RESEARCH ARTICLE

Whole-cell Lipase Catalytic Synthesis of Short-chain Fragrance Esters Using *Aspergillus flavus*

Santosh K. Rath¹, Gurpinder Kaur¹, Anirudh Sharma², Anmol Singh³, Ranjana Prakash¹, Sudip Mandal² and Nagaraja Tejo Prakash^{4,*}

¹ School of Chemistry & Biochemistry, Thapar Institute of Engineering & Technology, Patiala-147004, Punjab, India;
²University Institute of Pietschnology, Chandiganh University: ³Dr. B. C. Bay College of Pharmagy and *University Institute of Biotechnology, Chandigarh University; ³ Dr. B. C. Roy College of Pharmacy and AHS, Durgapur-713206, West Bengal, India; ⁴ School of Energy and Environment, Thapar Institute of Engineering & Technology, Patiala, 147004, Punjab, India* Sol K. Rath¹, Gurpinder Kaur¹, Anirud
dal² and Nagaraja Tejo Prakash^{4,*}
ol of Chemistry & Biochemistry, Thapar Inst
ersity Institute of Biotechnology, Chandigarh
13206, West Bengal, India; ⁴School of **Using Aspergillus flavus**

Santosh K. Rath¹, Gurpinder Kaur¹, Anirudh Sharma², Annoc

Mandal² and Nagaraja Tejo Prakash^{4,4}

¹School of Chemistry & Biochemistry, Thapar Institute of Engineering

²University Example 1 and the mistry of Biochemistry, Thapar Institute of

Stitute of Biotechnology, Chandigarh Univer.

West Bengal, India; ⁴School of Energy

Patiala, 147004, Punjab, India
 Abstract: Background: Fragrances and
 Santosh K. Rath¹, Gurpinder Kaur¹, Anirudh Sharma², Anmol Sing
Mandal² and Nagaraja Tejo Prakash^{4,*}

²*School of Chemistry & Biochemistry, Thapar Institute of Engineering & Tech*

²*University Institute of B* Note of Chemistry & Biochemistry, Thapar Institute of Engineering & Technology,
versity Institute of Biotechnology, Chandigarh University; ³Dr. B. C. Roy College
13206, West Bengal, India; ⁴School of Energy and Enviro

Abstract: *Background***:** Fragrances are the collection of unlike functional assemblies, most likely alcohols, esters, aldehydes, ketones, and acids in organic products/hydrocarbons. Short-chain aliphatic fragrance esters have immense applications as flavors in the food, pharmaceutical and cosmetic industries and also have remarkable commercial significance in cosmetics and personal care products like perfumes, face creams, shampoos, soaps, lotions, jams, jellies, *etc*. Bengal, India; ${}^{4}School$ of Energy and 1
147004, Punjab, India
Abstract: Background: Fragrances are the co
hols, esters, aldehydes, ketones, and acids in
grance esters have immense applications as f
and also have remark For exters, aldehydes, ketones, and acids in organic
nece esters have immense applications as flavors in
also have remarkable commercial significance in c
reams, shampoos, soaps, lotions, jams, jellies, *etc*
ective: Thi

*Objective***:** This study aimed to synthesize short-chain fragrance esters using a whole-cell lipase catalyst from Aspergillus flavus (RBD-01).

A R T I C L E H I S T O R Y

Received: July 18, 2022 Revised: December 01, 2022 Accepted: December 02, 2022

DOI: 10.2174/1570180820666230222145117

*Methods***:** The present study emphasizes the synthesis of artificial flavoring compounds by using a wholecell biocatalytic process, which can have wide significance. Herein, the preparation of ethyl alkanoates (ethyl propanoate to ethyl decanoate) was performed to investigate the flavors and fragrance excellence. The biomass from Aspergillus flavus (RBD-01) was used as a catalyst to facilitate the remarkable esterification activities towards the synthesis of important aroma esters with the help of a series of short-chain acids and alcohols. Faturia, 147004, Funjato, final
 Abstract: Background: Fragrances are the collection of unlike functional as

hols, esters, aldehydes, ketones, and acids in organic products/hydrocarbons

grance esters have immense appl This study aimed to synthesize short-chain fragrand

Short-chain fragrand

Short-chain fragrand

In the present study emphasizes the synthesis of artificity

In the present study emphasizes the synthesis of artification

I and also have remarkable commercial significance in cosmetics and personal care prod
face creams, shampoos, soaps, lotions, jams, jellies, *etc*.
Objective: This study aimed to synthesize short-chain fragrance esters usi ethyl decanoate) was performed to investigate the
spergillus flavus (RBD-01) was used as a catalyst
wards the synthesis of important aroma esters with
exanoate (4) among all synthesized alkanoates wa
sion rate. Further syn In Aspeciginas invias (KDD-01)
 Whods: The present study emphasizes the synthesis of artificial flavoring compounds by using

al biocadaly tie process, which can have wide significance. Herein, the preparation of ethyl

*Results***:** The ethyl hexanoate (4) among all synthesized alkanoates was found to have a fruity fragrance with a good conversion rate. Further synthesized alkyl hexanoates (4A-4I) were found to have good fruity/pineapple/berry flavors and significant aroma quality.

Conclusion: These results implied that whole-cell lipase of Aspergillus flavus (RBD-01) is a promising biocatalyst in the production of flavor aroma esters and can boost production in the food/cosmetic manufacturing industries. EXEC 4) among all synthesized alkanoates was found
Executive use of the synthesized alkyl hexanoates (4A-4I) v
and significant aroma quality.

applied that whole-cell lipase of Aspergillus flavus

of flavor aroma esters an

Keywords: Alkyl esters, *Aspergillus flavus*, whole cell catalyst, lipases, fragrance esters, biocatalyst.

1. INTRODUCTION

Fragrances are a collection of functional assemblies, most likely alcohols, esters, aldehydes, ketones, and acids in organic products/hydrocarbons [1, 2]. The less volatility and low molecular weight (˂ 400Da) of organic molecules have been found to be significantly effective due to their fragrance in today's generation [2]. Flavor esters are short-chain volatile fragrant compounds with pleasant fruity aromas and remarkable commercial significance in food, beverages, pharmaceuticals, cosmetics, and personal care products like perfumes, face creams, shampoos, soaps, lotions, jams, jellies, *etc*. [1, 3]. Since the reports on the production of fragrance

esters from natural plant and animal sources are reasonably limited due to resource constraints and biodiversity concerns, there is significantly more inclination of researchers to explore the synthesis of such compounds through the biocatalytic approach. So solid expositions in the set of the synthesis of important aroma esters with the help of a series of short-characteristics towards the synthesized alternation around to have a fruity fragrand conversion rate. Further sy For a whole-cell lipste of Asperginus havis (RDD-0)
For aroma esters and can boost production in the food
allyst, lipases, fragrance esters, biocatalyst.
esters from natural plant and animal s
limited due to resource const esters from natural plant and animal sources
limited due to resource constraints and biodive
there is significantly more inclination of rese
plore the synthesis of such compounds through
lytic approach.
Biocatalysis is a s

Biocatalysis is a specific function of pure enzymes or whole cells (bacteria, algae, fungi, plants, *etc*.) for organic compounds' chemical transformation, facilitating regio/chemo and stereo-selective specificity [4, 5]. This process has gained prominence as a green and sustainable alternative over chemical catalysis due to better substrate selectivity, milder reaction conditions, minimal steps for reaction, less energy utilization, and the formation of significantly less/no harmful by-products [4, 6]. Whole-cell biocatalysts are much preferred over purified enzymes due to their cost-effectiveness, ease of production, and the facilitation of a much greener Solon rate. Further synthesized aikyl nexanoates $(AA-4I)$ were found to have good

ry flavors and significant aroma quality.

results implied that whole-cell lipase of Aspergillus flavus (RBD-01) is a promising

coluction cell catalyst, lipases, fragrance esters, biocatalyst.

esters from natural plant and animal sources are reasonably

esters from natural plant and animal sources are reasonably

limited due to resource constraints and bio e synthesis of such compounds through the to
proach.
atalysis is a specific function of pure enzy
cells (bacteria, algae, fungi, plants, *etc.*) for
nds' chemical transformation, facilitating regio
eo-selective specificit Source the to resource constraints and biodiversity concerns,
 $\frac{1}{10}$ limited due to resource constraints and biodiversity concerns,

there is significantly more inclination of researchers to ex-
 $\frac{1}{10}$ limited d

^{*}Address correspondence to this author at the School of Energy and Environment, Thapar Institute of Engineering & Technology, Patiala, 147004, Punjab, India; E-mail: ntejoprakash@thapar.edu

approach [6, 7]. Additionally, whole-cell biocatalysts facilitate economically feasible catalytic processes where the enzyme isolation and purification steps can be easily omitted with much better efficiency in multistep reactions [8, 9]. The use of intracellular lipases in whole-cell biocatalysts is now widely exploited in food, beverages, agricultural, and cosmetics fields [10]. Though plants and animals are considered good sources of lipase production, high yields of lipases have been obtained from different microbial sources, which are widely applied in organic synthesis [11]. Among the established whole-cell biocatalyst systems, filamentous fungi have proven to be robust organisms for industrial applications. sources of lipase production, high yields cover
been obtained from different microbial sources of lipase production, high yields ourced
idely applied in organic synthesis [11]. Amored whole-cell biocatalyst systems, filame

Our earlier study demonstrated the importance of the whole cell catalyzed transesterification using *Aspergillus flavus* (RBD-01) to generate biodiesel from acid oil containing 55 % free fatty acids by conversion of alkyl esters to the extent of 98%. The results revealed that bio catalyzed transesterification process is a unique way to utilize acid oil as feedstock for the production of fuel-grade biodiesel [12]. In the present study, we further demonstrate the use of this fungal whole-cell catalyst for the generation of fragrance esters from aliphatic acid by esterification. Enzymatic expression of *A.flavus* as a whole-cell biocatalyst seems to exhibit a higher catalytic effect when compared with studies carried out by Garlapati *et al*. [13], leading to the synthesis of fragrance esters with a high yield of 94% for nonanol and 84% in case of ethyl decanoate. use of intracellular lipases in whole-cell biocatalysts is now

widely exploited in food, beverages, agricultural, and cos-

metrics fields [10]. Though plants and animals are considered

good sources of lipase production to be robust organisms for industrial applicant
er study demonstrated the importance of the atalyzed transesterification using Aspergilla
01) to generate biodiesel from acid oil contain
fatty acids by conversion of alkyl e are videly applied in equation timeterial conditions sources, which
tablished whole-cell biocatalyst systems, filamentous funging inoculated fla
have proven to be robust organisms for industrial applica-
the state of the Exerces the sum of alkyl esters to the

generate biodiesel from acid oil contain-

For estails revealed that bio catalyzed trans-

is a unique way to utilize acid oil as

duction of fuel-grade biodiesel [12]. In

further Figure 11 and the importance of the linear in the section of the section of the section of alkyl sters to the set and the original space of the section of alkyl sters to the set any of alkyl sters to the set and the set o For personal private incomes of this function. Enzymatic expression

ification. Enzymatic expression

ification. Enzymatic expression

ification. Enzymatic expression

ification is the synthesis of the synthesis of the

in We The results revealed that bio catalyzed transportants and process is a unique way to utilize acid oil as a continization of Esteriff the three forms transfer biddlessel [12]. In the proposition of fundations of fragmen For personal private use of the synthesis of fraction of the synthesis of fraction of the synthesis of fraction of the product of th

2. MATERIALS AND METHODS

2.1. Chemistry Methods

To carry out the experimental work, the laboratory scale culture media and other media constituents *viz.,* mycological peptone, Bushnell Haas broth (BHB), potato dextrose broth (PDB), and potato dextrose agar (PDA) were purchased from HiMedia laboratories, India. Other chemicals, such as aliphatic alcohols (methanol to decanol), aliphatic acids (propionic to decanoic), hexane, ethyl acetate, silica gel *100-200 mesh* for column chromatography, Bi-ammonium hydrogen ortho-phosphate $(NH_4)_2HPO_4$, were procured from SD Fine-Chem Limited, India. All reagents used were of analytical grade. The solvents used in the compounds' isolation were distilled before use to carry out the present work. The purified compounds were monitored by TLC on 0.25 mm silica gel 60 F254 plates (Merck) and visualized with 2% ceric ammonium sulfate solution as spraying reagents for detecting spots on the TLC under UV illumination. Confirmation of the compounds was initially done by column chromatography using silica gel 100-200 mesh stationary phase. \overline{H} NMR and ¹³C NMR spectra were recorded on a 400 MHz NMR spectrometer (400 MHz; JEOL JNM-ECS 400) with $CD₃Cl₃$ as solvent with TMS as an internal standard. The chemical shifts were represented in δ ppm and coupling constants in Hertz. The abbreviations used were as follows: s, singlet; bs, broad singlet; d, doublet; dd, double doublet; t, triplet; m, multiplet. The spectral data were consistent with the assigned structures. A high-resolution mass spectrum (HRMS) and MS were recorded on an Agilent system (Technologies 6540) instrument. Notice the distribution of the distribution of the set and the distribution with the distribution with the distribution with the distribution with the purification products were quantified by NMR spectroscoperol analytica

2.2. Preparation of Biomass for use as a Whole-cell Biocatalyst

The 2 to 3 discs (5 mm) were cut from freshly grown culture on PDA plates of *A. flavus* (RBD-01) and inoculated aseptically in 500 ml Erlenmeyer flasks containing 200 ml of sterile PDB. The fully grown fresh culture was then inoculated aseptically into 200 ml minimal media containing nitrogen (mycological peptone (0.5% w/v), (NH4)2HPO4 $(0.5\%$ w/v), and carbon source (cottonseed oil $(10\%$ v/v). The inoculated flasks were incubated at $28 \pm 2^{\circ}$ C on an orbital shaker set at 120 rpm. The culture was grown for about 120 h. Fungal biomass was separated from the culture broth by filtering through Whatman filter (No.2) paper, washed with hexane to remove the excess oil, and dried on absorbing paper. The partially dried biomass was crushed vigorously in liquid nitrogen to make a homogenous powder using a sterile pestle mortar.

2.3. Optimization of Esterification Reaction Conditions

Propionic acid was taken as a model aliphatic acid to optimize reaction conditions for the esterification. Furthermore, various aliphatic acids were taken to carry out stepwise modifications to achieve the optimum yield of alkyl esters (Table **1**).

2.4. Identification, Purification, and Quantification of Fragrance Ester

The product (aliphatic ester), thus obtained, was examined on thin layer chromatography (TLC) with hexane: ethyl acetate (98:2) as a mobile phase. The compound was then purified by column chromatography (99:1) and further confirmed on TLC plates by charring with anisaldehyde and potassium permanganate. All reactions were carried out in triplicate, and the obtained alkyl ester products were quantified using 1 H-NMR (JEOL 400 MHz, 1 H-NMR) with CDCl₃ as solvent. The chemical shifts were expressed in parts per million with tetramethyl silane (TMS) as the internal standard. ¹H NMR spectra were recorded with a pulse duration of 2.18 sec., with a relaxation delay of 4 sec, and 16 scans. All esterification products were quantified by NMR spectroscopy using the (Eq. **1**) proposed by Sharma *et al*. [14]: Solution the generation of tragrance esters

Note that the present to reacting the control of the synthetic expression

the conde-cell biocatalyst seems to exhibit a

calce the experimental with studies carried

al. [13], The product (aliphated on thin layer chromometer (98.2) as a model in the acetate (98.2) as a model in the purified by column chinary and purified by column chinary with the purchased from the purchased from the purchased eld of 94% for nonanol and 84%

THODS

Fragrance Ester

The product (aliphatic ester), thus obtained

ined on thin layer chromatography (TLC) w

acetate (98.2) as a mobile phase. The communital work, the laboratory scale
 For personal price of the phase of the triplicate, and the obtained alk

response both triplicate, and the obtained alk

assed from fied using ¹H-NMR (JEOL 40

ch as ali-

as solvent. The chemical shift

ds (propi-

mil work, the laboratory scale (98:2) as a mobile phase. The compound
purified by column chromatography (99:1) and fur-
tifutuents viz, mycological potansium permanganate. All reactions were carrier
B), potato dextrose broth million with tetramethyl silane (TMS)

and. ¹H NMR spectra were recorded w

and 2.18 sec., with a relaxation delay of 4

esterification products were quantified

py using the (Eq. 1) proposed by Sharr

re

re
 $C = 100 \times$ any estimation of the oriental and the obtained any ester products were quantic celus as solvent. The demical shifts were expressed in parts phatic acids (propi-

cals, such as ali-

as solvent. The chemical shifts were e

$$
C = 100 \times (Aea-CH_2/A\alpha-CH_2) \quad (1)
$$

Wherein:

C: conversion of acid to the corresponding alkyl ester.

 $AEA-CH₂$: integration value of the protons of the alkyl esters. For using the (Eq. 1) proposed by sharma et all.
 $C = 100 \times (A\text{E}\alpha - \text{CH}_2 / A\alpha - \text{CH}_2)$

Wherein:

C: conversion of acid to the corresponding
 $A\text{E}\alpha - \text{CH}_2$: integration value of the proto

seters.
 $A\alpha$ -CH₂: integr onversion of acid to the corresponding alkyl es
 κ - CH₂: integration value of the protons of the

CH₂: integration value of the methylene proton

ULTS AND DISCUSSION

rphology Study of Biomass

reported earlier, th

 $A\alpha$ -CH₂: integration value of the methylene protons

3. RESULTS AND DISCUSSION

3.1. Morphology Study of Biomass

As reported earlier, the dry biomass of the whole cell biocatalyst and the filtrate collected from the culture broth were taken up for analysis of lipolytic activity [14]. The culture had a characteristic morphology showing conidiophores of variable length, which were rough and spiny, and the bi-The C: conversion of acid to the corresponding alkyl ester.

The distribution
 \angle C: conversion of acid to the corresponding alkyl ester.
 \angle HHz
 \angle Acc-CH₂: integration value of the methylene protons
 \angle HHz

| | S. No. | Constant Parameter | Variable Parameter | Range | | | |
|--|--------|---|---|---|--|--|--|
| | Step 1 | The molar ratio of propionic acid/ethyl alcohol $1:1 + addi$ - tion of alcohol at 0 h and 36 h reaction time | Amount of biomass | 500 mg to 2000 mg | | | |
| | Step 2 | 1000 mg biomass + addition of alcohol at 0 h and 36 h reaction time | The molar ratio of ethanol | 0.5 to 2.0 | | | |
| | Step 3 | 1000 mg biomass + molar ratio of acid/alcohol 1:1.5 and 36 h reaction time | Time of addition of alcohol | 0 _h Two-step additions (supplemented at 0 h and 8 h, and the reaction continued up to 36 h). Three-step additions (supplemented at 0 h, 12) h, and 24 h, and the reaction continued up to $36h$) | | | |
| | Step 4 | 1000 mg biomass + molar ratio of acid/alcohol 1:1.5 addi- tion of alcohol at 0 h | Time of reaction | 6 h to 36 h | | | |
| | Jotbe | omass showed a cottony mass that turned from yellow to green over time (Fig. 1). | 60 Ester Yield (%) 50 $40 -$ $30 -$ | 2000 | | | |
| 700 1000 500 Amount of Riomace (mg) | | | | | | | |

Table 1. Optimization of parameters for esterification reaction using dried biomass.

Fig. (1). Morphological characteristics of fungal culture RBD-01 (*Aspergillus flavus*). (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

3.2. Effect of the Amount of Biomass

Variable amounts (500, 700, 1000, and 2000 mg) of biomass were used to determine the optimal levels that would facilitate the notable conversion of acid to esters. A maximum conversion (yield=48%) was observed when 1000 mg biomass was used in the reaction (Fig. **2**). Further increase in biomass extent to 2000 mg led to a decrease in the conversion of esters to 31%. A transformation of only 2% and 7% was also observed in the case of 500 mg and 700 mg of biomass, respectively (Fig. **2**). The optimized reaction condition suggested that an increase in the extent of biomass content beyond 1000 mg led to a decrease the product formation and reduced yield, which presumably was due to the increased viscosity of the reaction mixture in which homogeneous stirring could not take place. The observations on the excess biocatalyst not contributing to the increase in percentage conversion are further supported by the observations of Torres and Otero [15].

Fig. (2). Effect of biomass on esterification reaction.

3.3. Effect of Molar Ratio of Alcohol to Acid on the Esterification

The molar ratio of an acyl donor to an acyl acceptor plays a significant role in the esterification process. A maximum conversion of 52% was observed when propionic acid to ethyl alcohol molar ratio of 1:1.5 was used (Fig. **3**). The transformation of ester was observed to be 27%, 30%, and 7% in the case of 1:0.5, 1:1, and 1:2 acid/alcohol molar ratios, respectively (Fig. **3**). Higher concentrations of alcohol led to a decrease in the formation of ester due to the inhibition of enzyme activity. In general, the increased substrate concentration triggers product formation and accelerates the reaction rate [16]. The whole-cell catalyzed esterification reaction was greatly affected by the molar ratio of alcohol to acid concentration [3]. Fig. (2). Effect of blomass on ester

e RBD-01
 EXECT OF Molar Ratio of

terification

The molar ratio of an acyl d

a significant role in the estericonversion of 52% was observed

ethyl alcohol molar ratio of

transfor Note that the distributed or an explicited or an explicit of the decrease in the conversion of entire and contraction of the decrease in the conversion of the control of the conversion of this α and α and α and Forthcatton

Forthcatton

Forthcatter of an acyl donor to

a significant role in the esterification

conversion of 52% was observed widely

ethyl alcohol molar ratio of 1:1.5 w

transformation of ester was observed
 7% 3.3. Effect of Molar Ratio of Alcohol to Acid on the E

lead culture RBD-01

inticle). The molar ratio of an acyl donor to an acyl acceptor play

inticle). The molar ratio of an acyl donor to an acyl acceptor play

conver ethyl alcohol molar ratio of 1:1.5 was used
transformation of ester was observed to be 2
7% in the case of 1:0.5, 1:1, and 1:2 acid/alcc
os, respectively (Fig. 3). Higher concentrati-
led to a decrease in the formation of **EXECUTE INTO THE INTERT AND THE CONDETERT AND THE CONDUCT CONDUCT AND ANY ACCEPT (10.5 AMAXIMUM) conversion of 52% was observed when propionic acid to ethyl alcohol molar ratio of 1:1.5 was used (Fig. 3). The transformat** Exercise in the bring energy and the increased sense
enzyme activity. In general, the increased sense
ration triggers product formation and acceleration
ratio and acceleration [3].
as reported that, when added in excess, s Note that the case of 1:0.5, 1:1, and 1:2 acid/alcohol molar rati-

uld to a decrease in the formation of ester due to the inhibi-

xi-

uld to a decrease in the formation of ester due to the inhibi-

ump concentration tr

It was reported that, when added in excess, short-chain alcohols can modify the enzyme's hydrophilic end, resulting in denaturation [17]. Due to low molecular weight and higher polarity, methanol may easily diffuse and access the lipase enzyme localized in the cell membrane, thus catalyzing the reaction at a higher rate. However, the enzyme lipase is partially inactivated by the presence of an excessive amount of ethanol in the reaction system [17].

3.4. Effect of the Time of Addition of Alcohol

The time of alcohol addition also significantly influences the esterification reaction. Following the stepwise addition, alcohol was added in two-step and three-step intervals of 8 h and 12 h, respectively, to facilitate the reaction and determine the best yield of the esterification product. of molar ratio of acid/alcohol.
 For the Time of Addition of Alcohol

of alcohol addition also significantly influence

tion reaction. Following the stepwise additio

dded in two-step and three-step intervals of 8

spect For personal and addition also significantly influences

ction. Following the stepwise addition,

two-step and three-step intervals of 8 h

ly, to facilitate the reaction and deter-

f the esterification product.

n of al

One-time addition of alcohol at 0 h showed promising results with a yield of 52%. In two-step addition, alcohol was initially added at 0 h, followed by addition at 8 h, and the reaction was continued for 36 h. The final yield of the esterification product was only 2% compared to the one-time (0 h) addition of alcohol. In a similar pattern, a three-step addition, with a time interval from 8 h to 12 h between the successive additions of alcohol, did not enhance the ethyl ester yield. Comparatively, lower yields of products (3%) were obtained when the time interval of the addition of alcohol was 12 h, expectedly due to the reversible nature of the reaction. When the time interval of the addition of ethanol was greater than 0 h, although the alcohol was completely utilized in the reaction, the reaction was reversible, subsequently decreasing the product yield (Fig. **4**) [18]. 0

0.5 1.0 Molar ratio of acid/alcohol.
 Fig. (3). Effect of molar ratio of acid/alcohol.
 3.4. Effect of the Time of Addition of Alcohol

The time of acid/alcohol.

The time of acid/alcohol.

The time of acid/alcohol **3.6. Other Aliphatic**
 Effect of the Time of Addition of Alcohol

the time of alcohol addition also significantly influences

sterification reaction. Following the stepwise addition,

further extended to a

not was add Fortification product.

Sensitive and alip and alip

n two-step addition, alcohol was

wed by addition at 8 h, and the

6 h. The final yield of the esteri-

6 compared to the one-time (0 h)

lar pattern, a three-step addi The step of a step wise addition, the step wise addition, the step intervals of 8 h and eltand as an acyl accept
spectrol, to facilitate the reaction and deter-
spectrol, to facilitate the reaction product. The reaction w For personal prior of the esteri-

For personal private use of the esteri-

For personal private use of the esteri-

private of the successive

the ethyl ester yield.

The view of alcohol was 12 h,

privately use of the r

Fig. (4). Effect of time of addition of alcohol.

3.5. Rate of Reaction

In lipase-catalyzed reactions, the reaction time considerably influences the rate of reaction and product formation. The observations indicated that upon an increase in the reaction time, the extent of esterification increased up to 24 h, beyond which it noticeably decreased (Fig. **5**). The conversion percentage of ester increased from 2% at 6 h to 52% by 24 h. The reaction rate was thus directly dependent on the initial concentration of reactants and the time of reaction.

Fig. (5). Effect of time of reaction.

3.6. Other Aliphatic Acids as Acyl Donors

Based on the optimized conditions obtained with propionic acid and ethyl alcohol, the esterification reactions were further extended to a range of aliphatic acids as acyl donors and ethanol as an acyl acceptor using the dry biomass as the catalyst. The reaction was performed with 20 ml of the organic solvent containing 100 mM of each substrate (ethanol and aliphatic acid) in a 100 ml round bottom flask. To initiate the reaction, the whole cell catalyst was added to the reaction mixture, following the optimized conditions determined earlier, in an orbital shaking water bath at 37°C and 120 rpm for 72 h [19]. It was observed that the conversion of acid to esters was marginal in the case of short-chain aliphatic acids. The yield of ethyl propanoate (**1**) was about 5%, followed by ethyl butyrate (**2**) (2% yield) and ethyl pentanoate (**3**) (3% yield) (Fig. **6**). These esters, as reported previously [19], were highly volatile in nature. The yields of esters increased with the increasing chain length of acids, and yields of ethyl hexanoate (**4**), heptanoate (**5**), and octanoate (**6**) were 50%, 54%, and 50%, respectively. The conversion was observed maximum in the case of ethyl decanoate (84%) (**7**) (Fig. **6**). The yield of synthesized ethyl esters was calculated by analyzing the spectra obtained through NMR spectroscopy by applying the equation proposed by *Sharma et. al*. [14]. It was noted that the volatile nature of esters decreased with increasing the chain length of carbon (Table **2**). Yan Xu (2002) reported the synthesis of flavor ethyl esters of short-chain fatty acids using lipases wherein the whole cell and cell-free lipase from *Rhizopus chinensis* were used as catalysts for the esterification of hexanoic acid with ethyl alcohol. A maximum conversion of 96.5% was obtained in 72h in the case of whole-cell lipase as a catalyst and 84.9% yield in the case of cell-free lipase [20]. Abbas *et al*. (2003) reported using Mucor sp. lipase immobilized on Amberlite IRC 50 to synthesize aromatic esters of propionic, butyric, and caproic acids with methanol, ethanol, allyl butanol, isoamyl, geraniol, citronellol and farnesol alcohol used as acyl acceptors [21]. In addition, the change from fruity to waxy fragrance was evident with the change in the chain length (Table **2**). Note that the most alcohol was the most all the most are the most all the costs of the shape of the most all the costs of the most all the costs was marginal in the case of the most all the stering or any plane or any pla ethyl ester yield.

We were obtained at (3) (3% yield) (Fig. 100) was 12 h, e reaction. When

was greater than 0 yields of ethyl hexanos

lized in the reaction. We was observed maximum

was observed maximum

(7) (Fig. 6). Note that the distribution of energy of the distribution of sharp at the state at the state of products (3%) were obtained to the consider the energy of products (3%) were obtained or alcohol was 12 h, between the energy For the reaction of the reaction of the reaction of the contract of the CD (Fig. 6). The yield of synthetic (7) (Fig. 6). The yield of synthetic (7) (Fig. 6). The yield of synthetic troscopy by applying the equal al. [14] Note that the control of storber is increased with the increasing chain length of alcohol was 12 h, when the sime sime spectrom of the reason of θ or θ troscopy by applying the equation pr

al. [14]. It was noted that the volatil

creased with increasing the chain leng

Yan Xu (2002) reported the synthesis

of short-chain fatty acids using lipas

cell and cell-free lipas The visit of the section in the case of capture and through NMR spectra of the spectra of the spectra or that the volatile nature of esters denoted with increasing the equation proposed by *Sharma e* and $\frac{1}{2}$ (Y) (For short-chain ratiy actiss using inpases where
cell and cell-free lipase from *Rhizopus chine*
as catalysts for the esterification of hexanoic
alcohol. A maximum conversion of 96.5% w
72h in the case of whole-cell lipase The distribution of the synthesis of flavor (Table 2).

Tan Xu (2002) reported the synthesis of flavor ethyl esters

of short-chain fatty acids using lipases wherein the whole

cell and cell-free lipase from *Rhizopus chi* the case of cell-free lipase [20]. Abbas *et al.*
I using Mucor sp. lipase immobilized on Arto synthesize aromatic esters of propionic, l
roic acids with methanol, ethanol, allyl butan
eraniol, citronellol and farnesol al as catalysts for the esterification of hexanoic acid with ethyl
alcobol. A maximum conversion of 96.5% was obtained in
72h in the case of whole-cell Ipase as a catalyst and 84.9%
yield in the case of cell-free lipase [20]

Ethyl hexanoates (**4**) were further considered for the esterification reactions to synthesize diverse alkyl hexanoates (**4A-4J**) due to their significant fruity aroma. Esterification reaction was performed using hexanoic acid as the substrate and all primary alcohols (methanol - decanol) as acyl acceptors.

| 1 | Ethyl propanoate | 5.0 ± 0.11 | Strong ripe apple |
|------------------------|------------------|-----------------|-------------------|
| $\overline{2}$ | Ethyl butyrate | 2.0 ± 0.30 | Fresh red apple |
| 3 | Ethyl pentanoate | 3.0 ± 0.025 | Aniseed |
| $\overline{4}$ | Ethyl hexanoate | 50.0 ± 1.97 | Ripe fruit berry |
| 5 | Ethyl heptonate | 54.0 ± 1.54 | Ripe grapes |
| 6 | Ethyl octanoate | 50.0 ± 1.52 | Coconut |
| 7 | Ethyl decanoate | 84.0 ± 0.57 | Oily |
| $90 -$ 80 $70 -$ | For personal | | |

Table 2. The yield of ethyl esters from different acids and their nature of the aroma.

Fig. (6). Shows the comparison in ester yield from different acids with ethanol.

Fig. (7). Effect of chain length of alcohols on esterification of hexanoic acid.

3.7. Synthesis of Alkyl Hexanoates

The esterification reaction of hexanoic acid with all primary alcohols (methanol to decanol) was performed with lipase-catalyzed whole-cell biocatalyst to synthesize alkyl hexanoates (4A-4J) as per the previously outlined optimized conditions. The product formation was found to increase from methyl hexanoate (4A) to nonyl hexanoate (4I) with the increase in carbon chain length (Fig. **7**). However, decyl hexanoate (4J) was found to convert lesser than other alky hexanoates (Fig. **7**). The yield of alkyl hexanoates was quantified with the help of NMR spectroscopy as per the abovementioned equation proposed by Sharma *et al*. Maximum conversion was observed when nonanol (94% yield) was used as an acyl acceptor (Table **3**).

Keeping in view the fragrance-like ripe fruit berry of ethyl hexanoate (4B), all synthesized alkyl hexanoates (4A-4J) were tested for their aroma quality. From the results mentioned in Table **3**, it was observed that except decyl hexanoate (4J), all synthesized compounds (4A-4I) exhibited fruity pineapple/berry flavors with significant fragrance.

| S. No. | Alkyl Esters | Ester Yield (%) $(Mean + SD)$ | Fragrance |
|--------|------------------------|----------------------------------|-----------------------------|
| 4A | Methyl Hexanoate | 15.0 ± 0.54 | Strong pineapple |
| 4B | Ethyl Hexanoate | 50.0 ± 1.97 | Sweet pineapple |
| 4C | Propayl Hexanoate | 58.0 ± 0.32 | Sweet tropical pineapple |
| 4D | Butyl Hexanoate | 62.0 ± 0.68 | Fruity berry |
| 4E | Pentayl Hexanoate | 66.0 ± 1.46 | Fruity green apple |
| 4F | Hexayl Hexanoate | 68.0 ± 1.02 | Waxy, berry |
| 4G | Heptayl Hexanoate | 78.0 ± 0.27 | Green sappy |
| 4H | Octayl Hexanoate | 89.0 ± 0.97 | Coconut oily |
| 4I | Nonayl Hexanoate | 94.0 ± 1.57 | Flowery |
| 4J | Decayl Hexanoate | 64.0 ± 0.06 | Oily |

Table 3. The yield of alkyl esters of different alcohols with hexanoic acid and their nature of the aroma.

CONCLUSION

In summary, we demonstrated the use of a whole-cell biocatalytic process with *Aspergillus flavus* (RBD-01), facilitating the conversion of aliphatic acids with ethanol to obtain a variety of important alkyl aroma esters. The study revealed that the major factors which could influence esterification reactions are the length of the carbon chain of substrate/alcohol, substrate concentration, stirring speed and reaction time, the molar ratio of alcohols, and the amount of biocatalyst. The ethyl hexanoate (4) possessed a fruity fragrance with a good conversion rate among all synthesized alkyl esters. Good yields were obtained with almost all of the aliphatic acids, with the yield of esters increasing with the chain length of alcohol examined. The synthesized alkyl hexanoates (4A-4I) were observed to possess good fruity/pineapple/berry fragrances with aroma quality. The demonstrated synthesis reactions can thus be considered for further exploration in wide industrial applications, especially in the food/cosmetic industries. From different acids

CONCLUSION

In summary, we define the conversion of the private use of the conversion of the strate/alcohol, substrate/alcohol, Not determine the set of the set o biocatalytic process with *Asp*

cilitating the conversion of al

obtain a variety of important a

revealed that the major factors

fication reactions are the lengt

strate/alcohol, substrate conce

reaction time, the mola CONCLUSION

In summary, we demonstrated the use of a w
biocatalytic process with Aspergillus flavus (RBE

cilitating the conversion of aliphatic acids with e
obtain a variety of important alkyl aroma setes. T

revealed th Fraction Fractions are the length of the
strate/alcohol, substrate concentration
reaction time, the molar ratio of alcoh
biocatalyst. The ethyl hexanoate (4) grance with a good conversion rate
alkyl esters. Good yields wer Notain a variety of important alkyl aroma esters. The student

revealed that the major factors which could influence ester

fication reaction in expectation, substrate concentration, strimg speed a

reaction time, the mala alkyl esters. Good yields were obtained with a aliphatic acids, with the yield of esters increasing chain length of alcohol examined. The synthexanoates (4A-4I) were observed to fruity/pineapple/berry fragrances with aroma reaction time, the molar ratio of alcohols, and the amount of

biocatalyst. The einyl hexamoate (4) possessed a fruity frac-

alkyl esters. Good yields were obtained with almost all of the

aliphatic acids, with the yield

LIST OF ABBREVIATIONS

ETHICS APPROVAL AND CONSENT TO PARTICI-PATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data and supportive information are available within the article. For personal private used for studies that
SENT FOR PUBLICATION
of applicable.
ILABILITY OF DATA AND MATERIALS
ee data and supportive information are available. Example:

For personal and supportive information are available with

For personal private use of the set of the

Form of the set of the set of the set of t

FUNDING

This study is financially supported by CSIR, New Delhi, through awarding a Research Associateship [Grant no. 02(0309)17/EMRII]. **HUMAN AND ANIMAL RIGHTS**

No animals/humans were used for studies that are the MRC plass of this research.

CONSENT FOR PUBLICATION 1819

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data and supportive infor CONSENT FOR PUBLICATION SEER TO THE REST

Not applicable.

Not any interval and supportive information are available wi For personal private use of the personal private use of the contract of the co **ILABILITY OF DATA AND MATERIALS**

biocatalyst constructed

the data and supportive information are available within

the distributed or any or supported by CSIR, New Delhi, [11] $\frac{a}{B}$

arch Associateship [Grant no. deleteration]

T

conflict of interest, financial or $\begin{bmatrix} 13 \end{bmatrix}$ or $\begin{bmatrix} 13 \end{bmatrix}$ or $\begin{bmatrix} 13 \end{bmatrix}$ or $\begin{bmatrix} 13 \end{bmatrix}$ or $\begin{bmatrix}$

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

SKR would like to thank CSIR, New Delhi, for their generous funding for the award of Research Associateship [02(0309)17/EMR-II]. The authors would also like to acknowledge the analytical support provided by SAI Laboratories, TIET. Note thank the detailed support of interest

EDGEMENTS

The distributed by SSIR, New Delhi,

the mature of the industry.

III) the duration of lipse enzyme from a duration of lipse enzyme from a duration of lipse enzyme f biocatalyst

of interest, financial or

futp://dx.do

http://dx.do

http://dx.do

Prakash, Panance-base

using biocates

with the properties of the street of the street of the street of the street

Research Associateship
 NEREST

Note that the conflict of interest, financial or

Note ites of blend and biodiesel generated

bi mance-based derivant and the sum of also like to
the minimization of also like to
d by SAI Labora-
[15] Torres, C., October 2018, 40(21),
 $fects$, 2018, 40(21),
the freedstocks and chain
the sense of sum of also like to
glyc conflict of interest, financial or

Sharma, A.: Verma, A.: Luxani, V.; Melo,

Prakash, N.T.; Prakash, R. New proton nuc

Sharma, A.: Verma, A.: Luxani, V.; Melo,

Maxdoi.org/10.1021/ef4001506

Sharma, A.: Melo, J.S.; Tep

SUPPLEMENTARY MATERIAL

The 1H NMR spectral data of compounds **2-7** and **4A-4J** are provided in the supporting information section.

REFERENCES

- [1] SÁ, A.G.A.; de Meneses, A.C.; de Araújo, P.H.H.; de Oliveira, D. A review on enzymatic synthesis of aromatic esters used as flavor ingredients for food, cosmetics and pharmaceuticals industries. *Trends Food Sci. Technol.,* **2017**, *69*, 95-105.
- [2] Yan, H.D.; Zhang, Q.; Wang, Z. Biocatalytic synthesis of shortchain flavor esters with high substrate loading by a whole-cell lipase from Aspergillus oryzae. *Catal. Commun.,* **2014**, *45*, 59-62. http://dx.doi.org/10.1016/j.catcom.2013.10.018
- [3] de Souza, M.; dos Santos, K.; Freire, R.; Barreto, A.; Fechine, P.; Gonçalves, L. Production of flavor esters catalyzed by lipase B from *Candida antarctica* immobilized on magnetic nanoparticles. *Braz. J. Chem. Eng.,* **2017**, *34*(3), 681-690. http://dx.doi.org/10.1590/0104-6632.20170343s20150575
- [4] Humble, M.S.; Berglund, P. Biocatalytic promiscuity. *Eur. J. Org. Chem.,* **2011**, *2011*(19), 3391-3401.

http://dx.doi.org/10.1002/ejoc.201001664

- [5] Lin, B.; Tao, Y. Whole-cell biocatalysts by design. *Microb. Cell Fact.,* **2017**, *16*(1), 106.
	- http://dx.doi.org/10.1186/s12934-017-0724-7 PMID: 28610636
- [6] Garzón-Posse, F.; Becerra-Figueroa, L.; Hernández-Arias, J.; Gamba-Sánchez, D. Whole cells as biocatalysts in organic transformations. *Molecules,* **2018**, *23*(6), 1265.
- http://dx.doi.org/10.3390/molecules23061265 PMID: 29799483 [7] Reetz, M.T. Biocatalysis in organic chemistry and biotechnology: Past, present, and future. *J. Am. Chem. Soc.,* **2013**, *135*(34), 12480-12496. http://dx.doi.org/10.1021/ja405051f PMID: 23930719
- [8] Ban, K.; Hama, S.; Nishizuka, K.; Kaieda, M.; Matsumoto, T.; Kondo, A.; Noda, H.; Fukuda, H. Repeated use of whole-cell biocatalysts immobilized within biomass support particles for biodiesel fuel production. *J. Mol. Catal., B Enzym.,* **2002**, *17*(3-5), 157-165. http://dx.doi.org/10.1016/S1381-1177(02)00023-1
- [9] T, M.; S, T.; M, K.; M, U.; A, T.; H, F.; A, K. Yeast whole-cell biocatalyst constructed by intracellular overproduction of *Rhizopus oryzae* lipase is applicable to biodiesel fuel production. *Appl. Microbiol. Biotechnol.,* **2001**, *57*(4), 515-520.
- http://dx.doi.org/10.1007/s002530100733 PMID: 11762598 [10] de Carvalho, C.C.C.R. Whole cell biocatalysts: Essential workers from nature to the industry. *Microb. Biotechnol.,* **2017**, *10*(2), 250- 263.
	- http://dx.doi.org/10.1111/1751-7915.12363 PMID: 27145540
- [11] Bharathi, D.; Rajalakshmi, G.; Komathi, S. Optimization and production of lipase enzyme from bacterial strains isolated from petrol spilled soil. *J. King Saud Univ. Sci.,* **2019**, *31*(4), 898-901.
- [12] Sharma, A.; Melo, J.S.; Tejo Prakash, N.; Prakash, R. Fuel properties of blend and biodiesel generated from acid oil using whole cell biocatalyst. *Energy Sources Recov. Util. Environ. Effects,* **2018**, *40*(2), 148-154.

http://dx.doi.org/10.1080/15567036.2017.1406562

- [13] Sharma, A.; Verma, A.; Luxami, V.; Melo, J.S.; D'Souza, S.F.; Prakash, N.T.; Prakash, R. New proton nuclear magnetic resonance-based derivation for quantification of alkyl esters generated using biocatalysis. *Energy Fuels,* **2013**, *27*(5), 2660-2664. http://dx.doi.org/10.1021/ef4001506
- [14] Sharma, A.; Melo, J.S.; Tejo Prakash, N.; Prakash, R. Effect of feedstocks and chain length of alcohols on whole-cell-catalyzed generation of alkyl esters. *Energy Sourc. Recov. Util. Environ. Effects,* **2018**, *40*(21), 2612-2619. R, New Delhi, for their
 μ Bhama, A.; Melo, J.S.; Tejo Prakash, N.; Prakash, R

F Research Associateship

redstocks and chain length of alcohols on whole-ce

rs would also like to
 μ generation of ally lesters. *Dens*
	- http://dx.doi.org/10.1080/15567036.2018.1505979
	- [15] Torres, C.; Otero, C. Part I. Enzymatic synthesis of lactate and glycolate esters of fatty alcohols. *Enzyme Microb. Technol.,* **1999**, *25*(8-9), 745-752.

http://dx.doi.org/10.1016/S0141-0229(99)00117-9

- [16] Strohalm, H.; Dold, S.; Pendzialek, K.; Weiher, M.; Engel, K.H. Preparation of passion fruit-typical 2-alkyl ester enantiomers *via* lipase-catalyzed kinetic resolution. *J. Agric. Food Chem.,* **2010**, *58*(10), 6328-6333. The to the *fects*, 2018, 40(21), 2612-2619

I Labora-

(15) http://dx.doi.org/10.1080/1556

(15) Torres, C.; Otero, C. Part 1.

glycolate esters of fatty alcoh

25(8-9), 745-752.

(16) Strohalm, H.; Dold, S.; Pend

Prepa
- http://dx.doi.org/10.1021/jf100432s PMID: 20415422 [17] Xiao, M.; Mathew, S.; Obbard, J.P. Biodiesel fuel production *via* transesterification of oils using lipase biocatalyst. *Glob. Change Biol. Bioenergy,* **2009**, *1*(2), 115-125. http://dx.doi.org/10.1111/j.1757-1707.2009.01009.x For Form (18)

For personal private use only of the personal private constrained by

Preparation of passion functions of the personal private conduction
 For personal pass catalyzed kinetic resolution. *J*.
 For pass (15) Torres, C.; Otero, C. Part I, Enzymatic synthesis of lactate at glycolat estats of fatty alcohols. *Enzyme Microb. Technol.*, 199

25(8-9), 745-752,

http://dx.doi.org/10.1016/S0141-0229(99900117-9

Stection.

Stecti [17] Xiao, M.; Mathew, S.; Obbard, J.P. Biodiesel f
transesterification of oils using lipase biocataly
Biol. Bioenergy, **2009**, $I(2)$, $115-125$.
http://dx.doi.org/10.1111/j.1757-1707.2009.0100
Fukuda, H.; Kondo, A.; N Preparaton or passion ruris typical Zauky ester enantomes variables

pass-catalyzed kinetic resolution. J. Agric. Food Chem., 2010,

58(10), 6328-6333.

1171 Xiao, M.; Mathew, S.; Obbard, J.P. Biodiesel fuel production vi
	- [18] Fukuda, H.; Kondo, A.; Noda, H. Biodiesel fuel production by transesterification of oils. *J. Biosci. Bioeng.,* **2001**, *92*(5), 405-416. http://dx.doi.org/10.1016/S1389-1723(01)80288-7 PMID:
	- 16233120 [19] De Castro, H., De Oliveira, P., and Pereira, E., *Biotechnol Lett.*, **1997**, Vol. 19, no. 3, pp. 229-232. ransesterification of oils. *J. Biosci. Bioeng.*, 2001, 92(5), 4
ttp://dx.doi.org/10.1016/S1389-1723(01)80288-7 PMID:
6233120
De Castro, H., De Oliveira, P., and Pereira, E., *Biotech*
997, Vol. 19, no. 3, pp. 229-232.
Ku
	- [20] Xu, Y., Wang, D., Mu, X. Q., Zhao, G. A., and Zhang, K. C., *J Mol Catal B: Enzym.*, **2002**, Vol. 18, pp. 29-37.
	- [21] Abbas, H and Comeau, L, *Enzyme Microb. Technol.*, **2003**, Vol. 32, pp 589-595.

DISCLAIMER: The above article has been published, as is, ahead-of-print, to provide early visibility but is not the final version. Major publication processes like copyediting, proofing, typesetting and further review are still to be done and may lead to changes in the final published version, if it is eventually published. All legal disclaimers that apply to the final published article also apply to this ahead-of-print version. Note that $Biot. Bioenergy, 2009, 1/2), 115, 125.$

Note that $WbU/AXAobi.org/10.1111/1.1757-1707.2009.01009.x$

The Fukuda, H.; Kondo, A.; Noda, H. Biodiesel fuel production by

transesterification of oils. *J. Biosci. Bioeng.*, 2001, 92(