barring the present day Ethiopean and Oriental species, died by the middle of the Pleistocene as evidenced from the Siwalik fossils. Of late, three species were more or less happily thriving in the Indian subcontinent, *viz.* *Rhinoceros unicornis* Linnaeus, *R. sondaicus* Desmerest and *Dicerorhinus sumatrensis* (Fischer). Among them the *R. unicornis* was extensively distributed in the northern India[5], but dwindled to a vulnerable position, localized in the foothills of Nepal, Assam and West Bengal.

It appears that the Great Swampy Forest of Sunderban provided ideal shelter to both the greater and lesser rhinos along with massive swamp buffalo[6]. Unfortunately, these animals were systematically killed by the hunters in the 19th century. Baker[7], in spite of his interest in zoology, shot dead numerous rhinos in the lower Bengal, alleging that the species was multiplying too fast. Increase in predation of calves by tigers also contributed to their decline in numbers.

The fossil species from south India and Sri Lanka, excepting one unconfirmed molar of *R. unicornis* from Tamil Nadu[8], were of different species, *viz.* *R. deccanensis* from the Pleistocene Krishna Valley, *R. karnulensis* from the Ossiferous bed of Karnool, *R. sinhalensis* and *R. kegvena* from the Pleistocene Ratnapura, Sri Lanka. Jayakaran[9] reported one partial skull of rhinoceros from Tamil Nadu. But its taxonomic position could not be ascertained. Therefore, the present occurrence, is redolent of a more southern habitat of the great Indian rhino. This discovery may help present day planners interested in finding habitats for the species in some suitable pockets of its earlier domains.

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Influence of nitrogen status and mutation on the fatty acid profile of *Rhodotorula gracilis*

G. Venkateswaran, K. Shashi and Richard Joseph
Microbiology and Sanitation Discipline, Central Food Technological Research Institute, Mysore 570 013, India

We isolated the mutants of *Rhodotorula gracilis* that produce low levels of lipid. These mutants were deficient in ATP:citrate lyase, a key enzyme in lipid over-production. Additionally, these mutants were altered in the fatty acid profile, most pronounced alteration was seen in the mutant CFR-9 which produced only 25% of the lipid compared with the parent strain. In this variant a six-fold decrease in myristic, a two-fold decrease in palmitic and linolenic, but a four-fold increase in linoleic acid were seen.

SOME of the yeasts, mycelial fungi and algae are referred to as oleaginous organisms by virtue of their ability to accumulate large quantities of triacyl glycerol as storage lipid, which they most often do in carbon-rich and nitrogen-poor growth media[1]. This is an example of nutrition stress-induced modulation of physiology and metabolism in microorganisms, a phenomenon not so well understood so far. Besides such a quantitative change, qualitative changes in the liquid in terms of the composition of fatty acids have also been found in the oleaginous organisms cultured at non-optimal pH or temperature conditions[2–5]. Non-oleaginous microorganisms have also been known to respond to environmental stress by altering the fatty acid composition of their lipids. Ingram *et al.*[6] found that *Escherichia coli* grown in medium containing ethanol modifies its lipid by increasing the level of vaccenic acid (C18:1, Δ^{11}) with corresponding decrease in palmitic acid (C16:0). Changes in fatty acid composition of lipid were also detected when *E. coli* was cultured at a suboptimal temperature[7]. Beaven *et al.*[8] found that *Saccharomyces cerevisiae* exposed to high levels of ethanol produces a lipid with extensive modification, most pronounced in the increase of oleic acid (C18:1, Δ^{9}) and decrease in palmitic acid (C16:0).

The biological significance of the modification of fatty acid profile of lipid by the organism in response to changes in environmental factors is obscure. Presumably these organisms cope with environmental changes by suitable adjustment of their lipid composition. Our interest has been focused on finding genetic and biochemical factors influencing modification of fatty acid profile of *R. gracilis*. This yeast accumulates large quantities (about 60% w/w) of triacyl glycerol as storage lipid[9]. In the present studies, it was the objective to see if mutation could cause modification of fatty acid profile as this would be a first step in

attempts to understand the genetic basis for modification in fatty acids. Furthermore it is also our interest to use such knowledge to genetically manipulate this yeast for the production of tailor-made lipids.

All the chemicals were of analytical grade. Fatty acid standards, Sudan black-B, adenosine triphosphate disodium salt, coenzyme A disodium salt, NADH, tripotassium citrate, magnesium chloride and malic dehydrogenase were from Sigma.

Rhodotorula gracilis CFR-1 is a locally isolated strain, which was found to be capable of accumulating lipid up to 60% of its dry weight[9]. The organism being *R. gracilis* was confirmed by the National Collection of Yeast Cultures, Norwich, UK. It was routinely maintained on YEPD slants (yeast extract 10g, peptone 20 g, D-glucose 20 gl^{-1} of distilled water, pH 5.8). This culture was subjected to UV mutagenesis using Camac model UV-lamp and from among 20% survivors several variants that produced less lipid than the wild type strain were selected after microscopic observation of cells stained with Sudan black-B for visualizing oil globules inside cells adopting the method of Burdon[10]. For lipid production, the cultures were grown in the basal medium containing yeast extract 1.5 g NH_4NO_3 0.286 g, KH_2PO_4 0.75 g, $CaCl_2$ 0.4 g, $MgSO_4 6H_2O$ 0.4 g and D-glucose 40 gl^{-1} of distilled water, pH 5.8, under both nitrogen-limiting and nitrogen nonlimiting conditions. For nitrogen-limiting condition, NH_4NO_3 was added at the level of 0.286 $g\,l^{-1}$ and for nitrogen nonlimiting condition, it was added at 2.86 $g\,l^{-1}$ final concentration. Typically, 50 ml of the medium taken in 250 ml-Erlenmayer flask was inoculated with two loopsful of the yeast culture from slant and grown at 30°C on a gyrorotary shaker for 48 h. The culture was then centrifuged at 5000 rpm for 10 min, and the cells were washed with distilled water and filtered through a pre-weighed Whatman No. 42 filter paper. The filter paper containing cells was dried in an oven at 105±2°C for about 6 h and weighed to constant weights, and then taken up for estimation of the lipid.

Total lipid content of the cells was estimated by taking a known amount of dry cells obtained as above. It was wrapped in Whatman No.1 filter paper and hydrolysed with 1N HCl over boiling water bath for 1h. After removal of excess acid by washing, the filter paper packet was dried and the lipid extracted with petrolium ether (60–80°C BP) in the soxhlet apparatus. The solvent was distilled off prior to determination of lipid content by gravimetry. The fatty acid profile of the extracted lipid was determined by gas chromatography by using authentic fatty acid standards[11]. The relative percentage of fatty acids was determined using the Schimadzu C-R 3A integrator connected to gas chromatographic equipment. Enzyme assay: ATP:citrate lyase was assayed by coupled malic dehydrogenase method of Srere[12] as modified by Takeda *et al.*[13]

Description of two of the UV-induced variants of *R.gracilis*, namely CFR-9 and Rg (small), as compared to the wild type strain CFR-1 is given in Table 1. Table 1 also shows the amount of lipid produced by the cultures under both nitrogen-limiting and nonlimiting conditions and, the corresponding levels of the enzyme ATP:citrate lyase. The following three aspects of the data became apparent: i) The variant cultures Rg (small) and CFR-9 cultivated under the nitrogen-limiting condition produce much lower amounts of lipid per unit biomass, hence differing in this respect distinctly from the wild type CFR-1 strain; ii) cultured under the nitrogen nonlimiting condition, the differences in the lipid content of the variants from that of the wild type strain are very marginal, thus the variants become indistinguishable from the wild type under the nitrogen nonlimiting condition; iii) the ATP:citrate lyase level in the variants is much lower than in the wild type irrespective of whether the cultures were grown under nitrogen-limiting or nonlimiting conditions.

The data suggest that the variants Rg (small) and CFR-9 have sustained defect in the lipid accumulation process as a consequence of low levels of ATP:citrate lyase in them. This enzyme catalyses the conversion of

Table 1. Description of colour and colony morphology variants obtained after UV mutagenesis of *R. gracilis*

Strain	Description	Lipid (% w/w) in nitrogen-limiting condition	Lipid (% w/w) in nitrogen nonlimiting condition	ATP: citrate lyase (relative %)
R. gracilis CFR-1 (wild type)	Smooth, round orange red colonies	65	25.0	100
Rg (small)	*Small, smooth,* round orange red colonies	25	19.4	14
CFR-9	Smooth, round, *pale yellow* colonies	16	20.2	12

Table 2. Gas chromatographic analysis of lipid produced by *R. gracilis* CFR-1 and its variants in nitrogen-limiting and nitrogen nonlimiting media

	Fatty acid (w/w%) of the total lipid in					
	nitrogen-limiting medium			nitrogen nonlimiting medium		
Fatty acid	CFR-1	CFR-9	Rg (small)	CFR-1	CFR-9	Rg (small)
Myristic (C14 0)	1 8	0.3	1.1	1 3	0.5	0.7
Palmitic (C16·0)	30.0	16 9	26 4	17.7	14 5	13.7
Stearic (C18.0)	4 6	4.0	4.7	5.5	7.9	1 4
Oleic (C18:1)	54 6	43 6	46 9	51.8	47.8	71.7
Linoleic (C18:2)	7.0	30.0	15 4	16.3	23.8	6 3
Linolenic (C18:3)	1 0	0.5	0 2	2.5	1 4	1.4

citrate in the cytosol in the yeast cell to oxaloacetate and acetyl CoA, the latter being the precursor for fatty acid biosynthesis. Other workers[14–18] have found this enzyme only in oleaginous yeasts and not in other organisms. A rate limiting role for this enzyme in lipid overproduction has therefore been suggested. Extra and corroborative biochemical evidence to this idea has been obtained by us earlier[9]. In the present work we provide for the first time a genetic evidence to the relationship of ATP:citrate lyase to lipid overproduction in the variants of *R. gracilis* CFR-1.

At this stage we had no reason to expect that a change in the fatty acid composition would have occurred in the variants of *R. gracilis* CFR-1 because, the lower production of lipid in CFR-9 and Rg (small) *were thought to be due merely to defect in* overproduction of lipid (associated with ATP:citrate lyase). Data in Table 2 on the fatty acids composition of lipids extracted from the cultures have however been quite contrary to our expectations. Under both situations of the nitrogen status, significant changes in the fatty acid profile of the variants were discernible. In the cultures grown under nitrogen-limiting condition CFR-9 has a six-fold decrease in C14:0, a two-fold decrease in C16:0 and a two-fold decrease in C18:3 $\Delta^{9,12,15}$ but a four-fold increase in C18:2 $\Delta^{9,12}$ compared with the wild type CFR-1. In the Rg (small) a two-fold increase in C18:2 and a five-fold decrease in C18:3 $\Delta^{9,12,15}$ were seen.

In the cultures grown under nitrogen nonlimiting condition CFR-9 shows a two-fold decrease in both C14:0 and C18:3 $\Delta^{9,12,15}$. In the Rg (small) a significant increase in C18:1 Δ^{9} and decrease in C18:2 $\Delta^{9,12}$ were found.

The general conclusion from these observations is that, under either conditions of nitrogen status, the variants of *R.gracilis*, besides being low producers of total lipid, have altered composition of the fatty acids as well. The manner in which the fatty-acid composition has changed might suggest that there has been, in general, activation of processes of fatty-acid chain elongation and desaturation. This awaits confirmation by the measurement of the levels of the enzymes involved. It may appear unreasonable to expect that ATP:citrate lyase level can modify the fatty acid profile, since this enzyme provides primarily acetyl CoA, the precursor for fatty acid biosynthesis. However, questions could be raised as to whether factors related to acetyl CoA level *per se* would regulate the process of chain elongation and desaturation. On the other hand, variants of *R. gracilis* CFR-1 may also have sustained additional mutations which influence the chain elongation and desaturation processes quite independent of the mutation affecting ATP:citrate lyase levels. These questions will also need further examination. Although the present data underscore the role of nitrogen status in the lipid overproduction by oleaginous yeasts found by other workers[14,19,20], we *have obtained additional data that the nitrogen status* itself can also cause modification in the fatty acid composition in *R. gracilis*.

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Antifertility and abortifacient activities of vicolides B and D

Muzaffer Alam, T. Susan, S. Joy and A. B. Kundu
Captain Srinivasa Murti Drug Research Institute for Ayurveda,
A. A. Government Hospital Campus, Arumbakkam,
Madras 600 106, India

Vicolides B and D isolated from *Vicoa indica* DC. showed antifertility activity in rats. The inhibition of implantation and abortifacient activities of vicolide D at 200 mg per kg body weight were found to be 52.43% and 71.43% respectively. The antiprogestational activity of vicolide D was evident from its ability to affect the deciduoma formation in the uterus of pseudopregnant rats. The mixture of vicolides B and D in the ratio of 1:1 at a dose of 100 mg per kg body weight showed 28.08% inhibition of implantation and 70% antifertility activities. It had 50% and 62.5% abortifacient activity from day 8 to day 14 and from day 14 to day 21 respectively.

Vicoa indica DC. of the family Compositae is used by the tribal women of Bihar as an antifertility agent. Of the four vicolides isolated from this plant, vicolides B and D had shown (Figure 1) antifertility and abortifacient activities in albino rats[1–4]. Vicolide D had 71% antiimplantation activity at a dose of 100 mg per kg body weight[4]. Further antifertility activity of vicolide D was assessed at a dose of 200 mg per kg body weight besides its antiprogestational activity at a dose of 100 mg per kg body weight.

Vicolide B causes resorption of implants[1,2] whereas vicolide D prevents implantation[4]. The amount of vicolides B and D present in the plant varies with the maturity of the plant. Our observations showed that vicolide B is present in good quantity before the flowering stage while vicolide D dominates during the peak flowering period. The plant at preflowering stage is fit for antifertility use according to field reports from observations in the tribal areas.

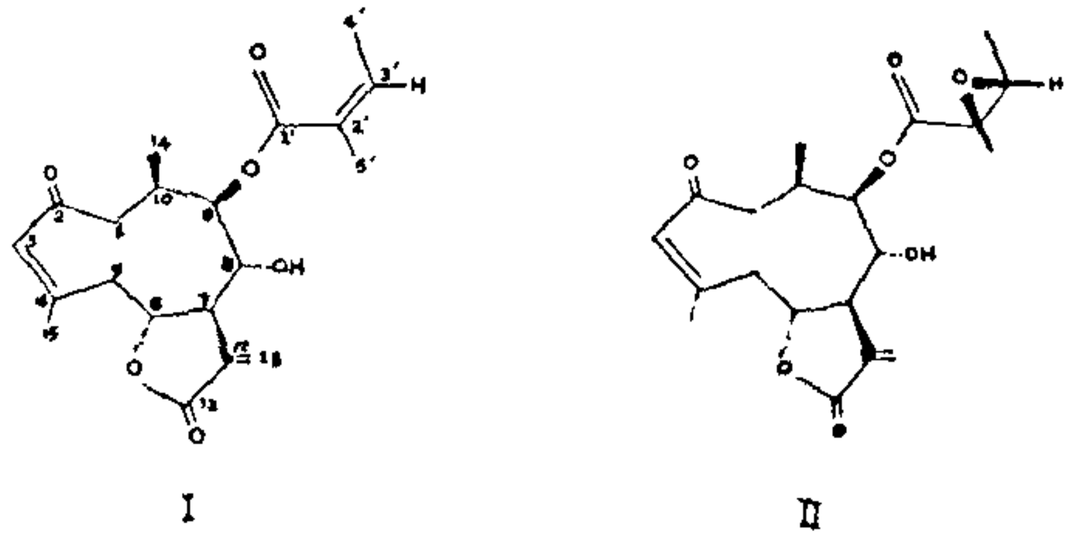
The fact that the whole herb is consumed by the tribals for contraception together with the difference in the degree and differential mode of action of the isolated vicolides prompted us to investigate the levels of combined activity of vicolides B and D.

The plant *Vicoa indica* DC. was collected from the neighbourhood of Madras city. Vicolides B and D were isolated according to the procedure detailed by Purushothaman *et al*[5,6].

Antiimplantation activity. Proven fertile female rats of Wistar strain weighing 150–200 g were screened for 2–3 oestrous cycles by examining the vaginal smears. The rats that showed normal cycles for two successive examinations were selected for the study. The method of Khanna and Chowdhuri[7] was followed with necessary modifications. The rats in prooestrous and oestrous stages were caged with fertile males in the ratio 3:1. The following day vaginal smears were examined and the appearance of sperm clusters in the smears was recorded as day 1 of pregnancy. Vicolide D was administered orally in suspension in 0.5% carboxy methyl cellulose at a dose level of 200 mg per kg body weight from day 1 to day 5. Control animals received vehicle only. On day 10 laparatomy was performed under light ether anaesthesia to examine uteri for implant number and size. Then the abdomen was closed and rats were allowed to recover and deliver after full term of pregnancy. Those rats that did not deliver were laparatomized on day 25 and uteri were examined for implantation sites. The number of implants present in vicolide-D-treated rats on day 10 was compared with the control group rats to determine the per cent of inhibition of implantation.

Abortifacient activity. The method of Khanna and Chowdhuri[5] was followed with necessary modifications as described earlier. Rats at day 1 of pregnancy were divided into two groups. The first group served as control and was fed vehicle only while the second group was administered vicolide D at 200 mg per kg body weight from day 14 to day 21 of pregnancy. Other experimental conditions were the same as detailed under antiimplantation study.

Antiprogestational activity. The antiprogestational activity of vicolide D was evaluated with reference to its ability to inhibit deciduoma formation in traumatized uterine horn of pseudopregnant rat. The experiment was carried out according to the method of Zarrow *et al*[8].



I II

Figure 1. Vicolides from *Vicoa indica* DC. I, vicolide B; II, vicolide D.