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CONVERSION OF COCONUT OIL TO METHYL KETONES BY TWO *ASPERGILLUS* SPECIES

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Key Word Index—*Aspergillus ruber*; *A. repens*; fungi; conversion; coconut oil; methyl ketones; shake culture.

Abstract—Isolates of *Aspergillus ruber* and *A. repens* have been grown on coconut oil as the sole carbon source in shake culture. Methyl ketones (C_5–C_{13}) were isolated by solvent extraction and analysed by combined gas chromatography and mass spectrometry. 2-Undecanone was the main volatile product reflecting the high concentration of dodecanoic acid in the original coconut oil. The reactivity of the individual short chain fatty acids as substrates for production of methyl ketones would appear to decrease with increasing molecular weight of the acid after taking into account the greater volatility of the lower molecular weight homologues. 2-Hexanone and 2-octanone were produced by all isolates in low concentration (< 1%). Nonanoic acid and 2-heptanone were converted into 2-octanone. Low concentrations of secondary alcohols were formed under aerobic conditions. It is suggested that the production of methyl ketones by partial β-oxidation is too closely related to mainstream metabolism to be of use in the biochemical taxonomy of the genus.

INTRODUCTION

High lipolytic activity has been found in many fungi, an observation which explains the increase in non-acylated fatty acids found in oil seeds and grain after fungal spoilage [1,2]. Previously we reported that 2-pentanone, 2-nonanone, 2-undecanone and the corresponding secondary alcohols were found in coconut after growth of four species of xerophilic fungi [3]. The fatty acid precursors, hexanoic, octanoic, decanoic, and dodecanoic, were converted by a modified β-oxidation to give reaction products one carbon atom less than that of the parent acid [3]. Considerable quantities of 2-hexanone and 2-octanone were isolated from coconut fermented by *Eurotium herbariorum* (Wiggers) Link, in addition to the expected homologues with an uneven number of carbon atoms [3]. *E. herbariorum* has been classified on the basis of small (< 5 μm) smooth lenticular ascospores with a trace of an equatorial furrow [4]. Other workers feel that *E. herbariorum* is better described as *E. rubrum* and *E. repens* [5]. This classification supercedes an older one where *Aspergillus ruber* Thom and Church and *Aspergillus repens* De Barry have been separated into two distinct species on the basis of similar ascospores but different colony growth, pigmentation and conidia [6].

The present study examines the occurrence of 2-octanone and 2-hexanone produced by different isolates of *A. ruber* and *A. repens* in suspension culture using coconut oil as the sole carbon source. An attempt was made to distinguish the two species biochemically.

RESULTS AND DISCUSSION

Under aerobic conditions the major volatile products from oxidation of coconut oil are the short chain aliphatic methyl ketones (C_5–C_{13}) (Table 1). Low concentrations (0.9%) of secondary alcohols were formed in this study unlike the higher yields which were obtained when oxygen was restricted [3]. In other investigations where vacuum distillation methods had been used to extract the volatile ketones, 2-heptanone and 2-nonanone predominated [3,7,8]. 2-Undecanone was the main product using a direct solvent extraction method reflecting the high concentration of dodecanoic acid in the original oil (Table 1). The results also indicate that there is a tendency for the rates of conversion of the fatty acids to decrease with increasing M_r so that tetradecanoic acid is virtually unreactive. It would seem that the low M_r ketones are lost, due in part to their greater volatility and in part to their greater solubility in water. There was no evidence for the production of methyl ketones of higher M_r than 2-tridecanone although 2-pentadecanone has been found in coconut oil [9]. The greater reactivity of octanoic acid was clearly demonstrated in a similar fermentation experiment using two different isolates of *A. repens* (CMI 298306-7) which failed to germinate after three days incubation at 25°. 2-Heptanone was the major reaction product (50%) but less than 0.02 mg ketones were produced. Similar results occurred when octanoic and decanoic acids were added separately to shake cultures of *Penicillium caseicolum*. 2-Heptanone was the main end product after incubation for 2 hr at 30° [10].

2-Octanone and 2-hexanone were produced by all isolates but in much lower yields than those reported previously [3]. The even numbered homologues can be derived from short chain fatty acids with an odd number of carbon atoms by deacylation and decarboxylation of the corresponding β-ketoacyl-CoA derivative. Heptanoic and nonanoic acids have been found in butter fat and coconut oil in low concentration [11,12]. In suspension culture nonanoic acid was converted to 2-octanone in 20% yield (Table 2). Dunlap and Perry [13] demonstrated an increase in the C_{11} and C_{13} fatty acids when *Mycobacterium* and *Achromobacter* species were grown

Table 1. Conversion of coconut oil (1.5 g) to aliphatic methyl ketones by isolates of *Aspergillus ruber* and *A. repens* after 3 day fermentation in shake culture at 25°

Fatty acid Carbon no.	Substrate* mg/100 mg oil	mmol/1.5 g	Products Methyl ketones†	μmol	% Yield
6:0	0.8	0.1	2-Pentanone	0.7	2.7
			2-Hexanone	0.1	
8:0	9.8	1.0	2-Heptanone	32.1	3.2
			2-Octanone	0.4	
10:0	6.3	0.6	2-Nonanone	26.9	4.9
12:0	49.5	3.7	2-Undecanone	49.7	1.3
14:0	16.7	1.1	2-Tridecanone	0.2	0.02
16:0	5.7	0.3			
18:0	2.2	0.1			
18:1	4.4	0.2			
18:2	0.3				

*Results are the mean of two esterifications and six analyses.

†Results are the mean of 12 fermentations using five different isolates of *A. ruber* and *A. repens*.

Less than 1% of the total volatiles isolated consisted of secondary alcohols.

The initial pH and a_w were 4.85 and 0.95 respectively.

on 2-pentadecanone as the sole carbon source. Addition of 2-heptanone to a 5 day suspension culture of *A. ruber* followed by a further 3 day incubation period resulted in *ca* 2% conversion to 2-octanone (Table 2). Methyl ketones may be metabolized in two ways. Firstly as terminal hydrogen acceptors where they undergo reduction to the secondary alcohol—the reaction rate appears to decrease with increasing M_r of the substrate [3], secondly they may be metabolized via a C_1 transfer mechanism [14] to form fatty acids which subsequently undergo deacylation and decarboxylation to form different ketones.

Low concentrations of 2-pentanone and 2-heptanone (5 μM) were formed in an 8 day suspension culture without added fatty acids but with 3% sucrose as the sole carbon source (Table 2). Similar ketones were found after 5 day growth of *A. clavatus* on a Czapek type medium where sucrose was again the sole carbon source [15]. Short chain fatty acids are known to inhibit growth and oxidative phosphorylation. Toxicity is pH dependent, the unionized acids shows greater toxicity than that of the anion [16]. If the rates of release of the individual fatty acids from the triacylglycerols is greater than the rate of complete β-oxidation of the individual fatty acids, the excess fatty acids may be converted into methyl ketones; this process is energetically wasteful. It is suggested that the synthesis of methyl ketones by certain fungi is either a response to the presence of short chain fatty acids in the environment [17], or a method of recycling CoA under unfavourable conditions for growth [17]. In this study oxygen was available to act as a terminal hydrogen acceptor. Under aerobic conditions it would be unnecessary to deacylate the β-ketoacyl-CoA derivative in order to regenerate CoA. Stokoe's original suggestion that the production of methyl ketones is a response to the presence of toxic short chain fatty acids (C_6–C_{12}) in the environment would make sense [17].

No clear biochemical differences were observed for the different isolates although some strains gave high yields and others low yields (Table 3). Clear differences in colony growth and pigmentation were observed for the two *Aspergillus* species. The synthesis of methyl ketones would appear to be too closely related to the mainstream metabolism of fatty acids to be of use in the biochemical taxonomy for this genus.

EXPERIMENTAL

Extraction of coconut oil. Desiccated coconut (Sri Lankan) was obtained as a gift from Geo Bassetts Ltd., Sheffield. Coconut

Table 2. Volatile products (μmol) arising from the addition of 2-heptanone and nonanoic acid to a 5 day suspension culture of *A. ruber* (CMI 298309) followed by a further 3 day incubation

Volatile products	2-Heptanone 356 μM	Nonanoic acid 286 μM	Control μM
2-Pentanone	11.1	9.9	5.5
2-Hexanone	0.3	1.5	ND
2-Heptanone	207.7	9.7	5.0
2-Octanone	17.0	60.9	ND
2-Nonanone	0.2	0.7	ND
Recovery (%)	65.0	28.9	

ND = not detected.

Table 3. Aliphatic methyl ketones (μmol) produced by fermentation of coconut oil (1.5 g) for 3 days at 25° by different isolates of *Aspergillus ruber* and *A. repens*

	A. repens			*A. ruber*	
CMI number	298304	298305	298308	298306	298309
2-Pentanone	2.6	1.8	2.3	ND	7.9
2-Hexanone	0.1	0.1	0.2	ND	0.1
2-Heptanone	31.3	16.7	45.0	0.3	79.8
2-Octanone	0.6	0.5	0.3	0.1	0.4
2-Nonanone	22.8	13.7	43.5	0.2	32.5
2-Undecanone	53.0	33.1	94.6	1.9	82.4
2-Tridecanone	ND	0.1	ND	ND	1.3

ND = not detected.
The results are the mean of two fermentations per fungal isolate.

(200 g) was extracted with dry Et_2O (4 × 200 ml) by soaking for 30 min at ambient temp., filtering (Whatman no. 1) and evaporating the combined filtrates to dryness under red. pres. Residual Et_2O was removed by N_2 and the oil stored at 4°.

Fermentation conditions. The following cultures were isolated during 1982–84 and deposited at the Commonwealth Mycological Institute. *A. ruber* (CMI 298303 and CMI 298309) both from Sri Lankan coconut; *A. repens* (CMI 298304) isolated from oats (U.K.); CMI 298305 from hazelnuts (Turkey); and CMI 298306–8 from rancid coconut [18]. Stock cultures were maintained at 4° on malt extract agar (Oxoid) to which sucrose (20% w/v) (MEA 20S) was added. The isolates were sub-cultured every 9 months. (a) *Shake cultures.* Fermentations were made at 25° in cotton-wool stoppered 250 ml Erlenmeyer flasks containing coconut oil (1.5 g) and a modified Czapek medium (45 ml) with the following composition per litre: $NaNO_3$ (2 g), KH_2PO_4 (1 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), KCl (0.5 g), $FeSO_4 \cdot 7H_2O$ (0.01 g), $ZnSO_4 \cdot 7H_2O$ (0.01 g), $CuSO_4 \cdot 5H_2O$ (0.005 g), and glycerol (200 ml). All reagents were Analar grade and media were made up with glass-dist. H_2O. Flasks were sterilized at 121° for 15 min before use. Spore suspensions of individual organisms were prepared from two slopes of 11-day-old culture on MEA 40S grown at 20° by washing × 3 with sterile H_2O (2 ml) and shaking for 10 sec. Spore suspension (3 ml) was added to each flask. Fermentations were performed in duplicate in an orbital shaker at 200 rpm at 25° for 3 days. The initial pH and a_w were 4.85 and 0.95, respectively. (b) *Suspension cultures.* Czapek glycerol medium (300 ml) containing sucrose (3% w/v) and yeast extract (0.01% w/v) was added to each of two micro-carrier pots (500 ml) and sterilized at 121° for 15 min. Spore suspension (5 ml) was added to each flask prior to incubation for 5 days at 60 rpm on a micro-carrier-stirrer and 25° until a dense pellet growth occurred. 2-Heptanone and nonanoic acid were added to separate flasks which were incubated for a further three days prior to extraction.

All glassware was washed with acid (0.02 M HCl) for 2 hr to inactivate any detergent, followed by tap and dist. H_2O (× 15).

Extraction of methyl ketones and secondary alcohols. (a) *Shake cultures.* CH_2Cl_2 (3 ml) and 6-undecanone (10 μl) (int. std) were added to each fermentation flask which was shaken for 100 sec and filtered through acid washed glass wool. The residue was rewashed (2 × 1 ml CH_2Cl_2) to dissolve the residual oil. The combined filtrates were shaken for 100 sec, centrifuged (20 min at 4500 rpm) and the lower CH_2Cl_2 layer removed, re-centrifuged under the same conditions and dried ($MgSO_4$) overnight before storage at 4°. (b) *Suspension cultures.* CH_2Cl_2 (1 ml) and 6-undecanone (20 μl) (int. std) was added to each flask. The contents were shaken for 100 sec, centrifuged for 20 min at 4500 rpm and the CH_2Cl_2 layer treated as before.

GC analysis of methyl ketones and secondary alcohols. The column (1.5 m × 4 mm i.d.) was packed with 10% (w/w) Carbowax 20M on acid washed Chromosorb WAW 80/100. N_2 was used as the carrier (40 ml/min). The injection port was maintained 250° and the column was programmed from 70° to 180° at 1.5° per min. The FID was kept at 220°. R_ts relative to 6-undecanone were determined and concns were obtained with reference to std mixtures of homologous Me ketones and secondary alcohols. Confirmation of identity was made by GC-MS using similar GC conditions.

GC analysis of fatty acids. The concn of individual fatty acids in the coconut oil was determined using the method of ref. [19]. Coconut oil (50 mg) and undecanoic acid (9 mg) (int. std) were esterified using MeOH. Chromatography was performed using two columns, 10% Carbowax 20M on acid washed Chromosorb WAW 80/100 (for short chain Me esters) and 10% DEGS on Supelco-port 100/120 (for long chain Me esters). Confirmation of identity was made with reference to std fatty acid Me esters (BDH and Sigma Chemicals) and the response factor with reference to undecanoic acid determined so that the low recovery of hexanoic and octanoic acids could be taken into account.

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