid oils into methyl ketones. These two compounds were selected because they represent the two of the three main classes of preservative. Sorbic acid is believed to interfere with cellular energy production whilst natamycin acts by altering the permeability the fungal cell membrane (Freese *et al* 1973, Lueck 1980).

Sorbic acid (2,4-hexadieneoic acid) is a straight-chain unsaturated fatty acid with a molecular weight of 112.13 (Brannen and Davidson 1984). Its solubility in water at 20°C is only 0.16 g per 100 ml. This increases with temperature and pH of the solution. The presence of other solutes decreases its solubility. Table 4.13 summarises the different solubility characteristics of sorbic acid. The compound is more soluble in a fat system than an aqueous. These chemical and physical

Table 4.13: Solubility characteristics of sorbic acid.*

Solvent	Solubility (%)	
	Sorbate	Potassium sorbate
Water 20°C	0.16	58.20
50°C	0.55	61.00
100°C	4.00	64.00
Ethanol 5%	0.16	57.40
100%	12.90	2.00
Acetone	9.20	0.10
NaCl solution		
5%	0.11	47.0
10%	0.07	34.0
15%	0.04	12.00
Corn oil 20°C	0.80	0.01
50°C	2.00	0.03

*Data from Branen and Davidson (1983).

properties all influence the applications and effectiveness of sorbic acid as a preservative.

Sorbic acid is primarily active against yeasts and moulds (Lueck 1980, Sofos and Busta 1981). It is also active against some bacteria (ICMSF 1980). Inhibition is usually fungistatic rather than fungicidal (Lueck 1980, Sofos and Busta 1981). The antimicrobial activity is associated with the undissociated molecule as with other short carbon chain length fatty acids (Gould *et al* 1983, Sofos and Busta 1983). Consequently, the antimicrobial action of sorbic acid is pH dependent and it increases as the pH of the substrate decreases.

It has been suggested that sorbic acid acts by inhibiting glycolytic and other enzymes (York and Vaughn 1964, Sofos and Busta 1981). It is also likely that sorbic acid acts as an uncoupler of oxidative phosphorylation (Freese *et al* 1973, Gould *et al* 1983). As the short chain fatty acids have hydrophilic and lipophilic properties it is generally accepted that they act as proton ionophores in charged membranes. This mechanism is discussed in Section 5.1.

Unlike most preservatives, natamycin is an antibiotic. It was first discovered by Dutch investigators working in South Africa who isolated it from *Streptomyces natalensis* (Struyk *et al* 1955). Natamycin was first used for the treatment of chronic infections caused by pathogenic moulds and yeasts (Raab

1972). It is only since the 1960s that natamycin has been used as a preservative in foods. Natamycin can be applied to food products in liquid slurries, pastes or as a solid. It has been used on the continent for the preservation of cheeses, sausage and salami (Moerman 1972, ICMSF 1980, Lueck 1980). Natamycin is not yet a permitted preservative for use in the United Kingdom (Preservatives in Foods Regulations 1989).

Natamycin belongs to the polyene macrolide group of antifungal antibiotics (Georgopapadakou *et al* 1987). It exhibits a crystalline structure which is virtually insoluble in water (0.01% w/v) and insoluble in fats or ketones (Struyk *et al* 1955).

The antifungal activity of polyene antibiotics is based on their ability to alter the permeability of the cytoplasmic membrane (Raab 1972). This is a two-stage process:

1. Binding of antibiotic to membrane
2. Change of permeability of the membrane

The binding of natamycin is dependent on the presence of fungal sterols. Bacteria are insensitive to polyenes because their membranes do not contain sterols (Raab 1972). The second step is responsible for the fungicidal activity of natamycin. Changes in cell membrane permeability lead to a loss of intracellular

potassium ions (K+). Extreme damage to the cell membrane can result in lysis.

Table 4.14 gives the results of a fermentation experiment when sorbic acid was incorporated into the growth medium where coconut oil was the sole carbon source. *Penicillium crustosum* was able to grow and convert coconut oil into methyl ketones in the presence of sorbic acid at concentrations of up to 1121 mg sorbic acid per kg of medium (10 mmolar). Sorbic acid is not normally included in foods at concentrations above 1000 mg Kg^{-1} (Preservatives in Foods Regulations 1989). The results in Table 4.14 show that at this concentration sorbic acid gave only 50 to 80% inhibition of growth. No growth or conversion of coconut oil took place in the presence of 2242 $mg.Kg^{-1}$ (20 mmolar) sorbic acid. However, the actual MIC of sorbic acid in this system was 1569 $mg.Kg^{-1}$ (14 mmolar). The MIC was derived from extrapolation of the dose response curve given in Figure 4.11 (Wyss *et al* 1945). This concentration is below 2000 $mg.Kg^{-1}$ which is the maximum permitted for incorporation into foods in the U.K, (Preservatives in Foods Regulations 1989).

The results shown in Table 4.15 show the effect of sorbic acid on the conversion of palm kernel oil into methyl ketones. The results were similar to those obtained when coconut oil was used as a sole carbon source. However, less growth took place on palm kernel

Table 4.14: The effect of sorbic acid on the conversion of coconut oil into methyl ketones by Penicillium crustosum.*

[Sorbic acid] ($mmol.l^{-1}$)	Isolate CMI 281919				Isolate CMI 300381			
	Biomass (mg dry wt)	Product - Total Ketones (mmol)	Molar Conversion (%)	Yield biomass ($\mu mol\ mg^{-1}$)	Biomass (mg dry wt)	Product - Total Ketones (mmol)	Molar Conversion (%)	Yield biomass ($\mu mol\ mg^{-1}$)
0	120.7±49.6	0.839±0.110	21.4±2.8	6.9±0.9	76.2±40.5	0.758±0.074	19.3±1.9	9.9±1.0
1	51.7±20.8	0.559±0.175	14.6±4.5	10.9±3.7	67.7±25.0	0.406±0.186	10.6±4.8	5.9±3.4
5	57.4±22.0	0.471±0.074	12.3±1.9	8.9±2.4	74.9±27.5	0.707±0.290	18.4±7.4	9.4±2.5
10	22.4±10.0	0.319±0.084	8.3±2.1	14.9±5.6	35.2±1.8	0.085±0.056	2.2±1.4	2.6±1.5
20	0	ND	0	0	0	ND	0	0

* Fermentations carried out in aerobic liquid shake culture at 200 rpm, 25 °C and pH 7 for 72 h. Results are the arithmetic mean of four fermentations and eight analyses (±S.D.).

Table 4.15: The effect of sorbic acid on the conversion of palm kernel oil into methyl ketones by Penicillium crustosum.*

[Sorbic acid] ($mmol.l^{-1}$)	Isolate CMI 281919				Isolate CMI 300381			
	Biomass (mg dry wt)	Product - Total Ketones (mmol)	Molar Conversion (%)	Yield biomass ($\mu mol\ mg^{-1}$)	Biomass (mg dry wt)	Product - Total Ketones (mmol)	Molar Conversion (%)	Yield biomass ($\mu mol\ mg^{-1}$)
0	30.5±7.7	0.180±0.136	6.2±4.7	5.9±4.5	30.9±3.5	0.405±0.130	13.9±4.5	13.1±4.2
1	47.1±3.1	0.380±0.199	12.8±7.3	8.1±4.2	24.8±5.2	0.471±0.353	15.9±11.9	19.0±14.2
5	41.4±4.6	0.202±0.064	6.8±2.2	4.9±1.5	18.9±6.5	0.222±0.096	7.5±3.2	11.7±5.1
10	12.5±1.7	0.114±0.049	3.8±1.6	9.1±3.9	10.5±3.7	0.145±0.029	4.9±1.0	13.8±2.8
20	0	ND	0	0	0	ND	0	0

* Fermentations carried out in aerobic liquid shake culture at 200 rpm, 25 °C and pH 7 for 72 h. Results are the arithmetic mean of four fermentations and eight analyses (±S.D.).

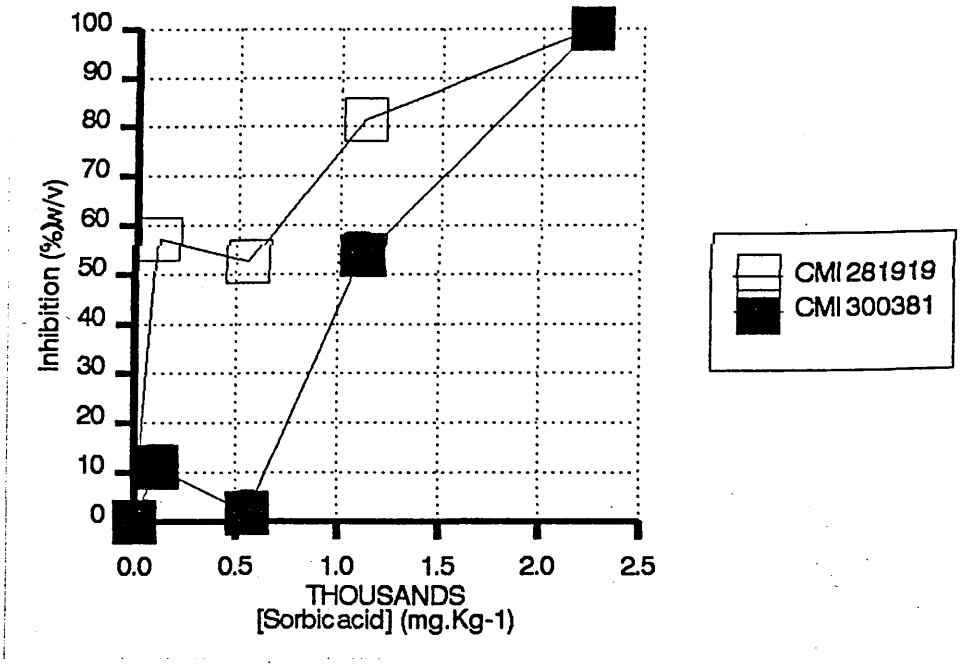


Figure 4.11 : Dose response curve for inhibition of growth of Penicillium crustosum by sorbic acid with coconut oil as a sole carbon source. Results are the arithmetic mean of four fermentations.

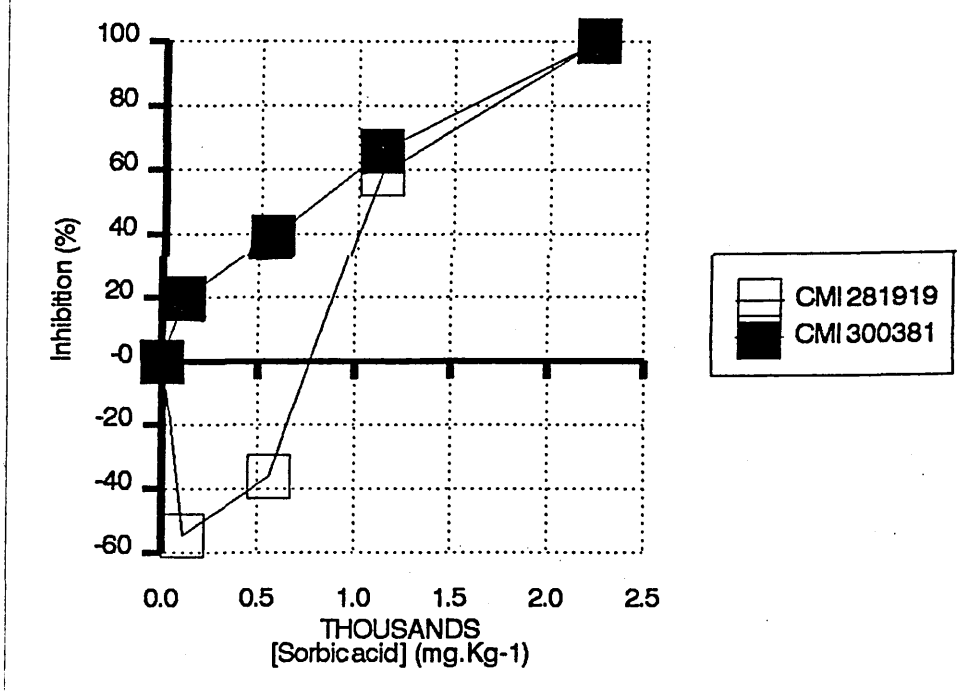


Figure 4.12 : Dose response curve for the inhibition of growth of Penicillium crustosum by sorbic acid with palm kernel oil as a sole carbon source. Results are the arithmetic mean of four fermentations.

oil than coconut oil at each concentration of sorbic acid. This confirmed that coconut oil was the preferred substrate to support the growth of *P. crustosum* (see Section 3.3). There was no evidence that sorbic acid inhibited growth at concentrations of up to 525 mg.Kg^{-1}(5 mmolar). The dose response curve given by Figure 4.12 suggested that 1 mmolar sorbic acid resulted in growth promotion. The dose response curve also indicates that the MIC of sorbic acid in this system was 1457 mg.Kg^{-1} (13 mmolar). This was slightly less than that recorded for sorbic acid when coconut oil was the sole carbon source.

A petroleum-like odour was detected in the headspace of fermentations that contained sorbic acid (5 or 10 mmolar). An experiment was performed to establish whether this odour was due to metabolism of the preservative by the fungus. Fermentations were carried out in micro-carrier culture with 4 mmolar sorbic acid and sucrose (3% w/v) as a sole carbon source. A petroleum-like odour was detected and the compound responsible was extracted from the suspension culture (Section 2.13). The volatile compound was identified as 1,3-pentadiene using gc/ms with known standards. The mass spectrum obtained for this compound is given in Figure 4.13. Identification was confirmed by proton nuclear magnetic resonance spectroscopy (^{1}H-nmr). Figure 4.14 gives the nmr spectrum for trans-1,3-

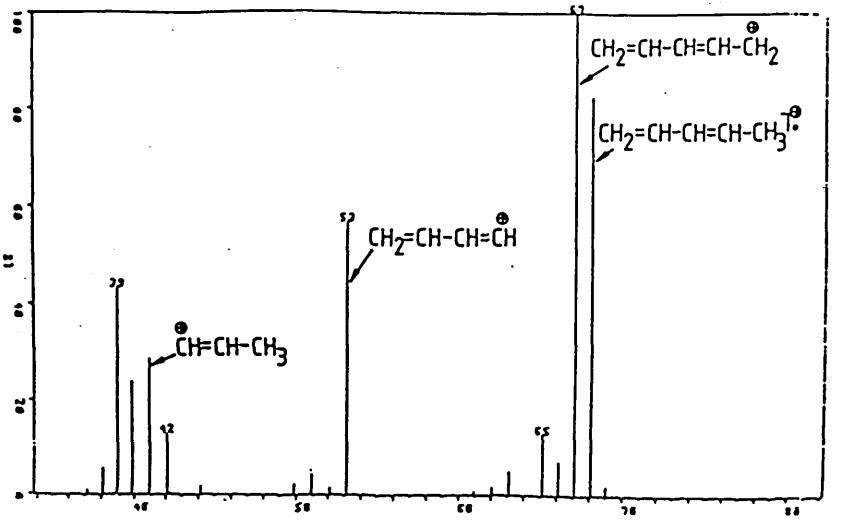


Figure 4.13 : Mass spectrum of trans-1,3-pentadiene produced after decarboxylation of sorbic acid by P.crustosum. Analyses were performed at 70 EV using a Carlo Erba series 2150 gas chromatograph coupled to a Micromass 30F mass spectrometer.

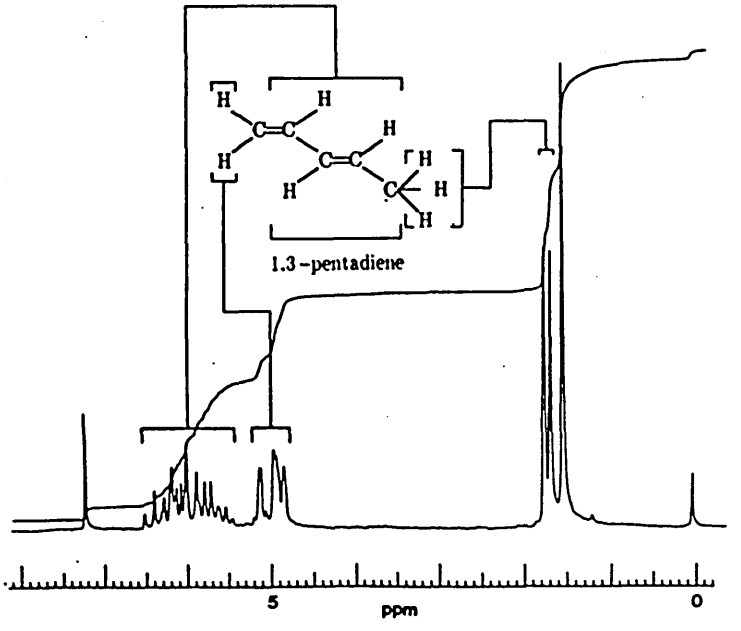


Figure 4.14 : NMR spectrum of trans-1,3-pentadiene produced after decarboxylation of sorbic acid. The unassigned peak is probably ethylene ($H_2C=CH_2$) which can be synthesized by P.crustosum (Pazout and Pazoutova 1989).

pentadiene produced from sorbic acid. This volatile compound was not detected in control fermentations that did not contain sorbic acid. It appeared that *P. crustosum* could decarboxylate sorbic acid. Several genera may have this ability (Hartog *et al* 1986). However, the decarboxylation of sorbic acid appears to be most widespread in the genus Penicillium (Marth *et al* 1966, Liewen and Marth 1985, Hartog *et al* 1986, Hatton and Kinderlerer 1988). Within a single species some strains have a greater resistence to sorbic acid than others (Liewen and Marth 1985). This subject is now the subject of further research within the group at Sheffield City Polytechnic (AFRC grant).

An experiment was carried out to compare the inhibition of fungal growth by sorbic acid with that of the saturated short chain fatty acids (C6 to C12). (See Section 3.4.) Figure 4.15 illustrates the dose response curves for both isolates of *P. crustosum* after a 72 h fermentation at pH 7.0. The MIC in this system was 1000 mg Kg^{-1} for both isolates. However, isolate CMI 281919 grew better than expected at relatively low sorbic acid concentrations (0 to 500 mg Kg^{-1}). It is possible that this isolate had the more efficient detoxification mechanism for the removal of sorbic acid from the environment.

The production of petroleum-like taints in foods is undesirable. For this reason sorbic acid may not be the

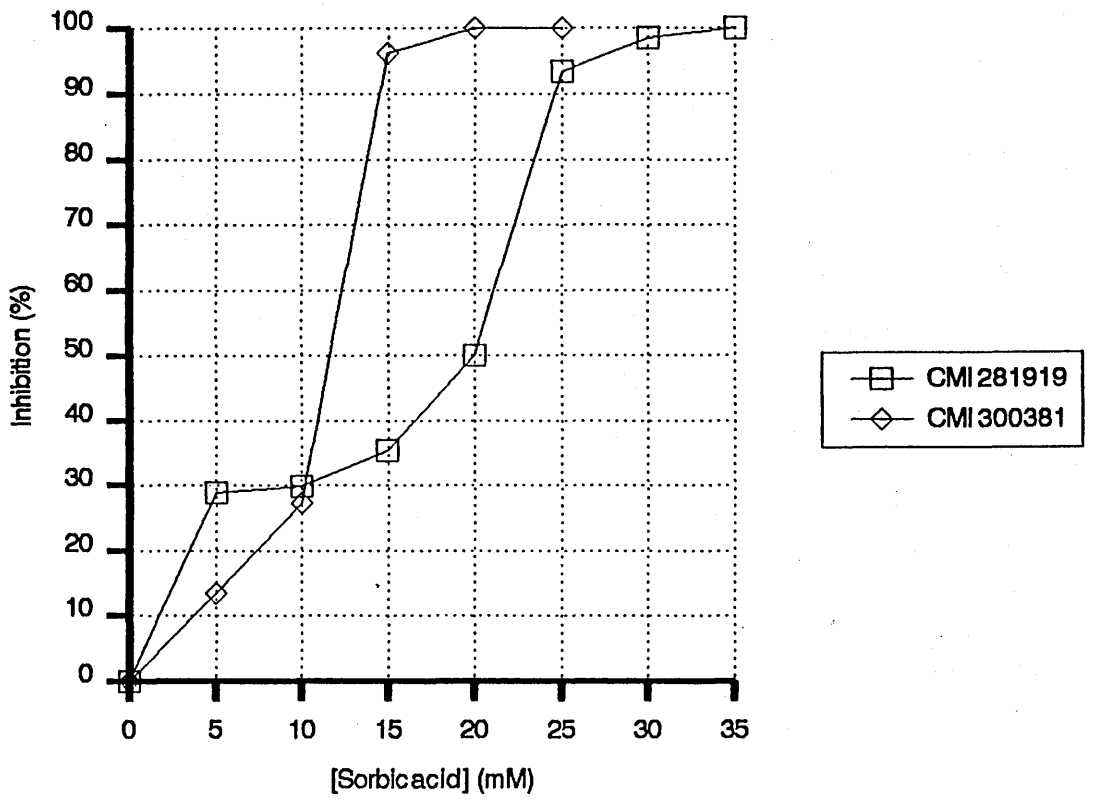


Figure 4.15 : Dose response curve for the inhibition of growth of two isolates of Penicillium crustosum by sorbic acid. Fermentations carried out in aerobic shake culture at 200 rpm, 25°C and pH 7.0 with sucrose as a sole carbon source. Results are the arithmetic mean of three fermentations; S.D.'s did not exceed 20%.

Table 4.16: The effect of natamycin on the conversion of coconut oil into methyl ketones by Penicillium crustosum.*

[Natamycin] (% w/v) x10^{-6}	Isolate CMI 281919				Isolate CMI 300381			
	Biomass (mg dry wt)	Product - Total Ketones (mmol)	Molar Conversion (%)	Yield ^{biomass} (μmol mg^{-1})	Biomass (mg dry wt)	Product - Total Ketones (mmol)	Molar Conversion (%)	Yield ^{biomass} (μmol mg^{-1})
0	120.7±49.6	0.839±0.110	21.4±2.8	6.9±0.9	76.2±40.5	0.758±0.074	19.3±1.9	9.9±1.0
1	94.3±1.9	0.372±0.034	9.5±0.9	3.9±0.3	95.7±5.3	0.387±0.093	9.9±2.4	4.0±0.7
5	79.6±11.2	0.316±0.057	8.1±1.5	4.0±0.3	80.9±21.0	0.328±0.072	8.4±1.8	4.1±0.6
10	68.8±7.7	0.311±0.065	7.9±1.7	4.5±0.7	57.8±8.5	0.350±0.045	9.0±1.2	6.1±0.4
50	12.8±4.8	0.142±0.021	3.6±0.5	12.1±3.3	6.6±0.7	0.115±0.020	2.9±0.5	17.9±4.6
100	0	ND	0	0	0	ND	0	0

* Fermentations carried out in aerobic liquid shake culture at 200 rpm, 25 °C and pH 7 for 72 h. Results are the arithmetic mean of four fermentations and eight analyses (±S.D.).

Table 4.17: The effect of natamycin on the conversion of palm kernel oil into methyl ketones by Penicillium crustosum.*

[Natamycin] ($\times 10^{-6}$ % w/v)	Isolate CMI 281919				Isolate CMI 300381			
	Biomass (mg dry wt)	Product - Total Ketones (mmol)	Molar Conversion (%)	Yield biomass (μmol mg^{-1})	Biomass (mg dry wt)	Product - Total Ketones (mmol)	Molar Conversion (%)	Yield biomass (μmol mg^{-1})
0	30.5±7.7	0.180±0.136	6.2±4.7	5.9±4.5	30.9±3.5	0.405±0.130	13.9±4.5	13.1±4.2
1	18.9±4.0	0.180±0.031	6.1±1.1	9.6±0.9	22.4±0.8	0.244±0.039	8.2±1.3	10.9±1.9
5	15.7±3.8	0.184±0.061	6.2±2.0	11.6±1.8	16.8±4.9	0.215±0.068	7.3±2.3	13.1±1.5
10	16.0±1.1	0.166±0.037	5.6±1.2	10.4±2.1	13.2±3.6	0.158±0.038	5.3±1.3	12.0±0.7
50	10.3±1.7	0.116±0.016	3.9±0.5	11.4±1.0	8.8±1.2	0.127±0.018	4.3±0.6	14.5±1.0
100	0	ND	0	0	0	ND	0	0

* Fermentations carried out in aerobic liquid shake culture at 200 rpm, 25 °C and pH 7 for 72 h. Results are the arithmetic mean of four fermentations and eight analyses (±S.D.).

preservative of choice for controlling fungal spoilage in the lauric acid oils. Its unsuitability is discussed in more detail in Chapter 5.

Natamycin was investigated to assess its suitability for the control of ketonic rancidity. Table 4.16 gives the results obtained when the antibiotic was incorporated into fermentations with coconut oil as a sole carbon source. Complete inhibition of fungal growth and ketone production was achieved with a natamycin concentration of only 1 mg. Kg^{-1}. The maximum yield biomass was recorded in the presence of 0.5 mg.Kg^{-1} of natamycin. The results in Table 4.17 show that natamycin was equally effective in inhibiting growth of *P. crustosum* on palm kernel oil.

4.5 The effect of glucose on fungal growth and ketonic rancidity

Glucose may be utilised as a primary carbon source by fungi via glycolysis and the T.C.A. cycle (Smith and Berry 1975). There is some evidence that the presence of low molecular weight fatty acids (< C12) can increase the rate of glucose oxidation by fungi (Rolinson 1954). Glucose has been reported to inhibit the conversion of Hexanoic (caproic) acid into 2-pentanone by *A. niger* (Lewis 1971). It appears that there may be a fatty acid sparing effect when fungi grow in the presence of both

Table 4.18: The effect of glucose on the conversion of coconut oil into methyl ketones by Penicillium crustosum.*

[Glucose] (% w/v)	Isolate CMI 281919				Isolate CMI 300381			
	Biomass (mg dry wt)	Product - Total Ketones (mmol)	Molar Conversion (%)	Yield ^{biomass} (μmol mg^{-1})	Biomass (mg dry wt)	Product - Total Ketones (mmol)	Molar Conversion (%)	Yield ^{biomass} (μmol mg^{-1})
0	120.7±49.6	0.839±0.110	21.4±2.8	6.9±0.9	76.2±40.5	0.758±0.074	19.3±1.9	9.9±1.0
3	128.6±9.2	0.996±0.064	26.0±1.7	7.8±0.8	173.1±11.3	1.068±0.238	27.8±6.2	6.2±1.2

* Fermentations carried out in aerobic liquid shake culture at 200 rpm, 25 °C and pH 7 for 72 h. Results are the arithmetic mean of four fermentations and eight analyses (±S.D.).

Table 4.19: The effect of glucose on the conversion of palm kernel oil into methyl ketones by Penicillium crustosum.*

[Glucose] (% w/v)	Isolate CMI 281919				Isolate CMI 300381			
	Biomass (mg dry wt)	Product - Total Ketones (mmol)	Molar Conversion (%)	Yield ^{biomass} (μmol mg^{-1})	Biomass (mg dry wt)	Product - Total Ketones (mmol)	Molar Conversion (%)	Yield ^{biomass} (μmol mg^{-1})
0	30.5±7.7	0.180±0.136	6.2±4.7	5.9±4.5	30.9±3.5	0.405±0.130	13.9±4.5	13.1±4.2
3	37.0±2.3	0.503±0.028	16.9±1.0	13.7±1.3	138.22±20.1	0.874±0.161	29.4±5.4	6.4±1.2

* Fermentations carried out in aerobic liquid shake culture at 200 rpm, 25 °C and pH 7 for 72 h. Results are the arithmetic mean of four fermentations and eight analyses (±S.D.).

sugars and fats. However, the effect of glucose on the conversion of the lauric acid oils into methyl ketones has not been studied.

Table 4.18 gives the results obtained when glucose (3% w/v) was added to the buffered Czapek fermentation medium containing coconut oil (1 g). Although there was an increase in the biomass and product, the actual yield biomass (product per unit biomass) did not alter. The increase in product concentration was due to an increased growth rate of the fungus. There was no promotion of ketone production by addition of glucose except that there was an increase in biomass. This demonstrated that the whole oils did not behave as free fatty acids. Lewis had found that glucose could inhibit the conversion of free acid into ketone (Lewis 1971).

Table 4.19 gives the results obtained when palm kernel was used as a substrate in the presence of glucose (3% w/v). Again there was an increase in growth rate and ketone production. The conversion rates of fatty acids into methyl ketones were more than doubled in the presence of glucose (a two-fold increase in ketone production was found in the presence of glucose).

4.6 The effect of light on fungal growth and ketonic rancidity

The effects of various forms of radiation on growth and development of fungi have been reviewed (Tan 1978). However, there is still very little information on the effect of visible light on fungal growth. Exposure to light has produced growth inhibition in *Penicillium* spp. (Chebotarev and Zemlyanukhin 1974). This was due to a decrease in the rate of cell wall synthesis (Chebotarev and Zemlyanukhin 1974). It was not established if the effect was direct or indirect. The effect of light on many fungi is to stimulate the production of spores (Leach and Trione 1986).

Table 4.20 gives the results obtained after fermentations with coconut oil were exposed to black light (Section 2.5.7). It appeared that the growth of isolate CMI 300381 may have been stimulated by this treatment. However, there was no evidence for increased conversion of coconut oil into methyl ketones. It is possible that individual strains of the same species may react differently to exposure to light.

When palm kernel oil was utilised as a sole carbon source there was an increase in the conversion of fatty acids into methyl ketones (Table 4.21). This was accompanied by a slight increase in biomass production.

Table 4.20: The effect of light on the conversion of coconut oil into methyl ketones by Penicillium crustosum.*

	Isolate CMI 281919				Isolate CMI 300381			
	Biomass (mg dry wt)	Product - Total Ketones (mmol)	Molar Conversion (%)	Yield $\frac{\text{product}}{\text{biomass}}$ (μmol mg^{-1})	Biomass (mg dry wt)	Product - Total Ketones (mmol)	Molar Conversion (%)	Yield $\frac{\text{product}}{\text{biomass}}$ (μmol mg^{-1})
Without light	120.7±49.6	0.839±0.110	21.4±2.8	6.9±0.9	76.2±40.5	0.758±0.074	19.3±1.9	9.9±1.0
With light	112.4±9.5	0.487±0.053	12.4±1.4	4.3±0.4	187.0±13.0	0.757±0.092	19.4±2.4	4.0±0.3

* Fermentations carried out in aerobic liquid shake culture at 200 rpm, 25 °C and pH 7 for 72 h. Results are the arithmetic mean of four fermentations and eight analyses (±S.D.).

Table 4.21: The effect of light on the conversion of palm kernel oil into methyl ketones by Penicillium crustosum.*

	Isolate CMI 281919				Isolate CMI 300381			
	Biomass (mg dry wt)	Product - Total Ketones (mmol)	Molar Conversion (%)	Yield ^{biomass} (μmol mg^{-1})	Biomass (mg dry wt)	Product - Total Ketones (mmol)	Molar Conversion (%)	Yield ^{biomass} (μmol mg^{-1})
Without light	30.5±7.7	0.180±0.136	6.2±4.7	5.9±4.5	30.9±3.5	0.405±0.130	13.9±4.5	13.1±4.2
With light	31.9±2.5	0.373±0.022	12.5±0.7	11.8±1.1	37.1±5.3	0.460±0.024	15.5±0.8	12.7±1.3

* Fermentations carried out in aerobic liquid shake culture at 200 rpm, 25 °C and pH 7 for 72 h. Results are the arithmetic mean of four fermentations and eight analyses (±S.D.).

4.7 The effect of oxygen removal on fungal growth

Filamentous fungi are not capable of strictly anaerobic growth (Smith and Berry 1975). To confirm this *P.crustosum* was grown in plate culture under either atmospheric or reducing conditions (Section 2.5.8).

No growth or methyl ketones were detected in the absence of oxygen. However, no ketones were detected in control experiments where growth had occurred. It is not known why this was the case but it could be attributed to a reduction in the fungal growth rate.

5. Final Discussion

5.1 Characterization of ketonic rancidity

Fatty acid composition of the oil is important in ketonic rancidity. Fermentations with coconut and palm kernel oils have established that ketonic rancidity occurs in fats and oils which contain short and intermediate carbon chain length fatty acids C6 to C14 (Hatton and Kinderlerer 1986). These observations are in contrast to beef tallow, cocoa butter, olive and hazelnut oils which contain only long carbon chain fatty acids (C16 and C18). No ketones were produced on fermentation of these oils (Section 3.2). The evidence presented in Chapter Three indicates that methyl ketones were produced as a result of primary metabolism and were not secondary metabolites as ketone production follows biomass (Figures 3.1 to 3.3).

The fatty acid composition of the oils is important in determining the growth rate of the fungus. The oils fall into three groups depending on their fatty acid composition. The first group contains fatty acids of short and intermediate carbon chain length (C6 to C14), the second contains saturated long chain acids (mainly C16 and C18) and the third group contains mono- and di- unsaturated acids (mainly C18). Growth was reduced on substrates in the first group. Maximum growth occurred in group three substrates

where the oils did not contain short carbon chain length fatty acids and were liquid at 25°C (hazelnut and olive oils).

In model experiments when simple triglycerides were fermented, similar results to those with whole oils were obtained except for glyceryl tridodecanoate. In this case dodecanoic (lauric) acid was far more reactive when present in a mixed glyceride (coconut or palm kernel oils) than in glyceryl tridodecanoate (Section 3.2). The high concentrations of 2-undecanone (0.14 to 0.58 mmol per g of substrate) produced when the lauric acid oils were fermented was due to the high concentration of dodecanoic acid in the substrate (Table 3.1). Minimum growth and ketone production were found when glyceryl tridodecanoate was used as a sole carbon source. Resistance to fungal attack was attributed to the low solubility of dodecanoic acid in water and high melting point (44.2°C) of this substrate.

In experiments using model substrates (Section 3.2) there was no evidence for production of ketones other than those containing one less carbon atom than the parent fatty acid. Chain length of the substrate fatty acid is important. The longest acid to undergo conversion into methyl ketones was tetradecanoic (myristic) acid which was converted into 2-tridecanone in extremely low yields (Section 3.2). Few references exist in the literature for the isolation and identification of 2-pentadecanone. Dartey and Kinsella (1973b) reported that *P.roquefortii* spores

could convert [U^{14}]-hexadecanoic acid into [U^{14}]-2-pentadecanone.

Conversion of the lauric acid oils into methyl ketones is an energetically wasteful reaction. When the lauric acid oils were fermented there was a reduction in the growth rate of the fungus compared to that on oils which did not contain short chain fatty acids (Section 3.2). It was unlikely that the fungus would produce methyl ketones unless their production contributed to survival of the organism.

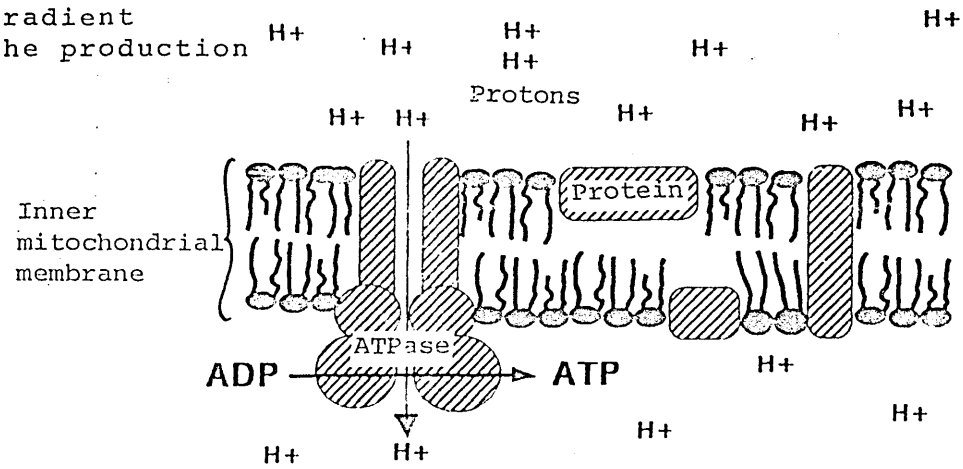
Many studies have been undertaken which demonstrate that short and intermediate chain length fatty acids (C6 to C14) inhibit fungal growth and oxygen uptake (Wyss *et al* 1945, Rothman *et al* 1946, Chattaway and Thompson 1956, Kabara *et al* 1972, 1977, Hunkova and Fencl 1977, 1978, Das and Bannerjee 1981). Conversion of these fatty acids into methyl ketones is an energetically wasteful process. A number of authors have suggested that this biotransformation may be a detoxification (Stokoe 1928, Lewis and Darnall 1970). Evidence is presented in Section 3.4 which establishes that short carbon chain length fatty acids (C6 to C12) are fungistatic. Minimum inhibitory concentrations are given in Table 3.9. The ability of these acids to inhibit growth would appear to be a function of chain length. On a molar basis, the order of toxicity was C11 > C10 > C12 > C6 > C8. Although it would be tempting to use fatty acids as preservatives in foods and pharmaceuticals (Kabara *et al* 1977), their odour would preclude this.

Fatty acids are known to act as uncouplers of oxidative phosphorylation in isolated mitochondria (Borst *et al* 1962, Hird and Weidemann 1966). Energy transducing membranes such as the inner-mitochondrial membrane are impermeable to protons (Mitchell 1972, Berry 1981). Weak lipophilic acids may cause protons to 'leak' back through this membrane in the direction of the proton gradient. Consequently the mitochondrion is unable to set up a proton gradient and the production of ATP is inhibited (Figure 5.1). The short chain aliphatic fatty acids could act as weak lipophilic acids.

Uncoupling activity for fatty acids was first proposed after experiments with isolated mitochondria (Borst *et al* 1962). An uncoupling mechanism has already been proposed to account for the antibacterial activity of preservatives such as propionic, benzoic and sorbic acids (Freese *et al* 1973, Sofos and Busta 1981). However, these preservatives are primarily antifungal. Therefore they must traverse the fungal cell membrane before they can interfere with energy production in the mitochondria.

Uncoupling activity accounts for the four categories of effect described by Hunkova and Fencl in 1977 (Section 3.4). Stimulation of oxygen uptake by sub-inhibitory concentrations of octanoic acid was first observed in *P. chrysogenum* (Rolinson 1954). Stimulation of respiration may be due to increased activity of the electron transport chain as it compensates for a partial collapse of the proton

(i) Proton gradient coupled to the production of ATP.



(ii) Proton gradient dissipated by fatty acid. ATP is hydrolysed to drive the ATPase as a proton pump.

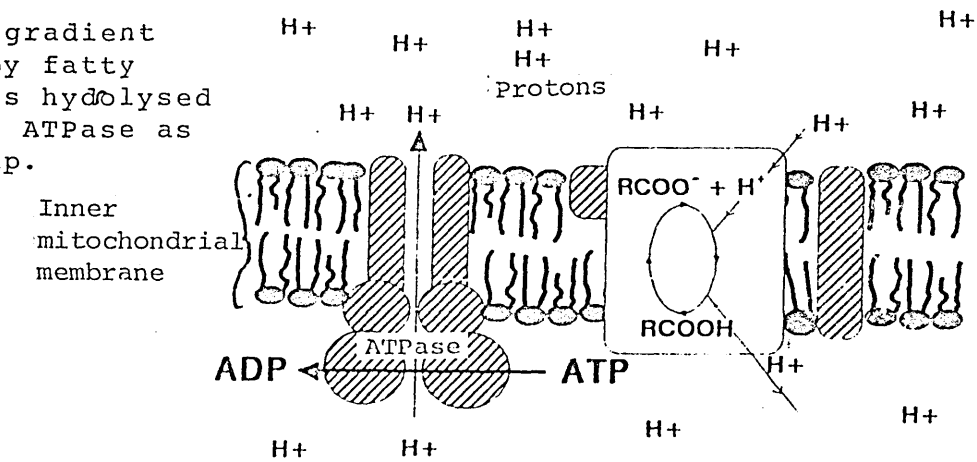


Figure 5.1 : Uncoupling of proton gradient from ATP production by fatty acid with lipophilic and hydrophilic properties.

gradient. Increasing the fatty acid concentration leads to less efficient utilization of substrate because energy is diverted into maintaining the proton gradient across a membrane which is no longer impervious to protons. Further increases appear to damage the mitochondria (Plates 3.5 and 3.6). Increased concentrations of undecanoic acid were reported to injure the cytoplasmic membrane of *T. rubrum* (Das and Bannerjee 1981). The fungicidal properties of short carbon chain length fatty acids may be related to cell-damage as well as uncoupling activity. A two-stage mechanism may be postulated to account for fatty acid toxicity, the first being uncoupling and the second disruption of the cell envelope.

Uncoupling activity may account for the abnormal mitochondrial structure in fungi grown on substrates containing short and intermediate carbon chain length fatty acids (Section 3.4). Although the mitochondrion is generally regarded as the site of fatty acid β-oxidation there was no evidence for this being the site for ketone production.

The results in Chapter Three suggest that detoxification is a more likely reason for ketone production than the recycling of coenzyme A suggested by Lawrence and Hawke (1968). The detoxification operates by removing free short carbon chain length fatty acids (C6 to C14) from the environment. Without such a mechanism the concentration of free fatty acids would increase until fungal growth ceased.

Elevated levels of free short chain fatty acids could eventually lead to lysis and death (Das and Banerjee 1981).

The results in Chapter Three can be summarised as characterising the following:

1. Ketonic rancidity occurs in fats and oils containing short and intermediate carbon chain length fatty acids (C6 to C14).

2. Methyl ketones are produced from simple triglycerides, free fatty acids and whole oils containing short and intermediate chain length fatty acids. Only ketones containing one less carbon atom than the parent fatty acid are produced.

3. The short and intermediate carbon chain length fatty acids are toxic to fungi. The order of toxicity was determined as C10 > C12 > C6 > C8.

4. Short and intermediate carbon chain length fatty acids may act as uncouplers of oxidative phosphorylation in the fungal mitochondrion.

5. The physical properties, (slip point and solubility) of the fatty acid and triglyceride affect toxicity.

6. Ketonic rancidity arises as a mechanism for the removal of short and intermediate carbon chain length fatty acids from the environment. This reaction apears to be

a characteristic of some fungal genera and is a detoxification reaction.

5.2 Control of ketonic rancidity

The preservation methods used to control ketonic rancidity are summarised in Table 5.1. They operate through the inhibition or slowing of fungal growth. Very few of the available preservation methods are based on the killing of micro-organisms (Gould *et al* 1983). The preserving factors in Table 5.1 are described as extrinsic factors (Section 1.6).

Not all the methods described in Table 5.1 were equally successful in controlling fungal growth and ketonic rancidity. The effectiveness of this environmental control is summarised in Table 5.2.

Increasing hydrogen ion concentration may have been more successful at inhibiting the fungus if a pH-stat had

Table 5.1 Classification of preserving factors

Preservation Factor	Mode of Achievement
Cold	Chill
Reduced a_w	Add solute (salt, sugar)
Acidify	Add acids
Restrict O_2	Vacuum or nitrogen pack
Add preservatives	Organic (sorbic acid)
	Antibiotics (natamycin)

(adapted from Gould *et al* 1983)

Table 5.2 Environmental factors used to control fungal growth. *

Environmental factor	Conditions which prevent growth
pH	pH was altered over range 4.0 to 8.0 but fungal growth took place after 3 d.
Temperature	<4° C or >37°C
Water activity (a_w)	0.91 (15% w/v NaCl) 0.92 (40% w/v Erythritol)
Oxygen	No growth in absence

* Most experiments were carried out in liquid shake culture at 200 rpm, 25°C and pH 7.0 for 72 h. Growth in the absence of oxygen was determined by radial growth on Petri dishes in an anaerobic jar at 25°C under H_2.

been used (Section 4.2). However, the experiment demonstrated the effectiveness of the fungal homeostatic mechanism which enabled the organism to grow over a wide range of pH values. This ability of fungi is well known and Table 5.3 gives reported pH limits for fungal growth.

There is some evidence that reducing pH can increase the toxicity of short chain fatty acids (Wyss *et al* 1945, Rolinson 1954, Lewis and Darnall 1970, Lawrence and Hawke 1968). However, the ΔpH shown in Figure 4.5 made it difficult to establish how pH affects fungal growth or conversion of triglycerides into methyl ketones.

Reducing the temperature was a more effective method than pH for controlling fungal growth and ketonic rancidity (Section 4.1). No fungal growth or ketones were detected after 72 h fermentation at 4°C (Table 5.2).

Table 5.3 The pH limits that permit fungal growth.*

Fungus	Minimum pH	Maximum pH
Aspergillus oryzae	1.6	9.3
Penicillium italicum	1.9	9.3
Penicillium variable	1.6	11.1
Fusarium oxysporium	1.8	11.1

*Data derived from ICMSF (1980)

Temperature control is generally regarded as a classical extrinsic parameter which acts on the dominant micro-organism to inhibit growth and spoilage (Mossel and Ingram 1955, Mossel 1983). However, the experimental evidence in Sections 4.3 and 4.4 suggests that temperature also affects implicit factors associated with the substrate.

The optimum temperature for fungal growth varied according to the fatty acid composition of the triglyceride (Table 4.7). This was in turn related to the melting point of the triglyceride and its component fatty acids. Fats, oils and triglycerides were apparently more susceptible to fungal attack and ketonic rancidity at temperatures where

they or their components were liquid. Phase-transition affects the resistance of oils and fats to fungal attack. This has not been reported previously and has implications for storage of fats and oils. Storage temperatures for fats and oils should be such that they are held in the solid phase.

Water activity (a_w) is an important extrinsic parameter affecting fungal growth (Section 4.3). Reduction of water activity was brought about by addition of solutes (NaCl and erythritol). The results indicated that both solutes functioned exclusively by reduction of the water activity. However, at a_w's greater than 0.95 coconut oil was more prone to spoilage than palm kernel oil due to the higher concentration of short chain fatty acids in coconut oil.

Penicillium crustosum did not behave as a xerophilic fungus, despite its having survived spoilage conditions in consumer returns of desiccated coconut (Kinderlerer and Clark 1986). The results in Section 4.3 show the fungus was unable to grow at an a_w of 0.90, whereas xerophiles are able to germinate and grow at an a_w of 0.85 (Pitt 1975).

Table 5.4 Effect of preservatives on fungal growth.*

Preservative	$10g_{10}$ MIC ($mg.Kg^{-1}$)	
	CMI 281919	CMI 300381
Natamycin	0	0
Sorbic acid	3.35	3.35
Sodium chloride	5.18	5.18
Erythritol	5.60	5.60

* Fermentations carried out in liquid shake culture at 200 rpm, 25°C and pH 7.0 for 72 h with coconut or palm kernel oil as a sole carbon sources.

The use of preservatives to control fungal growth and ketonic rancidity is summarised in Table 5.4. Osmotic regulators are included because salt and some sugars are often employed to preserve foods (ICMSF 1980). The large differences between the MIC's reflect the mode of action of each of the preservatives. Natamycin functions at the molecular level in fungal membranes (Raab 1972). Higher concentrations of sorbic acid are required to completely inhibit fungal growth via the uncoupling of oxidative phosphorylation (Freese *et al* 1978). Sodium chloride and erythritol function exclusively by reducing the availability of water for fungal growth (Section 4.3).

Although sorbic acid was effective in inhibiting fungal growth, its use is not recommended because of its

conversion into 1,3-pentadiene (Marth *et al* 1966, Hartog *et al* 1986, Hatton and Kinderlerer 1988). Production of pentadiene in foods is undesirable because it gives an unpleasant petroleum-like odour. Conversion of sorbic acid into 1,3-pentadiene is probably a detoxification mechanism for the removal of the preservative from the environment. Other fungal detoxification mechanisms have been reported. The yeast *Saccharomyces baillii* can excrete benzoic and sorbic acids from its cells (Warth 1977). *Chrysosporium xerophilum* may detoxify intermediate carbon chain length fatty acids by esterification with secondary alcohols (Kinderlerer *et al* 1988).

Natamycin was very effective in inhibiting fungal growth and ketonic rancidity. Several studies suggest that this compound is suitable for more widespread use as a food preservative (Lynch *et al* 1960, Levinskas *et al* 1964). In the light of these studies it may now be prudent to reassess natamycin for use in the United Kingdom.

Fungal homeostatic and detoxification mechanisms operate to permit growth under unfavourable environmental conditions brought about by changes in pH, a_w or preservative concentration (Gould *et al* 1983). These mechanisms are characteristics of the organism and can be termed implicit factors (Mossel and Ingram 1955, Mossel 1983).

The most important homeostatic principle is that the fungal mycelium reacts to maintain constant intracellular

water content and pH (Lewis and Darnall 1970, Gould *et al* 1983). This ability led to growth of *P.crustosum* over a pH range from 4.0 to 7.7 and a_w's from 0.91 to 0.99. Fungal detoxification reactions may produce compounds that give undesirable odours in contaminated foods. The production of methyl ketones from short carbon chain length fatty acids (C6 to C14) and conversion of sorbic acid into 1,3-pentadiene are examples of this problem.

Fungi possess a wide range of mechanisms which enable them to overcome many of the preservation techniques studied in Section 4. For this reason it would be unwise to depend upon a single preservative measure to control ketonic rancidity. It is common for food manufacturers to combine a number of preservation techniques in an effort to make them more effective and overcome microbial homeostatic mechanisms. The underlying principle is that intrinsic, extrinsic and implicit factors are all inter-related and should not be considered in isolation (Mossel 1983).

Combinations of reduced temperature and pH with appropriate levels of a_w are commonly used to extend the shelf-life of a product. Addition of preservatives may also improve the resistance of a food to spoilage. However, the selection of a preservative depends on its inherent characteristics. Organic acids such as sorbate are particularly affected by a_w, pH value, substrate availability and fat content.

The lauric acid oils should only be used where their unique properties are essential to the finished product (Section 1.2). Where they have to be used the conditions required to prevent ketonic rancidity may be summarised as follows:

1. Follow Good Manufacturing Practices (GMP) in any plant that processes or uses the lauric acid oils.

2. Store the lauric acid oils and their products at low temperatures (<4°C).

3. Store and package the lauric acid oils and their products in dark containers to exclude the light.

4. Pack in an inert atmosphere to exclude atmospheric oxygen.

These observations summarised in Chapter 5 suggest several areas where further research could be conducted. Some studies have already been undertaken. These include:

1. An investigation of the decarboxylation of sorbic acid by some members of the genus penicillium (AFRC grant held by J. L. Kinderlerer, Sheffield City Polytechnic).

2. The production of volatile methyl ketones by dermatophytes isolated from the feet of patients suffering from 'athletes foot'. This research may

well explain the 'cheese-like' odour of feet (collaboration between Sheffield City Polytechnic and The Royal Hallamshire Hospital, Sheffield).

3. An investigation into the effect of adding sunflower oil (mainly long carbon chain unsaturated fatty acids) to trilaurin to increase the rate of conversion of dodecanoic acid into 2-undecanone. (Undergraduate project, Janine Street, Department of Chemistry, Sheffield City Polytechnic).

The results in this study were obtained using fats or simple triglycerides as model substrates. Further work is required to confirm that they apply to a food system.

6. Conclusions

Fats and oils fell into three groups according to their fatty acid composition. Ketonic rancidity only occurred in fats and oils which contained short and intermediate carbon chain length fatty acids (C6 to C14). The lauric acid oils were particularly susceptible to ketonic rancidity as over 50% (w/w) of their constituent fatty acids are of short or intermediate carbon chain length. Coconut oil was the more prone to ketonic rancidity as it contained a higher proportion of short chain fatty acids than palm kernel oil.

Maximum fungal growth was detected on oils that contained a high proportion (>80% w/w) of long chain unsaturated fatty acids (olive and hazelnut oils). Less growth was detected on fats and oils that contained mainly long chain saturated fatty acids (beef tallow and cocoa butter). The fatty acid composition determined the physical properties (melting point, solubility in water) of fats or oils which influenced their susceptibility to fungal attack. The energy values of whole oils were similar and could not be used to account for the different fungal growth rates.

Fermentations with simple triglycerides confirmed the results using whole oils. There was no evidence for the production of ketones other than those containing one less

carbon atom than the parent fatty acid. Dodecanoic acid was less reactive as part of a simple triglyceride than in a mixed glyceride whole oil. This resistance of glyceryl tridodecanoate to fungal attack was attributed to the insolubility in water and high melting point of this substrate.

Experimental evidence suggested that the short and intermediate carbon chain length fatty acids (C6 to C14) present in the lauric acid oils could inhibit fungal growth. The most inhibitory fatty acid was decanoate (capric acid). This accounted for the greatest molar conversion of this acid in the lauric acid oils and the high yield (product per mg biomass) achieved when glyceryl decanoate was used as a sole carbon source. It is likely that the production of methyl ketones is the result of the operation of a detoxification mechanism for the removal of short and intermediate carbon chain length fatty acids from the environment.

Extrinsic factors were used to control fungal growth and prevent ketonic rancidity. In many cases homeostatic and detoxification mechanisms operated to permit fungal growth under unfavourable conditions. It was suggested that combinations of preservative measures should be employed to inhibit fungal growth and ketonic rancidity.

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