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# Biooxidation of Fatty Acid Distillates to Dibasic Acids by a Mutant of *Candida tropicalis*

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**Abstract: Fatty acid distillates (FADs) produced during physical refining of vegetable oil contains large amount of free fatty acid. A mutant of *Candida tropicalis* (M20) obtained after several stages of UV mutation are utilized to produce dicarboxylic acids (DCAs) from the fatty acid distillates of rice bran, soybean, coconut, palm kernel and palm oil. Initially, fermentation study was carried out in shake flasks for 144 h. Products were isolated and identified by GLC analysis. Finally, fermentation was carried out in a 2 L jar fermenter, which yielded 62 g/L and 48 g/L of total dibasic acids from rice bran oil fatty acid distillate and coconut oil fatty acid distillate respectively. FADs can be effectively utilized to produce DCAs of various chain lengths by biooxidation process.**

**Key words:** dibasic acids, *Candida tropicalis*, fatty acid distillate, biooxidation, fermentation

## 1 INTRODUCTION

Long chain dicarboxylic acids (DCAs) serve as raw materials for preparation of several important high value chemicals[1,2]. Normally these acids are produced from synthetic petroleum products. There are several chemical routes to obtain these bifunctional fatty acids. However, all of these routes involve several steps and thereby increasing the overall cost. Moreover, most of the DCAs produced by the methods are of short chain length. Microbial fermentation technology involves an alternative route for converting oleochemicals into high value compounds, but only a limited number of reactions are applied in industries[3]. Most of the reports available are on production of dibasic acids from hydrocarbons[4,5]. Fatty acids could also serve as an alternative substrate for oxidation[6-8].

Present trend is to substitute non-biodegradable ingredients present in lubricants, greases, functional fluids by fat based biodegradable materials and also to develop comparatively low cost technologies by choosing cheaper substrate for bio-transformation. Biorenewable raw materials like vegetable oil and fatty acids are interesting substitutes for petrochemical feed stocks in chemical industry[9,10]. Crude rapeseed oil and post refinery fatty acids produced oxalic acid under optimized pH conditions by a mutant of *Aspergillus niger*[11]. D. Fabritus *et al*.[12] utilized sunflower oil and rapeseed oil as substrate for bioconversion to dibasic acids in batch fed fermentations.

Fatty acid distillates (FADs) are a major by product produced during physical refining of fats and oils, which are a rich source of different long chain fatty acids and also available at a comparatively low cost than the hydrocarbons or industrially prepared pure fatty acids. So, the present study concentrates on using different FADs i.e. coconut oil (CNO), palmkernel oil (PKO), palm oil, rice bran oil (RBO), soybean oil (SBO) as raw materials to produce dicarboxylic acids of different chain length by a mutant of *Candida tropicalis* (M20).

## 2 EXPERIMENTAL

### 2.1 Yeast strain

A mutant of *C. tropicalis* (M20) obtained after several sets of mutations under ultraviolet (UV) radiation of the parent strain purchased from the reputed culture collection center of IMTECH, Chandigarh, India. A detail investigation was done about the ability of the mutant to utilize fatty acids of different chain length to produce dibasic acid of same chain length as the major product.

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### 2.2 Fermentation

For biooxodation study *C. tropicalis* was cultivated in 100 mL medium A (Composition: Glucose: 2.5 g/L, Urea: 5 g/L, Yeast extract: 0.5 g/L, $KH_2PO_4$: 0.1 g/L, $MgSO_4$: 0.04 g/L, $CaCl_2 2H_2O$: 0.02 g/L, $FeSO_4$: 0.002 g/L, pH of the solution $\simeq$ 6.5, in a 500 mL conical flask. 5 mL preculture was used as inoculum. After 48 h of growth, 10 g/L substrate was added to the medium. The flask was kept in a Biological Oxygen Demand (B.O.D) Shaker Incubator and the temperature was kept constant at 32℃. The pH of the medium was maintained at 6.5 for the first 48 h of fermentation (after substrate addition) and then subsequently raised to pH 7.5, where it was kept constant by periodical addition of 1N NaOH. Extraction and analysis was done after 144 h of fermentation.

### 2.3 Extraction and qualitative analysis of DCAs

To determine the production of dioic acid 10 mL of the medium was extracted thrice with equal volume of n-hexane. The hexane layer was passed through anhydrous $Na_2SO_4$ and evaporated to dryness for determination of unused substrates. The aqueous extract was then centrifuged at 10,000 × g for the separation of cell and other basic precipitated materials. Then the clear solution was adjusted to pH 2 by 6N HCl, boiled for few minutes. After cooling, medium was extracted thrice with equal volume of diethyl ether. Finally the ether layer passed through anhydrous $Na_2SO_4$ and evaporated to dryness to get a pale orange oily residue. The product obtained is the total amount of DCAs produced.

### 2.4 Quantitative estimation of DCAs produced

The residue obtained was dissolved in 10 mL of warm neutral alcohol (90%, v/v) and titrated with 0.04M NaOH. The data taken were the mean value of samples. The concentration of dicarboxylic acids was determined from the amount of 0.04M NaOH consumed, considering octadecanedioic acid as standard. The concentrations are presented as the total of all $\alpha,\omega$-dicarboxylic acids regardless of carbon chain-length.

### 2.5 Purification of the products

The oily residue obtained after drying over anhydrous sodium sulphate was purified by flash chromatography with the solvent system of tetrahydrofuran (THF): Petroleum ether[12].

### 2.6 Gas chromatographic method of analysis for dibasic acids

Samples esterified with $BF_3$-methanol were analyzed through a Hewlett Packard (HP) -5890A Gas Liquid Chromatography (GLC), connected with a HP-3390A data integrator. The GLC was fitted with a S.S. column (2m × 2 mm i.d.), packed with 10% SE-30 supported on Chromosorb-WHP (80/100 mesh), of HP make. The oven temperature was programmed from 100-230℃ at 7℃/min. the injector and detector block temperatures were maintained at 220℃ and 240℃ respectively. IOLAR-2 nitrogen was used as the carrier gas (flow rate 30 mL/min). The dibasic acid ester peaks were identified and calibrated with standard methyl esters.

### 2.7 Fourier transform infrared (FT-IR) spectroscopy

An FT-IR spectrum was recorded on a *JASCO* FT-IR 670 plus spectrometer to detect the formation of $\omega$-hydroxy acids in the products if any.

### 2.8 Analysis of the fatty acid distillates

Acid value, un-saponifiable matter and fatty acid composition of the fatty acid distillates are measured by standard methods.

### 2.9 Evaluation of the biotransformation of fatty acid distillates in a jar fermenter

Initially the data obtained from fermentation in shake flasks was evaluated in a 2 L jar fermenter (Model M-100, Tokyo. Rikakikai Co., Japan). The variables of fermentation conditions like pH of the conversion medium, concentration of the substrates added and time of fermentation to obtain maximum yield are optimized.

| | |
|---|---|
| Conversion medium | : Medium A |
| Innoculum | : 25 mL of preculture from growth medium (Medium II) of composition; Glucose: 1 g/L, Malt extract: 3 g/L, peptone: 5 g/L, Yeast extract: 3 g/L, pH $\simeq$ 6.5. along with oleic acid |
| Subtrate | : Rice bran oil FADs and Coconut FADs |
| Rate of addition of substrate | : Single time addition after 48 h of growth |
| Stirring speed | : 500-600 r.p.m. |
| Aeration | : 1 mL/min, alternately every after 1 min. |
| pH | : maintained at 6.5 for 48 h and then raised to 7.5 using 1N NaOH |
| Anti foam used | : silicon oil (automatically added according to requirement) |
| Sample collection | : 72 h, 96 h, 120 h and 144 h |

## 3 RESULTS AND DISCUSSIONS

Strains of *Candida tropicalis* are well known for their capability of oxidizing different alkanes and fatty acids to

dibasic acids of different chain length. In the present study different strains of *C. tropicalis* were screened to select an efficient mutant. Of the strains collected MT2 (Originally CT230, IMTECH, Chandigarh), showed highest productivity (0.5 g/L) of DCAs from oleic acid (10 g/L) and was chosen for a treatment with UV radiation. Fig 1 shows the improvement of the strain by multistage mutation with UV radiation. After several sets of mutation M20 was obtained which showed 20 fold better activity than the original strain.

During physical refining of rice bran, soybean, coconut, palm kernel and palm oils, fatty acid distillates of the respective oils are produced. The typical samples of fatty acid distillates collected for our bioconversion study are analyzed for their free fatty acid (FFA) content and unsaponifiable matter are tabulated in Table 1. The CNO and PKO FADs contains 71.3% and 61.2% FFA (as lauric) respectively whereas RBO, SBO and Palm FADs contains 80.5, 85.5 and 85% free fatty acids (as oleic) respectively. Thus the FADs containing such a high percentage of fatty acids, could be used as raw materials to produce dibasic acids of different chain lengths.

The detail fatty acid composition of the FADs are presented in Table 2. The fatty acid composition of the FADs corroborates with the original composition of their respective oil. RBO, SBO and Palm FADs can be utilized as the source for preparation of long chain dibasic acids ($C_{16}$ & $C_{18}$) and PKO and CNO could serve as the source of medium chain length dibasic acids ($C_8$ to $C_{12}$).

Table 3 shows biomass yield (after 48 h) and the product obtained from the substrates after 144 h of fermentation in the shake flasks. RBO, SBO and Palm FADs produced 4.2, 4.0 and 4.2 g/L dry biomass respectively whereas CNO and PKO FADs yielded 3.2 and 3.3 g/L respectively. Probably

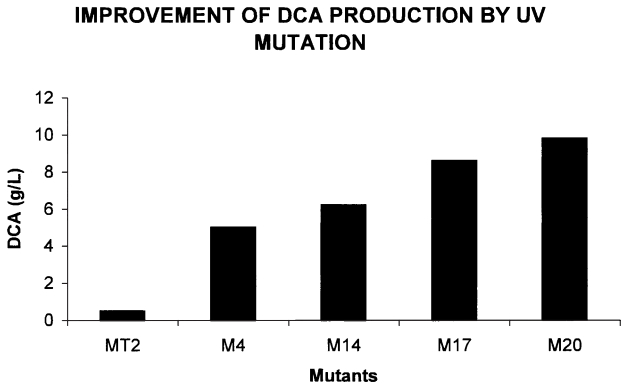


**Fig. 1** Multistage Mutation of the Wild Strain. (M4, M14, M17 & M20 are the best mutant obtained at each stage of mutation)

the low biomass content in presence of CNO and PKO FADs is due to minor toxicity of the low molecular weight fatty acids present in CNO and PKO FADs. RBO, SBO and Palm FADs produced 5.7, 5.5 and 5.7 g/L of total DCAs from 10 g of respective substrate after 144 h of fermentation. On the other hand CNO and PKO produced 3.7 and 3.4 g of total DCA under similar conditions. It can be stated from the observations that the FADs containing more proportion of long chain fatty acids i.e. $C_{16}$, $C_{18}$ are preferred as substrate for bioconversion by the mutant of *C. tropicalis* under study.

Table 4 gives the detail dibasic acid composition of the oxidized product obtained from the fatty acid distillates. Coconut oil FADs on biooxidation produces 36% of $C_{12}$ DCAs, 21% of $C_{18}$ DCAs, 19% and 14% of $C_{14}$ and $C_{16}$ DCAs respectively along with small amount of $C_8$ and $C_{10}$. Similarly PKO FADs produces 29%, 25%, 10% and 18% of $C_{12}$,

**Table 1** Analytical Characteristics of Coconut, Palm kernel, Soybean, Rice Bran Fatty Acid Distillates.

| Properties | Coconut | Palm | Palm kernel | Rice bran | Soybean |
|---|---|---|---|---|---|
| Free fatty acid (FFA) % | 71.3 | 85 | 61.2 | 80.5 | 85.5 |
| Unsaponifiable matter (%) | 0.5 | 0.5 | 1.2 | 14.5 | 2.8 |

**Table 2** Total Fatty Acid Composition of Coconut, Palm kernel, Soybean, Rice Bran Fatty Acid Distillates.

| | Fatty acids (%w/w) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 8:0 | 10:0 | 12:0 | 14:0 | 16:0 | 18:0 | 18:1 | 18:2 | 18:3 |
| Coconut | 4.5 | 8.6 | 45.5 | 17.2 | 9.9 | 1.4 | 10.8 | 2.1 | _ |
| Palm kernel | 11.2 | 10.7 | 38.6 | 24.1 | 7.7 | 1.6 | 4.3 | 1.8 | _ |
| Soybean | _ | _ | _ | _ | 22.6 | 1.4 | 17.4 | 55.5 | 5.1 |
| Rice bran | _ | _ | 1.7 | 1.5 | 29.3 | 1.2 | 37.5 | 28.8 | _ |
| Palm | | | - | 1.6 | 43.5 | 5.1 | 40.2 | 9.6 | - |

$C_{14}$, $C_{16}$ and $C_{18}$ DCAs respectively. On the other hand SBO, RBO and Palm fatty acid distillates mainly produce $C_{16}$ and $C_{18}$ dicarboxylic acids. The observation reveals that long chain fatty acids are more easily biooxidized to long chain DCAs than the shorter or medium chain fatty acids.

The biooxidation reaction has also been studied in a 2 L jar fermenter under optimum conditions using RBO and CNO FADs. The important process parameters like substrate concentrations, pH of the conversion medium and time of fermentation were already optimized after several trial runs using oleic acid as substrate (data not shown). The yield of oxidation products in the fermenter is shown in Table 5. Better conversion rate is observed in the fermenter under optimized conditions.

DCAs production from both RBO and CNO FADs is negligible within 72 h of study. The DCAs accumulation increases between 72 and 96 h, which give 15.1 g/L of total DCAs from RBO FADs and 10.6 g/L DCAs from CNO FADs. The maximum product formation is observed between 96 and 144 h of fermentation. At 144 h RBO FADs produce 62 g/L of total DCAs whereas CNO FADs gives 48 g/L of product. The lower yield from CNO FADs is also observed in the fermenter. The products are checked for the presence of any hydroxy acids by FT-IR spectroscopy. The spectroscopy graph (graph not shown) indicated the absence of any hydroxyl (-OH) group.

**Table 3** Bioconversion of Fatty Acid Distillates to Dibasic Acids.

| FAD | Dry biomass (g/L) | Residual lipid (g/L) | Product (g/L) |
|---|---|---|---|
| RBO | 4.2 | 2.1 | 5.7 |
| SBO | 4.0 | 2.3 | 5.5 |
| CNO | 3.2 | 3.0 | 3.7 |
| PKO | 3.3 | 3.5 | 3.4 |
| Palm | 4.2 | 2.3 | 5.7 |

Substrate used: 10g/L, Biomass recorded after 48 h of growth and DCA measured after 144 h of fermentation.

**Table 4** Dibasic Acid Composition of the Products by GLC Analysis .

| | Percent Dibasic Acid Composition | | | | | |
|---|---|---|---|---|---|---|
| SUBSTRATE | $C_8$ | $C_{10}$ | $C_{12}$ | $C_{14}$ | $C_{16}$ | $C_{18}$ |
| CNO FADs | 4 | 6 | 36 | 19 | 14 | 21 |
| PKO FADs | 5 | 4 | 29 | 25 | 10 | 18 |
| RBO FADs | | | | | 46 | 54 |
| SBO FADs | | | | 1 | 32 | 67 |
| PALM FADs | | | | 2 | 40 | 58 |

**Table 5** Bioconversion of FADs to DCAs with Time in a 2 L Jar Fermenter.

| | Yield of DCAs (g/L) with time (h) | | | |
|---|---|---|---|---|
| Substrate | 72 | 96 | 120 | 144 |
| RBO FADs | 2.34 | 15.1 | 43 | 62 |
| CNO FADs | Insignificant | 10.6 | 26 | 48 |

## 4 CONCLUSION

The present study clearly indicates the possibility of using SBO, RBO and Palm FADs as a good source of $C_{18}$ dibasic acid, which is an important industrial chemical. Similarly CNO and PKO could be an efficient producer of $C_8$ to $C_{12}$ dibasic acids. Moreover separation of different fractions of the FADs by fractional or molecular distillation, and concentrated fractions used as substrates will give dibasic acid of specific chain length depending on the composition of the substrate.

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