

# Single Cell Oil Production with *Cutaneotrichosporon curvatus* (syn, *Cryptococcus curvatus*) PY39 and Its Use for Biodiesel Preparation

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## Research

**Keywords:** Biodiesel, Culture conditions, *Cutaneotrichosporon curvatus* (syn, *Cryptococcus curvatus*), Fatty acid, Oleaginous yeast

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# Abstract

**Background:** Microorganisms including yeasts that are capable of accumulating lipids above 20 to 70% of their dry biomass are collectively named oleaginous. The oil produced from such microorganisms can be used as alternative sources of oils for human consumption, feedstocks for production of biodiesel, components in paints, coatings, detergents, cleaning products, cosmetics, plastics, rubber and intermediate products.

**Results:** In this study *Cutaneotrichosporon curvatus* (*Cryptococcus curvatus*) PY39, an oleaginous yeast, isolated from flower surface in Ethiopia, was grown under nitrogen-limited media. The capacity of this strain with respect to biomass production, lipid yield and lipid content was evaluated. To determine the optimal culture conditions for this oleaginous yeast, different carbon and nitrogen sources, carbon to nitrogen (C/N) ratio, pH, incubation temperature, shaking speed and incubation period were investigated. The optimal fermentation conditions were obtained as follows: 70 g/L of glucose as carbon source; combinations of ammonium sulphate and yeast extract as nitrogen sources at, respectively, 0.50 g/L and 0.47 g/L; initial pH of 5.0; temperature at 30°C, shaking speed of 225 rpm, and cultivated for 144 h. Under the optimized conditions, *C. curvatus* PY39 accumulated lipids up to 7.22±0.26 g/L on dry biomass basis. Such values correspond to lipid content of 48.66±0.60%. This strain was further grown on media containing peel mixtures of papaya and mango. Under the optimized conditions, *C. curvatus* PY39 exhibited a lipid yield and lipid content of 3.95±0.67 g/L and 35.02±1.63%, respectively. The fatty acid profiles were analyzed using gas chromatography. Data revealed the presence of significant amount of oleic acid (54.40±1.15%), palmitic acid (17.39±0.47%), stearic acid (13.16±0.93%) and low amount of other fatty acids in the extracted yeast oils which indicate that the fatty acid profiles were very similar to that of conventional vegetable oils.

**Conclusion:** The results of fatty acid profiles showed that the microbial lipids from the studied yeast can be good feedstocks for biodiesel production.

## Background

Microorganisms capable of accumulating lipids above 20 to 70% of their dry biomass are collectively named oleaginous [1]. The oil produced can be exploited as alternative sources of oils and fats for human consumption and, increasingly, for renewable energy (biodiesel) production [2] and components in paints and coatings, detergents, cleaning products, cosmetics, plastics, rubber, crop protection and intermediate products or synthons[3].

Oil accumulation by oleaginous microorganisms depends on the culture conditions such as optimization of carbon and nitrogen sources, C/N ratio, incubation period and temperature, pH, agitation and aeration rate [4, 5]. Optimizations of culture conditions are critical for increased biomass yield and lipid production by oleaginous microorganisms. Moreover, the data generated is useful for scale up efforts aimed at biodiesel production.

Optimization of cultivation parameters can result in higher lipid production by oleaginous yeasts and fosters their potential for industrial application. Optimization is an essential step of each industrial process because it can result in higher production and reduced cost of production [6].

Oleaginous microorganisms convert the carbon sources contained in various substrates into storage lipid under suitable culture conditions [7]. In order to reduce the cost of microbial oil production, low-cost raw materials like lignocellulosic hydrolysates such rice straw [9] and rice hull [7], glycerol [9, 10], sugar industry by-product such as molasses [11, 12, 13], effluent from steam of fish processing [14], whey [15], beer factory wastewater [16], starch wastewater [17], and products of fruit processing industry [18, 19] were investigated.

Papaya and mango are produced in vast amount worldwide. Various juice producing industries and local juice sellers produce a huge amount of different fruit wastes including fruit peels. The peels from these fruits are disposed as wastes. The utilization of such wastes is needed to be emphasized to save the resources and protect the environment from pollution [18]. Fruit waste has high level of sugars, including sucrose, glucose and fructose [20]. These fruit wastes are suitable for the production of SCO [18, 19], ethanol, biogas, lactic acid, enzymes and single cell protein [21]. Banana peel can also be used for the production of wine [22]. To improve the economic competitiveness of the bioprocess microbial oil (TAGs), the use of wastes that have low or even negative value can be used as natural substrates [23, 24]. These microbial oils can be used as feedstocks for the production of biodiesel [2].

This study aimed to investigate the effect of different cultivation factors on biomass production, lipid yield and lipid content of *C. curvatus* PY39. In addition, the potential of lipid production capacity of such selected oleaginous yeast was evaluated by cultivating it on mixtures of papaya and mango peels. Furthermore, the fatty acid profiles of this yeast were analyzed using gas chromatography.

## Results

### Effect of various parameters on biomass production and lipid yield

In the present study, the effects of various carbon sources (glucose, sucrose, maltose, lactose, galactose, xylose and glycerol) on biomass yield and lipid content by *C. curvatus* PY39 was tested using nitrogen-limited media. Maximum biomass ( $14.53 \pm 0.41$  g/L) and lipid content ( $48.66 \pm 0.60\%$ ) were attained by *C. curvatus* PY39 when glucose was supplied as a sole source of carbon (Table 1). On the other hand, *C. curvatus* PY39 gave the minimum lipid yield of  $8.38 \pm 0.26$  g/L and lipid content of  $24.70 \pm 0.48\%$  when grown in nitrogen-limited medium containing xylose.

Table 1

Effect of medium components on cell growth and lipid accumulation of *C. curvatus* PY39

Parameter	Biomass (g/L)	Lipid yield (g/L)	Lipid content (%)
Carbon source			
Glucose	14.53 ± 0.41 <sup>a</sup>	7.07 ± 0.27 <sup>a</sup>	48.66 ± 0.60 <sup>a</sup>
Sucrose	12.75 ± 0.46 <sup>b</sup>	4.70 ± 0.06 <sup>b</sup>	36.87 ± 0.66 <sup>c</sup>
Maltose	11.67 ± 0.41 <sup>c</sup>	4.66 ± 0.06 <sup>b</sup>	39.93 ± 1.36 <sup>b</sup>
Galactose	11.78 ± 0.99 <sup>c</sup>	3.76 ± 0.22 <sup>c</sup>	32.26 ± 0.89 <sup>e</sup>
Xylose	8.38 ± 0.26 <sup>d</sup>	2.07 ± 0.23 <sup>d</sup>	24.70 ± 0.48 <sup>d</sup>
Glycerol	11.21 ± 0.89 <sup>c</sup>	4.12 ± 0.65 <sup>c</sup>	36.76 ± 1.43 <sup>c</sup>
Glucose concentration (g/L)			
10	11.88 ± 1.10 <sup>b</sup>	3.42 ± 0.54 <sup>c</sup>	28.81 ± 2.02 <sup>d</sup>
30	13.40 ± 0.42 <sup>b</sup>	4.66 ± 0.63 <sup>bc</sup>	34.78 ± 2.64 <sup>b</sup>
50	14.35 ± 1.08 <sup>ab</sup>	4.38 ± 0.66 <sup>bc</sup>	30.52 ± 1.59 <sup>c</sup>
70	16.82 ± 2.27 <sup>a</sup>	6.80 ± 0.75 <sup>a</sup>	40.43 ± 1.73 <sup>a</sup>
90	15.04 ± 0.75 <sup>ab</sup>	5.39 ± 0.61 <sup>ab</sup>	35.84 ± 1.56 <sup>b</sup>
Nitrogen source			
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , YE	15.75 ± 0.25 <sup>b</sup>	6.35 ± 0.22 <sup>a</sup>	40.31 ± 0.79 <sup>a</sup>
NH <sub>4</sub> Cl, peptone	16.89 ± 0.23 <sup>a</sup>	6.21 ± 0.26 <sup>ab</sup>	36.76 ± 0.36 <sup>b</sup>
NH <sub>4</sub> Cl, YE	16.91 ± 0.40 <sup>a</sup>	6.10 ± 0.08 <sup>ab</sup>	36.01 ± 0.26 <sup>b</sup>
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , peptone	16.25 ± 0.12 <sup>b</sup>	6.15 ± 0.05 <sup>ab</sup>	37.84 ± 0.41 <sup>b</sup>
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , urea	15.10 ± 0.15 <sup>c</sup>	5.39 ± 0.11 <sup>b</sup>	35.70 ± 0.19 <sup>c</sup>
NH <sub>4</sub> Cl, urea	14.96 ± 1.21 <sup>d</sup>	5.18 ± 0.32 <sup>b</sup>	34.63 ± 0.56 <sup>d</sup>

All parameters are expressed as mean ± SD values in the same column, n = 3, within each column, means

are significantly different ( $p < 0.05$ ), unless they have a common letter.

YE- Yeast Extract

Parameter	Biomass (g/L)	Lipid yield (g/L)	Lipid content (%)
C/N ratio			
40	10.24 ± 1.13 <sup>d</sup>	2.22 ± 0.29 <sup>d</sup>	21.67 ± 1.8 <sup>f</sup>
60	10.75 ± 1.06 <sup>d</sup>	2.54 ± 0.34 <sup>d</sup>	23.62 ± 0.88 <sup>e</sup>
80	10.79 ± 0.54 <sup>d</sup>	3.52 ± 0.19 <sup>c</sup>	32.61 ± 1.48 <sup>d</sup>
100	11.55 ± 1.29 <sup>c</sup>	4.49 ± 0.61 <sup>b</sup>	38.86 ± 1.77 <sup>c</sup>
120	12.68 ± 1.23 <sup>b</sup>	5.12 ± 0.52 <sup>b</sup>	40.37 ± 0.56 <sup>b</sup>
140	13.97 ± 0.90 <sup>a</sup>	5.83 ± 0.33 <sup>a</sup>	41.79 ± 2.31 <sup>a</sup>
160	12.12 ± 0.66 <sup>b</sup>	4.89 ± 0.45 <sup>b</sup>	40.85 ± 2.94 <sup>b</sup>
All parameters are expressed as mean ± SD values in the same column, n = 3, within each column, means			
are significantly different (p < 0.05), unless they have a common letter.			
YE- Yeast Extract			

To know the optimum glucose concentration for maximum lipid content per dry biomass of *C. curvatus* PY39, various concentrations, i.e., 10, 30, 50, 70 and 90 g/L glucose concentrations were carried out. Results indicate that the optimum glucose concentration that led to maximum biomass and lipid content was 70 g/L leading to a biomass production of  $16.82 \pm 2.27$  g/L and lipid content of  $40.43 \pm 1.73\%$  (Table 1). On the other hand, the least amount of biomass and lipid content were attained by *C. curvatus* PY39, when the media contained 10 g/L glucose (Table 1). The lowest biomass and lipid contents were exhibited by *C. curvatus* PY39 in a nitrogen-limited medium with 10 g/L glucose viz.,  $11.88 \pm 1.10$  g/L and  $28.81 \pm 2.02\%$ , respectively. As shown on Table 1, 70 g/L glucose was the most favorable concentration for maximum lipid production by *C. curvatus* PY39 and hence was selected for further activities.

The effect of combined organic nitrogen (yeast extract, peptone and urea at 0.30 g/L concentration) and inorganic nitrogen sources  $(\text{NH}_4)_2\text{SO}_4$  and  $\text{NH}_4\text{Cl}$  at 0.30 g/L concentration by *C. curvatus* PY39, were determined in the nitrogen-limited media containing optimized glucose concentrations for each oleaginous yeast. As shown in Table 1, *C. curvatus* PY39 gave maximum biomass when it was grown on medium containing  $\text{NH}_4\text{Cl}$  and peptone in combinations as well as in the medium containing  $\text{NH}_4\text{Cl}$  and yeast extract. The biomass exhibited by *C. curvatus* PY39 when grown on a medium containing  $\text{NH}_4\text{Cl}$  and peptone was  $16.89 \pm 0.23$  g/L, while a biomass of  $16.91 \pm 0.40$  g/L was attained by this yeast when grown on medium containing  $\text{NH}_4\text{Cl}$  and peptone. This yeast gave maximum lipid content ( $40.31 \pm 0.79\%$ ) when  $(\text{NH}_4)_2\text{SO}_4$  and yeast extract were used in combinations as nitrogen sources.

Results also showed that the combined nitrogen sources that gave lowest biomass and lipid content were  $\text{NH}_4\text{Cl}$  and urea (Table 1) for *C. curvatus* PY39, leading to biomass and lipid contents of  $14.96 \pm 1.21$  g/L and  $34.63 \pm 0.56\%$ , respectively.

Based on the results in Table 1, the lipid content at different combinations of different nitrogen sources, one can understand that  $(\text{NH}_4)_2\text{SO}_4$  and yeast extract are the optimum nitrogen sources for *C. curvatus* PY39. Accordingly,  $(\text{NH}_4)_2\text{SO}_4$  and yeast extract were selected as optimal nitrogen sources for the yeast *C. curvatus* PY39 for further investigations.

The effect of C/N ratio on biomass production and lipid content of *C. curvatus* PY39 were studied by varying C/N ratios from 40 to 160 in nitrogen-limited media composed of 70 g/L of glucose, 0.5 g/L of yeast extract and various concentrations of  $(\text{NH}_4)_2\text{SO}_4$  as shown in Table 2 and adjusted pH of 5.5. To know the total nitrogen content of yeast extract, Kjeldahl method was used and the approximate result obtained was 20%.

Table 2  
The C/N ratio in nitrogen-limited medium

C/N ratio	Carbon source (glucose)	Nitrogen sources
40	70 g/L	0.5 g/L yeast extract and 2.83 g/L $(\text{NH}_4)_2\text{SO}_4$
60	70 g/L	0.5 g/L yeast extract and 1.74 g/L $(\text{NH}_4)_2\text{SO}_4$
80	70 g/L	0.5 g/L yeast extract and 1.18 g/L $(\text{NH}_4)_2\text{SO}_4$
100	70 g/L	0.5 g/L yeast extract and 0.85 g/L $(\text{NH}_4)_2\text{SO}_4$
120	70 g/L	0.5 g/L yeast extract and 0.61 g/L $(\text{NH}_4)_2\text{SO}_4$
140	70 g/L	0.5 g/L yeast extract and 0.47 g/L $(\text{NH}_4)_2\text{SO}_4$
160	70 g/L	0.5 g/L yeast extract and 0.35 g/L $(\text{NH}_4)_2\text{SO}_4$

The results of the effect of C/N ratio on biomass, lipid yield and lipid content by *C. curvatus* PY39 yeasts is also presented in Table 1. At C/N ratio of 140, *C. curvatus* PY39 exhibited its maximum lipid yield and lipid contents, i.e.,  $6.20 \pm 0.33$  g/L and  $41.75 \pm 2.31\%$ , respectively per dry biomass of  $14.85 \pm 0.90$  g/L. On the contrary, the least biomass, lipid yield and lipid content was exhibited by *C. curvatus* PY39 in a nitrogen-limited media with C/N ratio of 40 viz.,  $10.24 \pm 1.13$  g/L,  $2.22 \pm 0.29$  g/L and  $21.67 \pm 1.80\%$ , respectively. Based on the above result, C/N ratio of 140 was the optimum and selected value for *C. curvatus* PY39 and hence was used for further studies.

To know the best initial pH for maximum production of cell biomass and cellular lipid content in *C. curvatus* PY39, a nitrogen-limited medium with pH ranging from 4.0 to 7.0 were tested. Optimum pH

that led to maximum biomasses production were 5.0 ( $16.71 \pm 0.11$  g/L) and 5.5 ( $16.32 \pm 0.14$  g/L). Results indicate that the optimum pH that leads to maximum lipid yield and lipid content was 5.0. Results indicate that the optimum pH that led to maximum lipid yield and lipid content was 5.0 leading to lipid yield of  $7.05 \pm 0.14$  g/L and lipid content of  $42.19 \pm 0.50\%$  (Table 3). Results also showed that the pH that leads to the lowest biomass and lipid content was 7.0 for *C. curvatus* PY39, leading to biomass and lipid contents of  $12.15 \pm 0.05$  g/L and  $27.98 \pm 2.23\%$  (Table 3). The lipid yield and cellular lipid percentage content the dry biomass at different initial pH values gave a clear picture for the optimum pH values for this oleaginous yeast strain. Accordingly, pH 5.0 was selected as the optimum pH in the medium for *C. curvatus* PY39.

Table 3 Effect of temperature, initial pH, agitation speed and incubation period on cell growth and lipid

accumulation of *C. curvatus* PY39

Cultivation conditions	Biomass (g/L)	Lipid yield (g/L)	Lipid content (%)
pH			
4.0	13.30±0.28c	3.95±0.25d	29.70±1.14e
4.5	14.48±0.34b	5.45±0.25c	37.65±1.54c
5.0	16.71±0.11a	7.05±0.14a	42.19±0.50a
5.5	16.32±0.14ab	6.64±0.05b	40.67±0.27b
6.0	14.97±0.07b	5.65±0.01c	37.82±1.05c
6.5	14.94 ±0.37b	4.82±0.65cd	32.26±0.60d
7.0	12.15±0.05e	3.39±0.16e	27.98±2.23f
Temperature (oC)			
20	12.87 ±0.64a	3.89±0.34c	30.22±1.02d
25	13.05 ±0.37a	4.61±0.42b	35.32±0.73b
30	14.97±0.03a	6.67±0.48a	45.49±0.67a
35	11.66 ±0.62b	3.77±0.18c	32.33±0.34c
40	8.17 ±0.13c	1.92±0.17d	23.52±0.36e
Agitation speed (rpm)			
100	11.44±0.41c	3.31±0.25d	28.93±0.77f
125	12.49±1.17b	4.39±0.16c	35.16±1.36e
150	12.58±0.35b	4.98±0.57c	39.58±2.44d
175	12.75±1.27b	5.12±0.42c	40.18±0.86c
200	12.98±0.36b	6.33±0.19b	48.77±0.44b
225	13.75±0.77a	6.93±0.66a	50.41±2.36a
Incubation period (h)			
0	1.25±0.45f	0.66±0.02f	5.28±0.51h
24	9.26±1.24e	2.04±0.15e	22.03±0.58g
48	11.75±0.69d	3.26±0.53d	27.74±0.65f
72	12.98±0.53c	4.35±0.24c	33.51±1.39e
96	13.78±1.01b	5.26±0.63b	38.23±2.64c
120	15.75±0.86a	7.22±0.26a	45.88±0.50a
144	13.75±0.56b	5.60±0.14b	40.75±1.50b
168	12.68±1.55c	4.60±0.29c	36.28±2.72d

All parameters are expressed as mean± SD values in the same column, n= 3, within each column, means are significantly different ( $p < 0.05$ ), unless they have a common letter.

Biomass and cellular lipid content of oleaginous yeast, namely *C. curvatus* PY39 was tested by growing each at temperatures of 20, 25, 30, 35 and 40°C in a nitrogen-limited culture media. As it is presented in Table 3, the best temperature for maximum biomass yield was 30°C for *C. curvatus* PY39 that led to biomass production of  $14.97 \pm 0.03$  g/L. Results also indicated that the optimum temperature for maximum lipid content by *C. curvatus* PY39 was 30°C that led to lipid content of  $45.49 \pm 0.67\%$  (Table 3). On the other hand, this strain gave the least amount of biomass, lipid yield and lipid content at temperature of 40°C. Based on this, *C. curvatus* PY39 gave biomass ( $8.17 \pm 0.13$  g/L) and lipid content

( $23.50 \pm 0.36\%$ ). Since the maximum lipid content was observed at the temperature of  $30^{\circ}\text{C}$  by this oleaginous yeast, this temperature was selected for further experiments.

The effects of agitation rate on cell biomass production, lipid yield and lipid content by the *C. curvatus* PY39 were determined under nitrogen-limited conditions by varying the agitation speed from 100 to 225 rpm is depicted in Table 3. A sharp increase in biomass and lipid yield as well as lipid content was exhibited by *C. curvatus* PY39 when the agitation speed was increased from 100 to 225 rpm. This strain gave maximum lipid yield ( $5.50 \pm 0.66$  g/L) and lipid content ( $40.00 \pm 2.36\%$ ) per dry biomass of  $13.75 \pm 0.77$  g/L at the agitation speed of 225 rpm.

Results from Table 3 also indicate that the agitation speed that led to the lowest biomass and lipid content was 100 rpm for *C. curvatus* PY39 leading to biomass production, lipid yield and lipid contents of  $11.44 \pm 0.41$  g/L and  $2.96 \pm 0.25$  g/L, respectively. Based on the facts generated, all further experiments were carried out in nitrogen-limited media at agitation speed of 225 rpm for *C. curvatus* PY39.

Biomass production, lipid yield and cellular lipid content of *C. curvatus* PY39, were tested at 0, 24, 48, 72, 96, 120, 144 and 168 h in nitrogen-limited media separately. It was apparent that, there was a sharp increase in biomass production, lipid yield and lipid content from 0 to 144 h by *C. curvatus* PY39. Maximum lipid yield of  $7.22 \pm 0.26$  g/L was attained by *C. curvatus* PY39 which corresponds to a lipid content of  $46.01 \pm 0.50\%$  per biomass of  $15.69 \pm 0.86$  g/L at 144 h incubation (Table 3).

## **Estimation of total sugar in peel mixtures of papaya and mango**

Total sugar present in fruit peel mixture was estimated at absorbance of 490 nm with different concentration of working standard of glucose solution depicted in Fig. 2. Percentage of total sugar in the fruit peel sample mixture was 13.20%.

## **Production of single cell oil using peel mixtures of papaya and mango**

Biomass, lipid yield and cellular lipid content of *C. curvatus* PY39 was tested by growing them on peel mixture of papaya and mango hydrolysate that were inoculated 10% v/v ( $\sim 7.94 \times 10^8$  cells/mL) seed cultures. The results obtained in this study were considerable and showed high potential of biomass, lipid yield and lipid content by the investigated *C. curvatus* PY39. *C. curvatus* PY39 when grown on peel mixtures gave biomass, lipid yield and lipid content of  $11.28 \pm 0.61$  g/L,  $3.95 \pm 0.67$  g/L and  $35.02 \pm 1.63\%$ , respectively.

## **Discussion**

Biomass production, lipid accumulation and fatty acid profile of oleaginous microorganisms are affected by the carbon source available during fermentation [5, 25]. For the production of maximum cell biomass and lipid in oleaginous yeasts, medium with an excess of carbon source and limited amount of nitrogen sources has a significant influence [26]. In this study, glucose, sucrose, maltose, galactose, xylose and glycerol were tested as carbon sources for the production of cell biomass and cellular lipid content. As it can be seen in Table 1, glucose as a carbon source gave maximum biomass and cellular lipid content by *C. curvatus* PY39. Considerable biomasses and lipid contents were also attained by this strain when grown both on sucrose and maltose. Ratledge [27], Saxena [28], Zhao et al. [29], Ahmad et al. [30] and Enshaeieh et al. [31] also reported glucose as the best carbon source for production of SCO.

On the other hand, lowest biomass and cellular lipid content were exhibited by this strain when xylose was used as a carbon source (Table 1). For economic reasons however glucose is not recommended for industrial production of SCO. Even though this oleaginous yeast gave lowest biomass and lipid content when xylose was supplied as a carbon source, it is still recommended for industrial production of SCO because it is obtained from cheap cellulosic wastes.

To evaluate the performance of different carbon substrates including wastes, glucose is often used as a comparison basis. Therefore, in this study we investigated the effect of glucose concentration, ranging from 10 to 90 g /L, for biomass and SCO yields by *C. curvatus* PY39. From the evaluated glucose concentrations, higher lipid production was exhibited by *C. curvatus* PY39 when this oleaginous yeast was grown in nitrogen-limited media containing 70 g/L of glucose (Table 1). When the tested yeast grew on media containing lower concentrations of glucose (especially 10 g/L and 30 g/L glucose), it yielded considerable biomass and lipid. Thus, it is recommended that such lower glucose concentrations should be used for industrial production of SCO. This saves cost of production. Previous researchers also reported different glucose concentrations for maximum production of biomass, lipid concentration and lipid content by various oleaginous yeasts. Accordingly, Enshaeieh et al. [32] reported 75 g/L for maximum production of biomass (12.7 g/L), lipid yield (7.13 g/L) and lipid content by *Rhodotorula* sp.110, while Kraisintu et al.[33] reported 70 g/L as the optimum glucose concentration for *Rhodospiridium toruloides* DMKU3-TK16 for maximum production of biomass, lipid yield and lipid content. Media containing concentrations of 80 and 90 g/L glucose were the best for maximum production of biomass, lipid yield and lipid content by *Torulasporea globosa* YU5/2 [34] and *Rhodotorula mucilaginosa* F [35], respectively.

In this study, the effect of combined organic and inorganic nitrogen sources on biomass production, lipid yield and cellular lipid content by *C. curvatus* PY39 was determined in nitrogen-limited media. The results showed that combinations of  $(\text{NH}_4)_2\text{SO}_4$  and yeast extract were the most favorable nitrogen sources for the maximum lipid production by *C. curvatus* PY39 among the different combinations tested as nitrogen compounds (Table 1). However, this yeast gave lowest biomass and cellular lipid content when it was grown in a media containing  $\text{NH}_4\text{Cl}$  and urea as nitrogen sources. These differences may be due to the assimilation preferences of some nitrogen sources over the others [36]. According to previous researchers, different nitrogen sources supported different oleaginous yeasts either in combination or

alone. *Trichosporon fermentans* gave maximum biomass and lipid content when it was grown in media containing urea and peptone, respectively [37]. On the other hand, Kraisintu et al.[33] reported that *Rhodospiridium toruloides* DMKU3-TK16 gave the highest biomass when the yeast was grown in a medium containing yeast extract and  $(\text{NH}_4)_2\text{SO}_4$  as nitrogen sources, while higher cellular lipid contents of 53.71 and 53.10% were produced, when peptone was provided with  $\text{NH}_4\text{Cl}$  and with  $(\text{NH}_4)_2\text{SO}_4$ , respectively. Maximum biomass (17.57 g/L), lipid yield (6.29 g/L) and lipid content (35.78%) were exhibited by *Rhodotorula* sp. 110 when yeast extract and  $(\text{NH}_4)_2\text{SO}_4$  were used in combination [32].

For the production of lipid by oleaginous microorganisms, there must be nutrient imbalance culture medium and specifically the C/N ratio has to be high [4, 38, 39]. In the current study, the selected oleaginous yeasts exhibited maximum biomass, lipid yield and lipid content at the C/N ratio of 140 (Table 1). Such optimum C/N ratio that enables *C. curvatus* PY39 to accumulate maximum lipid was found to be high. This means that, in a high C/N ratio medium, the depletion of the nitrogen levels is rapid and that sugar would selectively go in for lipid synthesis, thereby accounting for high lipid yield [40, 41]. Other researchers also reported C/N ratio as an important parameter for maximum lipid production and found optimum C/N ratios for the yeasts they tested and hence 77, 163 and 140 were found to be the optimum C/N ratios for maximum biomass and lipid production by *Rhodospiridium toruloides* ATCC 10788[42], *Trichosporon fermentans* [37] and *Rhodospiridium toruloides* DMAKU3-TK16 [33], respectively.

pH is one of the most important physiological factors affecting cell growth and lipid production[43]. In this study, highest biomasses were obtained when the pH media were kept at 5.0 and 5.5, while maximum lipid yield and lipid content were attained by *C. curvatus* PY39 when the pH values of 5.0 and 5.5 (Table 3), indicating that the tested oleaginous yeast prefer acidic pH to grow and accumulate lipids [4, 5, 44]. Previous studies also reported similar pH values for different oleaginous yeasts. Accordingly, 5.0 was the optimum and selected pH for *Lipomyces starkeyi* [45] and *Rhodotorula glutinis* EF081370 [46] for maximum production of biomass and lipid, while 5.5 was the best pH for *Rhodospiridium toruloides* DMKU3-TK16 [33], *Cryptococcus curvatus* NRRLY – 1511 (EI-[11] for maximum production of biomass and lipid. pH of 6.0 was the optimum value for maximum production of biomass and lipid yield by *Trichosporon fermentans* [47] and *Trichosporon cutaneum* [48].

Cellular lipid accumulation is critically affected by the culture temperature. Temperature affects all living organisms and controls the growth rate, lipid synthesis and alters the composition of fatty acids at cellular level [5, 25]. In the current study, *C. curvatus* PY39 exhibited maximum biomasses at temperatures of 20, 25 and 30°C, the biomasses exhibited at these temperatures were significantly the same. Maximum lipid content was attained by *C. curvatus* PY39 at the growth temperature of 30°C. Such results show that temperature of lipid synthesis and cell growth temperature are not consistent [5]. Maximum lipid content obtained at 30°C was in line with the results reported by Hong et al. [43], Syed et al. [49] and Mamatha [25]. It was evident in this research work that at a temperature of 40°C, the least biomass, lipid yield and lipid content were exhibited by this strain. An increase in temperature will

increase enzyme activity. If the temperature is too high, enzyme activity will diminish (enzymes may be denatured). Lowering temperature will decrease enzyme activity. Such effects in turn might influence on biomass production and lipid yield.

Agitation and aeration have great importance in aerobic culture to increase biomass and hence lipid production [50]. In the current study, the optimum agitation speed for maximum biomass production and lipid content by *C. curvatus* PY39 was at 225 rpm. This is because the higher the agitation speed, the oxygen that dissolve in the medium become higher and it increases growth and lipid content as energy metabolism and synthesis of lipid components need oxygen [6]. Too low shaking speed of the flask culture would cause low dissolved oxygen in the medium, limits cell growth and synthesis of oil, whereas too high rotation speed could cause the shear stress to become larger and reduces cell growth and synthesis of oil [5]. Other researchers also investigated optimum agitation speeds for different oleaginous yeasts. Accordingly, 180 and 200 rpm were optimum agitation speeds for *Rhodotorula glutinis* [51] and *C. curvatus* NRRLY - 1511 [11], respectively.

Different oleaginous microorganisms require different culture time (incubation period) for maximum biomass production and lipid accumulation [11, 33, 43, 51]. In the current study, *C. curvatus* PY39 exhibited highest biomass and lipid content towards the latter incubation periods (144 h). In the previous studies, researchers have found that there is a close relationship between the length of incubation period and the best time for the synthesis of lipids [5]. There were reports of oleaginous yeasts during the whole growth period, the accumulation of oil was very little in the early stage because of the nutrients and cell growth, most oil (grease) was used to the synthesis of cell membrane, the lipid would have increased significantly and reach the peak in the stable phase [5]. Other researchers also reported different optimum incubation periods for maximum production of lipid by different oleaginous yeasts. Incubation period of 72 h was optimum for maximum biomass and lipid production both in *R. glutinis* [51] and *C. curvatus* NRRLY - 1511 [11]. On the other hand, *Rhodospiridium toruloides* DMKU3-TK16 exhibited maximal lipid yield and lipid cellular lipid content after 168 h of cultivation [33].

Fruit based industries produce large volume of wastes, these wastes pose increasing disposal and pollution problems and represent a loss of valuable biomass and nutrients [52]. However, these carbohydrate rich wastes can be tuned into valuable substrates for the commercial production of lipids and thus can be regarded as available option for meeting the growing demand for lipids. For economic reasons, low-cost feedstock substrates such as peels of orange, banana, potato, sugarcane bagasse, corn stalk, tomato waste, rice bran, rice straw and rice hull was used as a carbon source for the production of SCO [8, 7, 35, 53]. On the other hand, such fruit wastes have limitations such as their seasonal availability, substrate preparation cost, high water content and spoilage problems. Still with limitations they can be alternative feedstocks.

Microbial lipids have long been considered as an alternative to sources of conventional oils. To produce such lipids, oleaginous microorganisms, especially oleaginous yeasts have to be grown on cheap substrates for economic reasons. Taking this into account, *C. curvatus* PY39 was cultivated under

nitrogen-limited media containing papaya and mango peel mixtures for the production of lipid. Peel mixture of papaya and mango hydrolysate were found to be rich in carbohydrate content and supported the growth of *C. curvatus* PY39. The results obtained in this study are in agreement with the research works by Tinoi and Rakariyatham [54] on utilization of pineapple waste hydrolysate for lipid production by *Rhodoturula glutinis* and Rattanapoltee and Kaewkannetra [55] on heterotrophic growth of a microalga, namely *Scenedesmus acutus* that cultivated on pineapple peels.

The type of species and growth conditions such as temperature, pH, type of substrate (medium of composition), variation in C/N ratio, and oxygen availability influence the fatty acid profiles and the efficiency of lipid accumulation [56, 57]. In this study, the fatty acid profiles of *C. curvatus* PY39, was evaluated using GC. It was observed that the fatty acid compositions are qualitatively similar but slightly differ quantitatively.

*C. curvatus* PY39 exhibited a predominant amount of oleic acid and low amounts of other fatty acids (linoleic acid, palmitic acid, linolenic acid, palmitoleic acid, palmitic acid, arachidic acid and behenic acid). The fatty acid profile determined in the current study displayed preferential synthesis of high percentage of saturated and monounsaturated fatty acids in the *C. curvatus* PY39. The concentration of saturated and monounsaturated fatty acids of *C. curvatus* PY39 added up  $85.24 \pm 2.63\%$ . In previous studies, researchers have also reported oleic acid as the major fatty acid in SCOs followed by palmitic acid and low amount of other fatty acids [30, 58, 59]. The fatty acids that were exhibited by the four strains were not only similar to fatty acid profiles of other oleaginous yeasts but also they fit well with the fatty acid profiles of different vegetable oils such as rapeseed, soybean, palm, and sunflower [60, 61]. From these comparisons, one can see that there are similarities with the relative abundance of fatty acid profiles with slight differences. This again confirmed that the lipids from these oleaginous yeasts are good oil feedstock for biodiesel production. In addition, the main components of fatty acid profiles of the four oleaginous yeasts are C16:0, C18:0, C18:1 and C18:2 which is similar to cacao-butter [62]. Thus, such fatty acids can be used for human consumption.

## Conclusion

Culture conditions played a critical role on the growth rate and lipid accumulation by *C. curvatus* PY39. Comparing the results with previous data, it is clear that there are some differences across species and strains and it is, therefore, important to investigate the optimal parameters for each strain tested. This study also showed the capacity of *C. curvatus* PY39 to utilize peel mixtures of papaya and mango hydrolysate as a media for the production of biomass and lipid. Thus, peel mixtures of papaya and mango hydrolysate could be used as cheap substrates for the cost-effective production of SCO which is used as a feedstock for the preparation of biodiesel. The ability to utilize fruit peel hydrolysate is very important from an environmental point of view. This research work provides an insight to cultivate oleaginous yeasts on other lignocellulosic wastes. *C. curvatus* PY39 have been observed to realize its ability to produce higher concentrations of saturated and monounsaturated fatty acids (with oleic acid

the most dominant) than those of unsaturated fatty acids. This makes this oleaginous yeast the most promising candidates for biodiesel production.

## Methods

### Yeast strain

In this study, 200 samples were collected from soil, plant surfaces (leaves, flowers and fruits), traditional oil mill wastes, and dairy products (cheese, milk and yoghurt) in Ethiopia. Yeast isolation was performed according to the methods of Pan et al. [24]. Three hundred and forty yeast colonies were isolated from these samples. It was found that the yeast strain PY39, which was isolated from flower contained oil content of  $46.51 \pm 0.70\%$  w/w. This strain was identified as *C.curvatus*, using both conventional (morphological and physiological) and molecular (sequencing both ITS domains and D1/D2 domains of the large subunit) by Jiru et al. [63]. The accession number for this strain is KX525704.

### Inoculum Preparation

A loopful of yeast cells from growing on Yeast Malt (YM) extract agar slants, containing glucose (10 g/L), peptone (5 g/L), yeast extract (3 g/L), malt extract (3 g/L) and agar (20 g/L), were inoculated into a sterilized nitrogen-limited medium containing, glucose (50 g/L),  $(\text{NH}_4)_2\text{SO}_4$  (0.31 g/L), yeast extract (0.50 g/L),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (1.5 g/L),  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (0.1 g/L),  $\text{KH}_2\text{PO}_4$  (1.0 g/L),  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (0.035 g/L),  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  (0.011 g/L),  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$  (0.007 g/L),  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  (0.002 g/L),  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$  (0.0013 g/L) and  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (0.001 g/L). The culture was allowed to grow for 24 h at 30°C, pH 5.5 at 200 rpm. An inoculum of 10% v/v ( $\sim 7.94 \times 10^8$  cells/mL) was added to the nitrogen-limited cultivation medium.

#### Optimization of culture conditions for biomass production and lipid accumulation

The influence of carbon sources (glucose, sucrose, maltose, galactose, xylose and glycerol), glucose concentration (10, 30, 50, 70 and 90 g/L), nitrogen sources [ $(\text{NH}_4)_2\text{SO}_4$  and yeast extract,  $\text{NH}_4\text{Cl}$  and peptone,  $\text{NH}_4\text{Cl}$  and yeast extract,  $(\text{NH}_4)_2\text{SO}_4$  and peptone,  $(\text{NH}_4)_2\text{SO}_4$  and urea,  $\text{NH}_4\text{Cl}$  and urea], C/N ratio (40, 60, 80, 100, 120, 140 and 160), pH (4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0), temperature (20°C, 25°C, 30°C, 35°C and 40°C), agitation speed (100, 125, 150, 175, 200 and 225 rpm) and incubation period (0, 24, 48, 72, 96, 120, 144 and 168 h) were tested in a nitrogen-limited media. One parameter was tested at a time. Dry biomass, lipid yield and lipid content were determined. Cultures were grown in 250 mL Erlenmeyer flasks containing 50 mL media. For determination of C/N ratio, total nitrogen content of yeast extract was determined using Kjeldahl apparatus according to the instructions published by Krishna and Ranjhan [64].

### Dry Cell Weight Determination

In series of the propagation processes, cell harvest and biomass determination was done according to Pan et al. [24]. Cells were harvested by centrifugation at 5000 × g for 15 min, washed twice with distilled water, frozen at -80°C and freeze dried overnight to constant weight. The dry biomass was determined gravimetrically.

## Determination Of Lipid Content

Lipid extraction was done following the method described by Folch et al. [65], with some modifications. Freeze dried biomass was ground with a pestle in a mortar and 1 g of sample was extracted with 3.75 mL solvent mixture of chloroform and methanol (2:1) overnight. The solvent mixture was transferred into a clean separating funnel through Whatman No1 filter paper. Then 1.25 mL of the solvent mixture was added through filter paper into separating funnel. This was followed by washing with 0.75 mL of distilled water. The solvent/water mixture was left overnight to separate into two clear phases. The bottom phase was collected and the solvents evaporated under vacuum. Diethyl ether was used to transfer the extract into pre-weighed glass vials and the solvent evaporated. The dry lipids were weighed and lipid content calculated.

$$\text{Single cell oil content} = \frac{\text{Single Cell Oil Weight (g/L)}}{\text{Cell Dry Weight (g/L)}} \times 100$$

## Production of single cell oil using peel mixture of papaya and mango

### Substrate Collection And Preparation

Fresh papaya and mango peels were collected from fruit processors within the Arat Kilo area, Addis Ababa, Ethiopia. The peels were thoroughly washed with water to remove attached foreign materials. Then the peel mixture was homogenized and blended for 5 min. A 2% (v/v) sulphuric acid at a solid to liquid ratio of 1: 10 (w/v) was added in to the fruit peel mixture for hydrolysis. A 2 mm screen was used to sieve the resulting slurry before being stored at 20°C for subsequent use. The diluted peel mixture then was boiled, allowed to cool and sedimentation of insoluble materials occurred. The sediments were removed by decantation. The resulting mixture was centrifuged with a high speed at 5000×g for 10 min for further removal of insoluble materials. The supernatant solution was separated from the pellet. The pellet was discarded and the supernatant was used for the cultivation purpose.

## Determination of total sugar in papaya and mango peel mixtures

**Sample preparation:** For determination of total sugar, 100 mg of peel mixture of papaya and mango was homogenized and taken in boiling tube and hydrolyzed by adding 5mL of 96% sulphuric acid in it. Boiling tubes were kept in water bath for 3 h and then removed from water bath and cooled to room temperature. After cooling, it was neutralized by adding solid sodium carbonate until effervescence ceases. Then whole volume was made up to 100 mL by adding distilled water and centrifuged. Then the supernatant was used for total sugar estimation.

**Total sugar estimation:** Total sugar contained in fruit peel mixture was analyzed by the method of DuBois *et al.* [66] which is based on the phenol - sulphuric acid reaction. The standard curve for glucose was prepared by adjusting the final concentration to 0.2 mg/mL as 0, 0.02, 0.04, 0.06, 0.08, 0.1, 0.12, 0.14, 0.16, 0.18, and 0.2. The final volume of each tube was made 2 mL by adding distilled water. The amount of fruit peel sample used was 0.2 mL. One mL of 5% phenol and 5mL of 96% sulphuric acid was added one by one in each tube and vortexed well so that the phenol and sulphuric acid get mixed thoroughly with working standard. After 10 min all the tubes were placed in water bath at 25°C for 15 min. At last the absorbance was read at 490 nm using spectrophotometer (JENWAY model 6405). The total sugar in the fruit peel mixture was calculated following the protocols developed by Sadasivam and Manickam [67].

Absorbance corresponds to 0.2 mL of fruit peel sample test = x mg of glucose

$$100 \text{ mL of peel sample solution contain } = \frac{x}{0.2} \times 100 = \% \text{ of total sugar present}$$

## Production of single cell oil from oleaginous yeasts peel wastes

Production of SCO was tested in a nitrogen-limited media as listed in the previous chapters except the carbon source. In this case the carbon source used was fruit peel mixture and the amount used was estimated according to the method of DuBois *et al.* [66] and Sadasivam and Manickam [67]. Fruit peel mixture of 53% (~70 g/L) was used. The fermentation medium was inoculated with 10% (v/v) of the liquid seed culture.

All the optimized parameters were applied here for the production of SCO by each of this oleaginous yeast using peel mixtures of papaya and mango.

## Analysis of fatty acids profiles using gas chromatography

To determine the fatty acid composition of the lipids, the extracted lipids were transferred to GC vials, dissolved in chloroform and methylated with trimethylsulphonium hydroxide (TMSOH) [68]. The vials were then sealed and vortexed for approximately 5 sec. Fatty acid methyl esters were subsequently analyzed on a Shimadzu GC-2010 gas chromatograph with a flame ionization detector. An injection volume of 0.5 µL of sample was added into a SGE-BPX-70 column (length of 50 m and inner diameter

0.22 mm). The injection port had a temperature of 250°C and a split ratio of 1:10. The column temperature was 200°C. Hydrogen gas was used as a carrier gas at a flow rate of 40 mL/min. The total program time was 4.50 min per sample with a column flow rate of 1.37 mL/min. Peaks were identified by reference to authentic standards.

## Statistical analysis

All experiments were done in triplicate. One way-ANOVA was performed to calculate significant differences in treatment means. SPSS version 20.0 software was used for interpretation of the data. Mean separations were performed by Tukey *post hoc* tests. A *p* value < 0.05 was considered significant.

## Abbreviations

SCO: Single Cell Oil; GC: Gas Chromatography; TAGs: Triacylglycerols

## Declarations

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### Authors' contributions

TM performed the experiments as part of his doctoral work.

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### Availability of data and materials

The sequence dataset generated for this isolate is available in the NCBI Short Read Archive repository (Accession Number KX525704).

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

## Competing interest

Not applicable.

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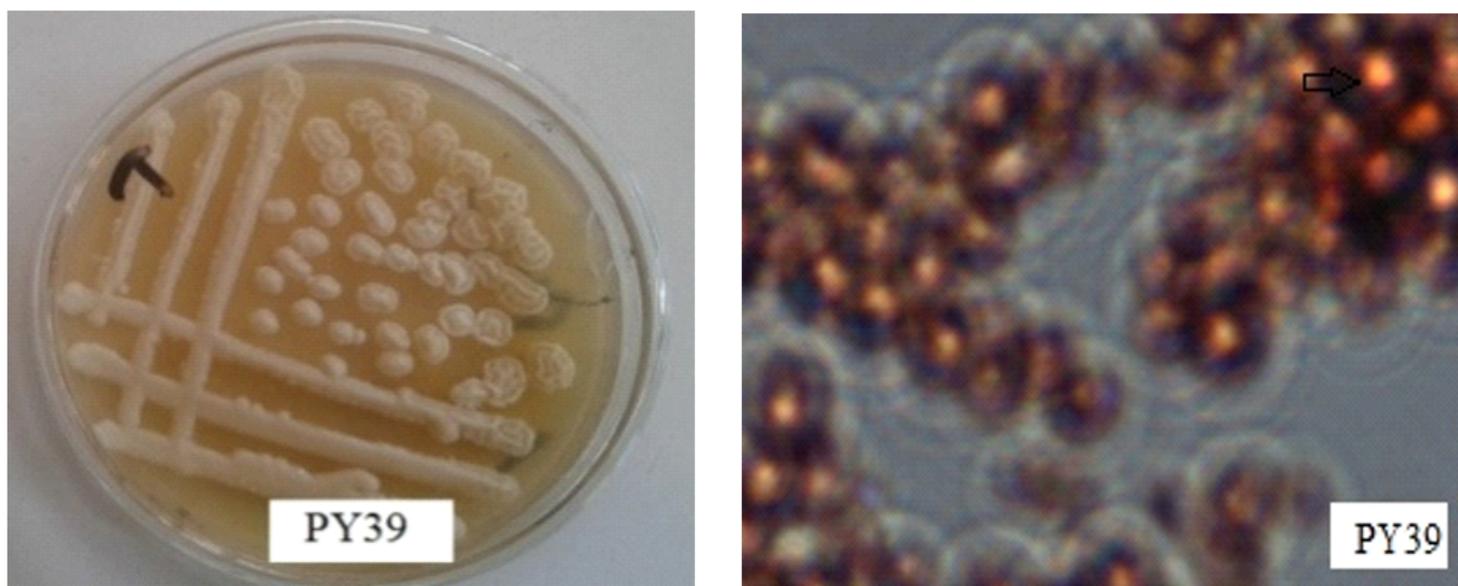
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## Figures



**Figure 1**

a) Colony morphology of PY39 and b) Sudan III stained cells of PY39.

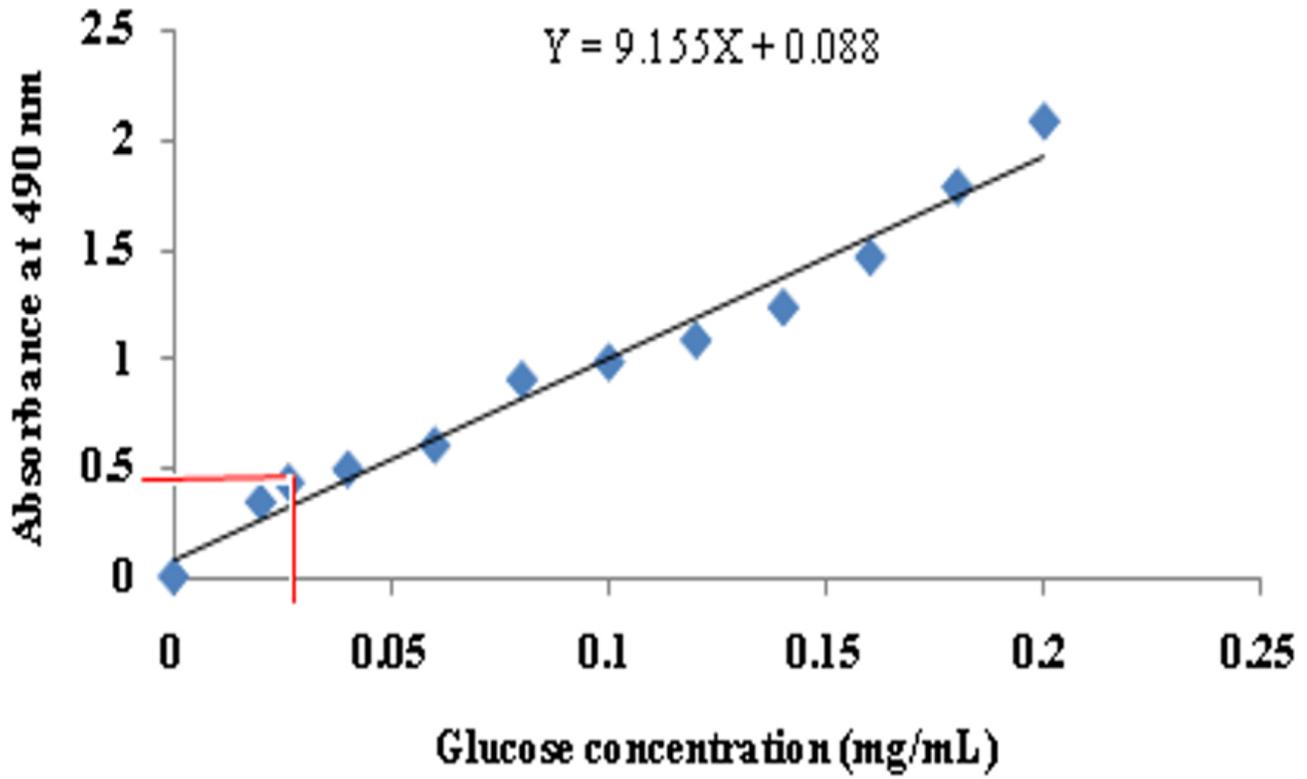


Figure 2

Standard curve of glucose for the determination of total sugar in peel mixtures of papaya and mango.