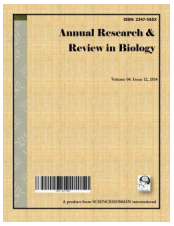

Annual Research & Review in Biology

22(3): 1-8, 2018; Article no.ARRB.38360
ISSN: 2347-565X, NLM ID: 101632869

Microbial Oil Production under Optimum Condition by Oleaginous Yeast

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Authors' contributions

This work was carried out in collaboration between all authors. Author DMM designed the study, prepared the figures and tables and performed the statistical analysis. Authors FHAEZ and HKEM managed the analyses of the study, managed the literature searches and wrote the first draft. All authors wrote the protocol, read and approved the final manuscript.

Article Information

DOI: 10.9734/ARRB/2018/38360
Editor(s):
(1) Tony Gutierrez, School of Life Sciences, Heriot-Watt University, UK.
(2) George Perry, Dean and Professor of Biology, University of Texas at San Antonio, USA.
Reviewers:
(1) Gisel Chenard Díaz, Federal University of Rio de Janeiro, Brazil.
(2) Azhari Muhammad Syam, University of Malikussaleh, Indonesia.
(3) Tatang Sopandi, University of PGRI Adi Buana Surabaya, Indonesia.
Complete Peer review History: http://www.sciencedomain.org/review-history/22724

Original Research Article

Received 23rd October 2017
Accepted 3rd January 2018
Published 13th January 2018

ABSTRACT

Two oleaginous yeast strains identified as *Issatchenkia orientalis* D5 and *Candida tropicalis* S5 were evaluated for oil production under optimized growth factors. Both strains were inoculated in 2L of optimal fermentation broth medium for 9 days. Sample was taken periodically (24 hrs.), The data exhibited that cell biomass was gradually increased along with time sample to reach maximum values of cell dry weight (11.22 & 13.30 gL^{-1}) after 8 and 9 day at consumed sugar equal to 79.52 and 156.29 gL^{-1} for *Candida* and *Issatchenkia*. It was found that *Candida* gave the highest values of lipid weight, lipid content, lipid yield, conversion coefficient and lipid productivity after 6 days being 2.16 gL^{-1}, 34.17%, 2.70%, 2.73% and 0.36 gL^{-1}/day, while *Issatchenkia* recorded the highest lipid concentration and lipid yield after 4 days of the fermentation period being 3.24 gL^{-1} and 2.06%. The specific growth rate, doubling time, multiplication rate and number of generation were 0.317 day^{-1}, 2.18 days, 0.458 day^{-1} and 1.37 for *Candida* and reached to 0.275 day^{-1}, 2.52 days, 0.396 day^{-1} and 0.793 for *Issatchenkia*. The data proved that the strains *Issatchenkia orientals* and *Candida tropicalis* could be used as feedstock producers for microbial lipid production.

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Keywords: Oleaginous yeast; fermentation; microbial lipid.

1. INTRODUCTION

Microbial lipids are promising resource instead of traditional oil sources in the production of biodiesel, and other products due to similar chemical composition and energy value [1].

Oleaginous microorganisms belonging to the genera of algae, bacteria, yeast and fungi have the ability to accumulate lipids under specific cultivation conditions [2], in the conditions of nitrogen limitation the accumulate lipids can increase up to 70% by dry weight of biomass. Oils derived from microbial sources are also defined as Single-Cell Oils (SCO). Single cell oil is a kind of lipid producing by oleaginous microorganisms as the supplementary source of conventional oil and fat [3]. Oleaginous organisms can store lipids in oil vacuole of cell in the form of triacylglycerols [4].

The term of biolipids, including triacylglycerol produced by oleaginous yeast, have been confirmed to be one of the important raw materials for production of biodiesel. The biodiesel quality depens upon the fatty acid composition of the biolipids [5]. Production of biolipids by oleaginous yeast are suitable for biodiesel, because the composition of fatty acid satisfies important criteria i.e., chain length and saturation degree. However, the composition of fatty acid of biolipids is strain specific, thus it is important to select oleaginous yeast strains to ascertain their suitability for biodiesel production [6].

The lipids accumulated by oleaginous yeast are mainly composed of long-chain fatty acids, including oleic acid (C18:1), palmitic acid (C16:0), linoleic acid (C18:2) and stearic acid (C18:0), which are similar to the composition of plant oils and can be converted into biodiesel by enzymatic or inorganic catalysis [7,8]. The previous fatty acids could be used as nutritional complement for medical application and can be used for biodiesel production.

Although, oils produced by oleaginous microorganisms are considered as potential alternatives for biodiesel production due to their similar fatty acid composition to the vegetable oils which contain mainly C_{16} and C_{18} fatty acids. [9,10,11,12]. However, the cost of microbial oil production is still higher than those of vegetable and animal oils. Therefore, finding strategies to improve the economics of microbial oil production process is require.

The current study aims at application of optimum culturing, growth and fermentation requirements for maximizing oil production by oleaginous yeast *Candida tropicalis* and *Issatchenkia orie*.

2. MATERIALS AND METHODS

2.1 Strains Growth

Out of 76 yeast isolates, two potential isolates in oil production were identified as *Candida tropicalis* S5 and *Issatchenkia orientals* D5 in a previous study conducted by [13]. The two isolates were grown and stored on YPDA medium which composed from:(gL^{-1}) glucose, 20; peptone, 10.0 g; yeast extract, 5.0 g; agar 20.0 g., then incubation at 28- 30°C for 48 h. and stored in a refrigerator at 4°C until use. The stock cultures were renewed once every 2 months.

Isolates (S5, D5) were prepared by inoculation of 250 ml of conical flask volume containing 100 ml of inoculum's medium [14] (glucose, 20 gL^{-1} ; peptone, 10 gL^{-1} ; Yeast extract , 10 gL^{-1} and pH 6.0) with a loop of the tested yeast. The inoculated flasks were incubated on orbital shaker (150 rpm) for 24 hours at 28-30°C. Under aseptic condition, the content of the flask ($6x10^9$ cfu/ml) was centrifuged at 10000 rpm for 15 min and then the pellet was used as a standard inoculum for 100 ml of productive medium. Growth characteristics, lipid production and sugar consumption of the two selected yeast strains were studied using 5L Erlenmeyer flask containing 2L of optimal fermentation N-limited medium as that used by [15]: (gL^{-1}) (Glucose 40, $(NH_4)_2SO_4$, 1.0; KH_2PO_4, 7.0; NaH_2PO_4, 2.0; $MgSO_4.7H_2O$ 1.5); Yeast extract, 1.0). Each flask inoculated with 5 ml broth culture of both strains, then incubated at 28-30°C under shaking conditions (150 rpm) for 9 days on incubator shaker under optimized growth culturing requirements of carbon and nitrogen sources, temperature and C/N ratio for maximizing lipid production yield. Samples (50 ml) were taken periodically every 24 hrs to determine the dry biomass, lipid content and sugar consumed. The relation between time (h) and cell dry weight (gL^{-1}) was plotted using Excel program. The lipid content was extracted by the method of [16], which is modified by [17]. Sugar consumption, sugar utilization efficiency, yield factor, lipid yield, conversion coefficient and lipid

productivity were estimated. Specific growth rate (μ), doubling time (t_d), multiplication rate (MR) and number of generation (N) were also calculated.

2.2 Determination of Yeast Biomass

An aliquot of 10 ml of the culture was harvested by centrifugation at 5000 rpm for 10 minutes at 4°C. The harvested biomass was washed twice with 10 ml of distilled water and dried at 60°C to constant mass. The dry biomass was then determined gravimetrically.

2.3 Estimation of Residual Sugar

The total amount of residual sugar (as glucose) was determined in the supernatant of yeast cultures using 3,5 dinitrosylcilic acid reagent as described by [18].

2.4 Specific Growth Rate (μ)

The specific growth rate (μ) and doubling time (t_d) were calculated from the exponential phase according to [19] using the following equation:-

$$\mu = (\ln A - \ln A_o)\, t^{-1}$$

Where:

- μ = Specific growth rate (h^{-1})
- A = Amount of growth after t time
- A_o = Amount of growth at the beginning of logarithmic phase
- T = Time of the logarithmic phase

2.5 Doubling Time (t_d)

$$t_d = \ln 2.\ \mu^{-1}$$

2.6 Number of Generation (N)

Number of generations was calculated using the following equation according to [20]:

$$N = t/t_d \quad \text{or} \quad N = (\log A - \log A_o)/\log 2$$

Where,

- N = Number of generations
- T = the period of exponential (h)
- t_d = doubling time (h)
- A = Amount of growth after t time
- A_o = Amount of growth at the beginning of logarithmic phase.

2.7 Multiplication Rate (MR)

Multiplication rate was calculated according to the following equation [20].

$$MR = N\, t^{-1} \quad \text{or} \quad MR = \mu/\ln 2$$

Where,

- MR = Multiplication rate
- N = Number of generation
- T = Time of the logarithmic phase
- μ = Specific growth rate (h^{-1}).

2.8 Yield Factor (Y)

Yield factor = amount of growth (dry weight) per 100 unit of sugar consumed [21].

2.9 Sugar Utilization Efficiency (SUE)

Sugar utilization efficiency = consumed sugar gL^{-1}/ initial sugar gL^{-1} [22].

2.10 Lipid Content

Lipid content= lipid weight (gL^{-1})/Cell dry weight (gL^{-1}) × 100 [15].

2.11 Conversion Coefficient (C.C)

Conversion coefficient of lipid yield = lipid weight (gL^{-1}) X 100 / consumed sugar (gL^{-1}) [22].

2.12 Lipid Productivity

Lipid productivity= lipid weight (gL^{-1})/ fermentation time (h^{-1}) [23].

2.13 Statistical Analysis

Statistical analysis was carried out by standard error "SE" according to [24]. LSD (Least significant difference) test was used to compare the significant difference between means of replicates. The level of statistical significance was marked at $P < 0.05$. The CoStat 6.4 program version was used for the analysis.

3. RESULTS

Results in Tables 1&2 show the growth parameters of tested oleaginous yeast strains

namely *Candida tropicalis* S5 and *Issatchenkia orientalis* D5. It was observed that cell biomass in both strains gradually increased along with the time course. This increase was parallel associated with consumed sugar to reach maximum values of cell dry weight (11.22 & 13.30 gL^{-1}) after 8 and 9 days for both strains. The consumed sugar was slightly increase by increasing time of fermentation to reach its maximum at the 8^{th} (79.52 gL^{-1}) and the 9^{th} day (156.29 gL^{-1}) for *Candida tropicalis* S5 and *Issatchenkia orientalis* D5, respectively. It is worth to mention that the rate of sugar consumption was varied between the two strains. The data in Tables 1&2 denote that the amount of consumed sugar by *Issatchenkia* (142.91 gL^{-1}) was almost double that consumed by *Candida* (79.07 gL^{-1}) at the maximum lipid production. Consequently, the conversion coefficient by *Candida* (2.73%) was surpass that detected for *Issatchenkia* (2.26%). Additionally, the values of yield factor (biomass yield %) continuously increased for both strains through the time course and reached to the maximum values (14.10 & 8.51%) at the 8^{th} and the 9^{th} day for *Candida* and *Issatchenkia*, respectively. The same trend was observed with respect to lipid yield characters, since it was found that *Candida tropicalis* S5 realized the highest values of lipid weight, lipid content, lipid yield, conversion coefficient and lipid productivity after 6 days. Their assigned values were 2.16 gL-1, 34.17%, 2.70%, 2.73% and 0.36 gL^{-1}/day, respectively. On the other hand, the second yeast strain *Issatchenkia orientalis* D5 recorded the highest values of accumulated lipid and lipid yield after 4 days of beginning the fermentation being 3.24 gL^{-1} and 2.06%, respectively, as well the values of other parameters, lipid content, conversion coefficient and lipid productivity.

Table 3 illustrated by Fig. 1 manifested the growth kinetics of the two tested yeast, strains, calculated from exponential phase. The data showed that the values of the specific growth rate, doubling time, multiplication rate and number of generation were 0.317 day^{-1}, 2.18 days, 0.458 $day^{-1}$and 1.37 for *Candida* compared to 0.275 day^{-1}, 2.52 days, 0.396 day^{-1} and 0.793 for *Issatchenkia.* It should be mention that all the previous recorded values for *Candida* were higher than those of *Issatchenkia*, except the value of doubling time. It was also observed that, there was a high positive correlation between cell dry weight and lipid weight along with incubation time (Fig. 1B&C). From the regression analysis of lipid weight against incubation time course, two straight line equations for both strains were obtained (Fig. 1C) being $y = 0.2596\ e^{0.358x}$and $y = 0.7495\ e^{0.385x}$ for *Candida tropicalis* S5 and *Issatchenkia orientalis* D5, respectively. These equations revealed that the specific production rate of lipid by *Candida* strain was lower (0.358 day^{-1}) than that observed by *Issatchenkia* strain (0.385 day^{-1}). It means that the lipid content of the first strain reached the double value of lipids after 1.94 days, compared to 1.80 days for the second strain.

Table 1. Biological activity of *Candida tropicalis* S5 grown in optimized N-limited medium in shaking batch culture at 30°C under optimum culture conditions

Time (day)	Cell dry weight (gL^{-1})	Lipid weight (gL^{-1})	Lipid content (%)	Consumed sugar (gL^{-1})	SUE (%)	Yield factor (%)	Lipid yield (%)	C.C (%)	Lipid productivity (gL^{-1}/day)
Zero	1.58 J	0.10 G	6.32 I	0.00 I	0.00 I	0.00 J	0.12 H	0.00 H	0.00 D
1	1.78 I	0.15 G	8.42H	67.70 H	84.62 H	2.63 I	0.18 G	0.22 G	0.15C
2	2.38 H	0.52 F	21.84 E	73.13 F	91.41 F	3.25H	0.65 F	0.71 F	0.26 B
3	3.42 G	0.84 E	24.56 D	72.25 G	90.31 G	4.73G	1.05 E	1.16 E	0.28 B
4	4.55 F	1.16 D	25.49 C	76.79 E	95.99 E	5.92 F	1.45 D	1.51 D	0.29 B
5	4.68 E	1.50 C	32.05 B	78.66 C	98.33 C	5.95 E	1.87 C	1.90 C	0.30 B
6	6.32 D	2.16 A	34.17 A	79.07 B	98.84 BC	7.99 D	2.70 A	2.73 A	0.36 A
7	8.76 C	2.06 B	23.51 D	79.49 A	99.36 AB	11.02 C	2.57 B	2.59 B	0.29 B
8	11.22 A	2.13 AB	18.98 F	79.52 A	99.40 A	14.10 A	2.66 A	2.67 A	0.27 B
9	9.92 B	1.17 D	11.79 G	78.20 D	97.75 D	12.68 B	1.46 D	1.49 D	0.13 C
LSD	**0.027**	**0.077**	**0.93**	**0.29**	**0.541**	**0.056**	**0.077**	**0.056**	**0.056**

Values in the same column with the same letter have no significant difference while those have different letters show a significant difference at p≤ 0.05.

Initial glucose 80 gL^{-1} Lipid content (%) = lipid weight (gL^{-1}) /cell dry weight (gL^{-1}) X 100

Sugar utilization efficiency (SUE) % = consumed sugar (gL^{-1}) /initial sugar (gL^{-1}) X 100 Yield factor (%) =cell dry weight (gL^{-1})/consumed sugar (gL^{-1}) X 100

Conversion coefficient (%) = lipid weight (gL^{-1}) / consumed sugar (gL^{-1}) X 100 Lipid yield (%) = lipid weight (gL^{-1}) / initial sugar (gL^{-1}) X 100

Lipid productivity ($gL^{-1}day^{-1}$) = lipid weight (gL^{-1}) / time of fermentation (day)

Table 2. Biological activity of *Issatchenkia orientalis* D5 grown in optimized N-limited medium in shaking batch culture at 30°C under optimum culture conditions

Time (day)	Cell dry weight (gL^{-1})	Lipid weight (gL^{-1})	Lipid content (%)	Consumed sugar (gL^{-1})	SUE (%)	Yield factor (%)	Lipid yield (%)	C.C (%)	Lipid productivity (gL^{-1}/day)
Zero	3.28 H	0.10G	3.04 G	0.00 F	0.00 F	0.00 G	0.06G	0.00 I	0.00G
1	3.30 H	0.50 F	15.15 D	138.50 E	87.93 E	2.38 F	0.32F	0.36 G	0.50C
2	4.58 G	1.50 C	32.75 C	142.14 C	90.24 CD	3.22 F	0.95 C	1.05 C	0.75B
3	5.72 F	2.53 B	44.25 B	140.32 D	89.09 DE	4.08 E	1.61B	1.80B	0.84 A
4	5.82 E	3.24 A	55.67 A	142.91 C	90.73 C	4.07 E	2.06A	2.26 A	0.81A
5	6.30 D	0.98 D	15.55 D	146.70 B	93.14 B	4.29 E	0.62F	0.66 D	0.19D
6	8.26 C	0.84 E	10.17 E	147.53 B	93.66 B	5.60 C	0.53 E	0.57 E	0.14E
7	8.22 C	0.80 E	9.73E	156.74 A	99.51 A	5.24 D	0.51E	0.51 F	0.11E
8	9.40B	0.46 F	4.89 F	156.77 A	99.53 A	6.00 B	0.29D	0.29 H	0.06F
9	13.30A	0.43 F	3.23 G	156.29 A	99.23A	8.51 A	0.27F	0.27 H	0.05F
LSD	**0.094**	**0.094**	**0.078**	**1.62**	**1.61**	**0.275**	**0.019**	**0.056**	**0.055**

Values in the same column with the same letter have no significant difference while those have different letters show a significant difference at p≤ 0.05.

Initial glucose 157.5 gL^{-1}Lipid content (%) = lipid weight (gL^{-1}) /cell dry weight (gL^{-1}) X 100

Sugar utilization efficiency (SUE) % = consumed sugar (gL^{-1}) /initial sugar (gL^{-1}) X 100

Yield factor (%) =cell dry weight (gL^{-1})/consumed sugar (gL^{-1}) X 100

Conversion coefficient (%) = lipid weight (gL^{-1}) / consumed sugar (gL^{-1}) X 100

Lipid yield (%) = lipid weight (gL^{-1}) / initial sugar (gL^{-1}) X 100

Lipid productivity ($gL^{-1}day^{-1}$) = lipid weight (gL^{-1}) / time of fermentation (day)

Table 3. Growth parameters of *Candida tropicalis* S5 and *Issatchenkia orientalis* D5 strains grown in optimized nitrogen- limited medium at 30°C during 9 days under shaking conditions

Growth parameters	*Candida tropicalis* S5	*Issatchenkia orientalis* D5
Specific growth rate (μ) (day^{-1})	0. 317	0.275
Doubling time (td) day	2.18	2.52
Multiplication rate (MR) (day^{-1})	0.458	0.396
Number of generations (N)	1.37	0.793

Specific growth rate (μ) h^{-1}=Log A-Log A_0 / ($t-t_0$)

Doubling time (t_d) h =ln2/ μ

Multiplication rate (MR) = 1/td or MR= μ /ln2

Number of generation (N) = t/t_d

4. DISCUSSION

Oleaginous yeasts are able to store large quantities of TAGs in the form of lipid bodies in the cells. Typical lipid contents range from 20% to 76% depending species and culture conditions [25].

The similarity of lipid in microorganisms such as fungi and yeast has been a lot of attention because it can be used as a feedstock for biodiesel production and many other industrial purposes. Yeast that can accumulate lipid more than 20% of their biomass are called as oleaginous [26]. Lipid of oleaginous yeast known as triacylglycerols is rich in fatty acids similar to that of plant oil. Optimization the culture conditions are very needed for elimination the time consuming for cost effective production.

The current data manifested that the maximum oil yield was obtained after 6 days by *Candida* and after 3 days by *Issatchenkia* and then oil yield decreased afterwards. Similar data were obtained by [27] through their work on *Yarrowia* sp. and *Torulaspora glaobosa,* respectively. Actually, the values of sugar utilization efficiency were found to be parallel with that trend of lipid parameters in both *Candida and Issatchenkia*. Similar trend was observed by [28] through their work on *Candida viswanath*.

It could be concluded that the maximum lipid productivity by both yeast strains were realized when glucose used as carbon source. These results are similar to those obtained by [29] who stated that the yeast strain *Cryptococcus musci* had the best lipid productivity at 0.37 gL^{-1}/day of glucose consumption.

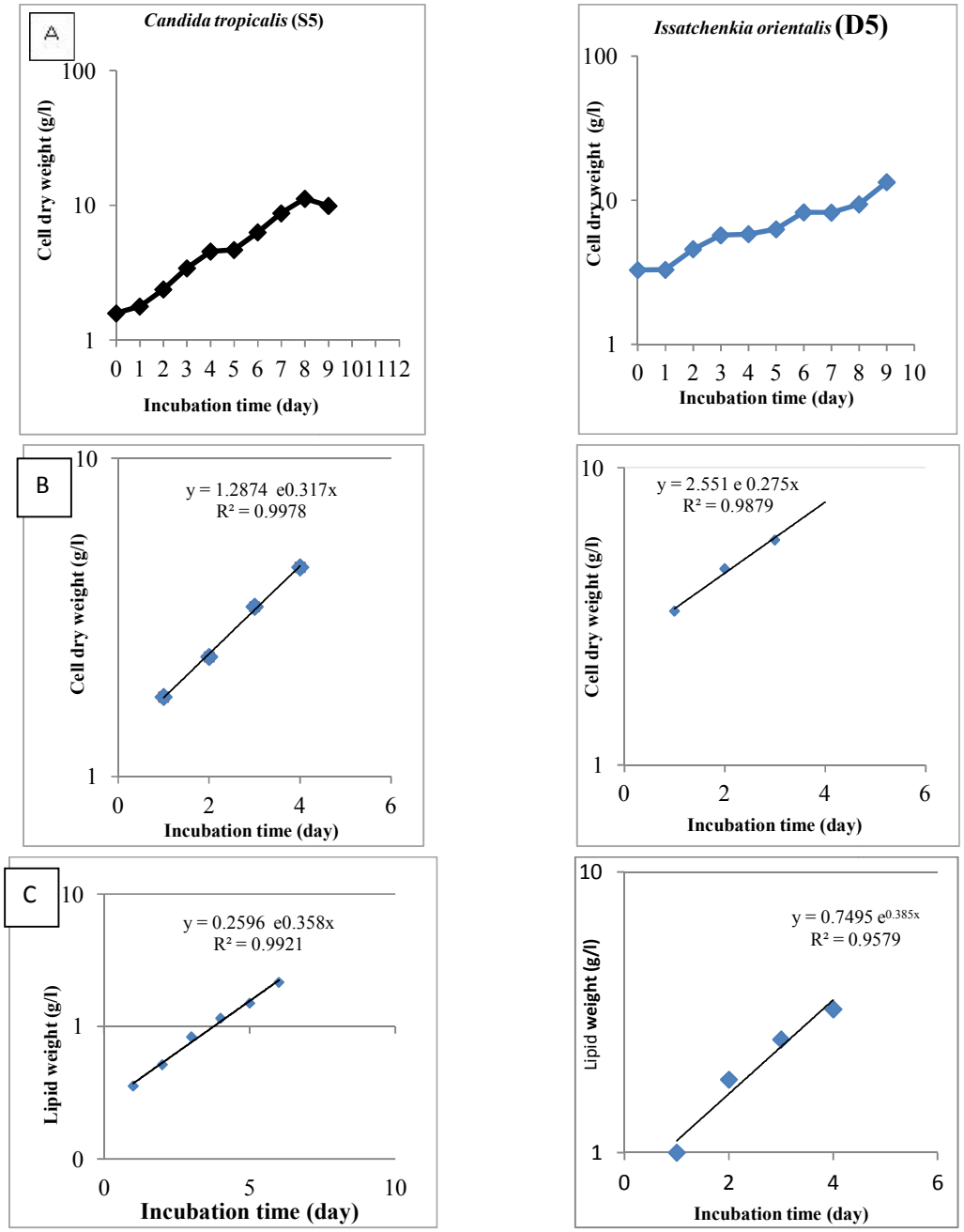

Fig. 1. Growth curve (A), correlation coefficient between cell dry weight and incubation time (day) (B) and correlation coefficient between lipid weight and incubation time (day) (C) of *Candida tropicalis* S5 & *Issatchenkia orientalis* D5 on semi logarithmic scale

It is worth to mention that both tested yeast strains showed approximately the same growth parameters except that number of generation which was longer for *Candida* compared to that value recorded to *Issatchenkia*. Meanwhile, the cell propagation potentiality of yeast strain *Candida* was faster more compared to *Issatchenkia*. Through the exponentially phase the doubling time increased due to the deficient in medium's constituents, particularly N which is not sufficient to support yeast growth.

Time to harvest cell biomass for lipid extraction could be considered to get the maximum lipid

yield through the fermentation process as clearly observed from the current data. These results are generally in accordance with that obtained by [30] who reported that the yeast cell dramatically increased in the logarithmic growth phase deduced to using lipids as constituent in membrane lipid synthesis to support cellular growth in size first. When cells reach their maximum size, they return to accumulate lipids as an oil bubble inside the cell. Cell lipids content reached the highest value at stationary phase and upon nutrient depletion, fatty acid accumulation rate decreased gradually until cells exit out of starvation, occasionally, accumulated lipids will degraded to free fatty acid rapidly. Based on aforementioned, harvesting cell time is better at early stationary phase to prevent lipids degradation.

The values obtained for conversion coefficient of the current work were inferior to those previously reported by [31] who showed that the lipid coefficient of *Rhodosporidum toruloidus* was 10 g lipid per 100 g of consumed xylose. It was observed that the lipid value per medium volume per day was closely correlated with production efficiency. Consequently, it is considered that the most important parameter is lipid productivity since, high lipid productivity increases yield per harvest volume and decreases the production cost. Based on the obtained data.

5. CONCLUSION

Out of 76 yeast isolates, two yeast strains were identified as *Candida tropicalis* S5 and *Issatchenkia orientals* D5 could grow and accumulate lipid under optimal conditions. Maximum lipid productivity found when glucose used as carbon source in both strains. The growth kinetics of the two strains was calculated from exponential phase. The two newly strains could play a key role in the economic production of biodiesel as feedstock producers.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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