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Oleaginous Yeast from Sago Waste Water : Screening and Characterization of *Candida trophicalis* for Biolipid Production

[1]Ashika, S., [2]Kiruthika, T., [3]Ashokkumar, K., [4]H.M. Suraj and [*1]Sivakumar Uthandi
[1]Department of Agricultural Microbiology, TNAU, Coimbatore
[2]Department of Bioenergy, AEC&RI, TNAU, Coimbatore
[3]Department of Plant Molecular Biology & Biotechnology, TNAU, Coimbatore - 641 003.
[4]Department of Biotechnology, School of Agriculture, PRIST University, Tanjore - 613 403.

An investigation was made to identify and screen an efficient oleaginous yeast, which could accumulate high level of lipid in its biomass by using sago processing waste water as substrate. A total of three isolates obtained from sago waste water sample were screened for their ability to utilize starch by amylase secretion. Of which, the isolate ASY1, identified as *tropicalis* by 18s rRNA sequencing was found to be positive for amylase activity. The yeast isolate ASY1 was cultivated in production medium and sago waste water for 84 h. Later, kinetic analyses of various parameters like pH, biomass, amylase, starch, protein and cellular lipid were carried out. The comparative data revealed that the lipid content in production medium and sago waste water was 48.96 and 29.41%, respectively. The starch utilization was found to be 12.58 and 4.38 g.L^{-1} in production medium and sago waste water, respectively. Therefore, *Candida tropicalis* isolate ASY1 (Accession number: MF668256) could be a potential candidate for next generation biodiesel production.

Key words: Oleaginous yeast, Sago waste water, Biolipid, Biodiesel production.

Cassava (*Manihot esculenta*) is a native plant from South America that has a huge nutritional importance in the tropics, where its root is one of the main sources of carbohydrates (Okudoh *et al.,*2014 and Byju *et al.,* 2016). The starch content in the root varies between 20 and 32 % and it depends up on the region, climate, soil and cultivation method. The water content in the roots at harvest is around 60 % (Chavalparit *et al*., 2009). The starch extraction process is water intensive, which generates a polluting wastewater stream. Studies assessing the cassava starch processing have reported a consumption of 11 to 20 m^3 of freshwater and a generation of 12 m^3 of effluent stream for each ton of processed cassava (Kamaraj *et al.,* 2006; Colin *et al.,*2007).

Modern factories involved in processing cassava to starch in Southern Asia reported a water consumption value of 4.3 m^3 per ton of (Chavalparit *et al*., 2009). The effect in a water course after the discharge of the effluent would be a dramatic drop in the levels of oxygen, due to its high content of organic compounds. The analysis of cassava effluent also confirms high values for both biochemical and chemical oxygen demand (BOD and COD). Besides, the organic pollutants, cassava wastewater presents other hazards making this effluent unique. Cassava toxicity arises from a glycoside called linamarin present in every part of the plant, which by hydrolysis yields glucose, acetone and hydrocyanic acid (Fukushima *et al.,* 2016). The toxic and polluting potential is aggravated by the fact that linamarin is very soluble in water. Therefore, the discharge of the untreated effluent from cassava processing can pose a serious threat to the fauna from the surrounding water courses. The concentrations of organic matter and linamarin in the resulting effluent are higher in the production of cassava flour than in the cassava-to-starch process, as the latter uses more water and the pollutants are thus, more diluted. So the waste water can be viably utilized for microbial lipid production, which in turn can alleviate the contamination caused by cassava processing waste water in soil and water bodies. In this investigation principal focus was given to identify efficient oleaginous yeast strains that can use different carbohydrate substrates to aggregate microbial oil, under stress conditions particularly, lipids.

*Corresponding author`s email: usivakumartnau@gmail.com

Material and Methods

Isolation and screening of oleaginous fungi for lipid production

Sago processing waste water sample was collected from Salem, Tamil Nadu, India and analysed for different parameters following APHA method (APHA, 2012). Serially diluted waste water was plated in Yeast extract agar medium and incubated at 30°C for 48 h. Based on colony morphology, yeast isolate was picked up, sub cultured and purified.The colonies on YPD plates were transferred onto soluble starch agar medium flooded with iodine (Mohanta*et al.,* 2008). Subsequently, lipid accumulation was identified in pure yeast isolate by Nile-red (Sigma) staining method (Kimura *et al.,* 2004).

Molecular identification of yeast isolate

DNA from the selected yeast isolate was extracted by Bust n' Grab method (Harju *et al.,* 2004) and its concentration and purity were observed by Nano drop spectrophotometer and agarose gel electrophoresis. Polymerase chain reaction (PCR) amplification of 18s rDNA gene was carried out using a master cycler gradient thermo cycler (Eppendorf, Germany). PCR reactions were performed in 25-µL volumes containing 1 µL of DNA, 12 µL of PCR Master mix (Applied Biosystems, UK), 2 µL each forward and reverse primers and 8.0 µL of PCR water (Sigma, Ireland). The forward primer was NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and rever sprimer wasNL4(5'-GGTCCGTGTTTCAAGACGG-3'). Thermo cycle patterns were as follows: 94°C for 3 min (one cycle); 94 oC for 1 min, 50°C for 1 min, 72°C for 2 min (40 cycles); and 72°C for 10 min (one cycle). PCR products with a correct size were confirmed by 1.2 per cent agarose gel electrophoresis.

The sequencing of purified PCR products containing 1.5 - 1.6 kb of 18s rDNA was carried out by means of automated sequencing **(**Euro Fins Biotech Pvt. Ltd., Germany**).** The sequence data generated were subjected to homology search through Basic Local Alignment Search Tool (BLAST) of National Centre for Biotechnological Information (NCBI).

Growth curve determination

The isolated yeast strain ASY1 was cultured in YPD medium and optical density (OD) was measured at every 12 h in a spectrophotometer (Model: Systronics, India) at 600 nm. Clear YM medium was used as blank for the OD measurement. For getting OD values between 0.1-0.5, the cell suspensions were diluted 10, 20 or 100 times.

Sago waste water treatment with yeast strain ASY1

Yeast isolate ASY1 obtained from sago waste water was inoculated into YPD broth and incubated at room temperature (30±2°C) for 24 h and transferred to 250 mL Erlenmeyer flasks containing 50 mL of culture medium, which consisted of (g/L): starch, 30; yeast extract, 1.5; KH_2PO_4, 7; Na_2HPO_4, 5; $MgSO_4$, 1.5; $FeCl_3$, 0.08; $ZnSO_4$, 0.01; $CaCl_2$, 0.1; $MnSO_4$, 0.1; $CuSO_4$, 0.1; and CO $(NH_3)_2$, 0.1. The pH value of the medium was 6.0. The flasks were incubated at room temperature (30±2°C) on a rotary incubator shaking at 140 rpm for 84 h. Shake-flask cultures in 250 mL Erlenmeyer flasks contained 50 mL of sterilized sago wastes based medium and the pH was adjusted to 6.0. The cultures were initiated with 10% of a 24 h old seed culture (approximately 10^7cells / mL) and incubated at room temperature (30±2°C) on a rotary shaker at 140 rpm for 84 h.

Analytical methods

After incubation microbial cells were harvested from the media by centrifugation and washed with distilled water three times and dried at 60°C in a hot air oven until a constant weight was obtained (Xue *et al.,*2008). A known weight was taken, and then lipid extraction was performed using chloroform - methanol method (Folch *et al.,* 1957). The supernatant was utilized for analyzing pH, dissolvable starch (Dubios *et al*.,1956), amylase (Bernfeld, 1955) and protein (Bradford method). All experiments were performed in triplicates. The statistical significance between the treated and non-treated waste water was evaluated by one-way ANOVA (analysis of variance) and Duncan's multiple range tests ($p<0.05$) using the DSTAT version 6.0 software.

Results and Discussion

The data on proximate analyses of sago processing waste water, collected from Salem are presented in Table 1. Three yeast isolates were obtained from sago waste water on YPD plates; one among them was screened for production of amylase using starch plate method, which resulted in clear zone of starch hydrolysis in the Petri dishes after iodine treatment (Fig.1). Thus, the yeast isolate

Table 1. Physiochemical characteristics of sago processing wastewater

Parameter	Measure
Colour	Dirty white
pH	4.1
Temperature (°C)	26.7
Salinity (ppt)	2.02
Electrical conductivity (dsm^{-1})	2.67
Starch ($mg.L^{-1}$)	7067
Total soluble solids (TSS) ($mg.L^{-1}$)	1472
Dissolved oxygen (DO) ($mg.L^{-1}$)	6.4
Total biological oxygen demand (BOD) ($mg.L^{-1}$)	4800
Total chemical oxygen demand (COD) ($mg.L^{-1}$)	7200
Total Kjeldahl nitrogen (TKN) ($mg.L^{-1}$)	117.4

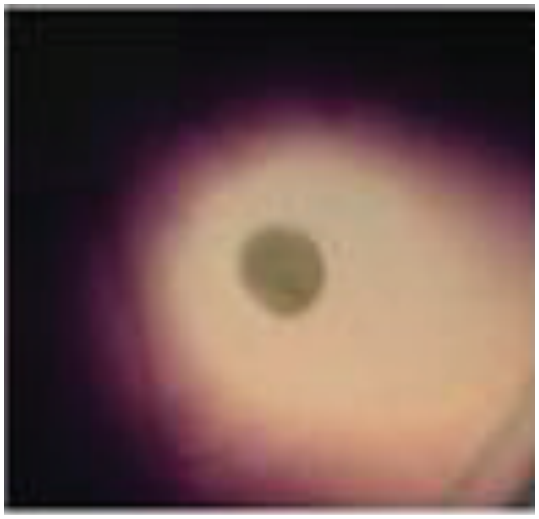

Fig. 1. ASY1 showing positive for amylase activity

Candida trophicalis ASY1 was found to be positive for amylase production, as determined by the width of zone of hydrolysis formed around the yeast colonies on starch agar medium. The yeast isolate ASY1 showed a zone of hydrolysis of 1.5 cm width.

Nile Red (NR) fluorescence was observed only in highly hydrophobic compartments of the cell, such

as lipid bodies (Greenspan *et al*., 1985). Oleaginous yeast is known to accumulate high levels of lipids, when carbon is in surplus and a key nutrient like nitrogen or phosphorous becomes restrictive. These accumulated lipids or SCOs get deposited as intracellular lipid bodies (LBs) which can be easily detected by a fluorescent probe, Nile red. Kimura *et al*. (2004) have visualized LBs using this approach. Oleaginous microbes viz., *Lipomycess tarkeyi* IFO10381, *Rhodosporidium toruloides* IFO-0559, *Cryptococcus curvatus* IFO-1159, *Mortierella isabe llina* varying in size, number and shape have been detected by Ratledge *et al*. (2002). In the present study, the yeast isolate from sago waste water grown under carbon rich and nitrogen limiting conditions revealed a variable number of oval and ellipsoidal size when observed through fluorescent microscopy (Fig. 2).

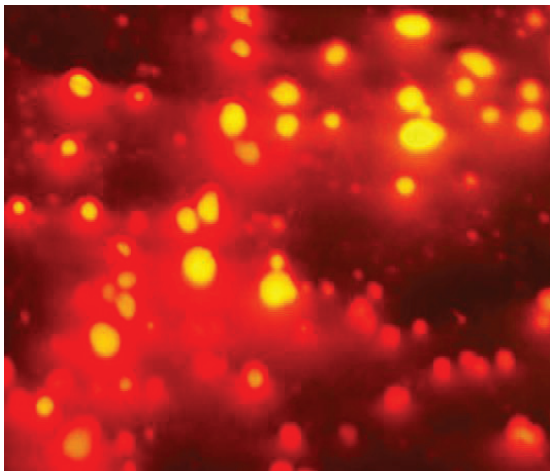

Fig. 2. Microscopic images of oleaginous yeast stained with Nile red showing lipid accumulation

Identification of oleaginous yeast isolates

The potential isolate ASY1 possessing the highest lipid content was selected for molecular identification. Fig.2. Microscopic images of oleaginous yeast stained with Nile red showing lipid accumulation PCR

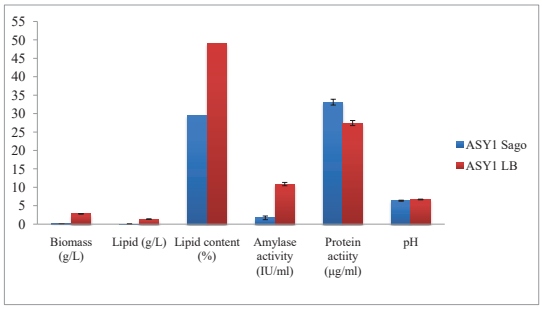

Fig 3. Growth of Yeast strain ASY1 (OD) in production medium and sago waste water

products were sequenced and the yeast isolate ASY1was identified as *Candida trophicalis* (Accession number: MF668256) (Fig.4).

Growth of Candida trophicalis

Candidate yeast was cultured in 250 mL shake flasks with pre-culture medium and cultivated at 25 and 30°C. Optical density was measured at 600 nm for every 12 h. Appropriate OD readings were used for drawing a growth curve to identify, the point of time suitable for harvesting the yeast culture. According to the test results, the best harvesting time was determined to be around 84 h for *Candida trophicalis* ASY1. Qier Sha (2013) reported that the best harvesting time was around 42 h for *Yarrowia lipolytica*, 44 h for *Rhodotorula glutinis* and 75 h for *Lipomyces lipofer* and *L. starkeyi*.

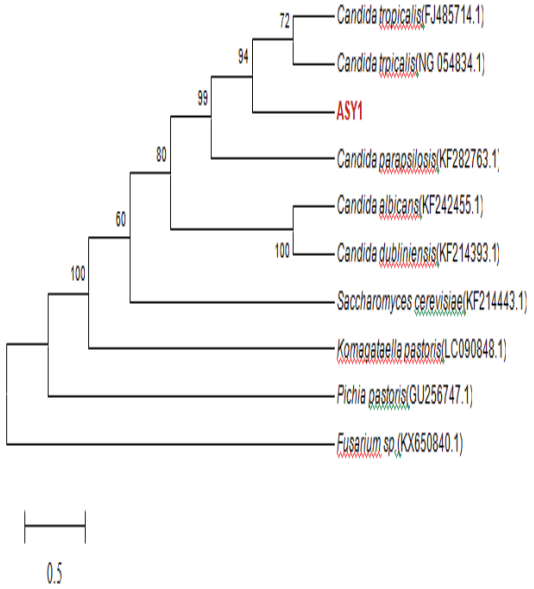

Fig 4. Phylogenetic tree constructed based on homology of NL region

Cultivation of oleaginous yeast for bio-lipid production in sago waste water

The oleaginous yeast, *Candida trophicalis* was inoculated in to the production medium and sago waste water and incubated for 84 h. Culture medium was withdrawn from the experimental flasks and subjected to parametric analyses. *Candida trophicalis* ASY1 in production medium recorded a maximum biomass of 2.8 $g.L^{-1}$, with the maximum lipid yield of 1.364 $g.L^{-1}$ registering starch utilization of about 12.58 $g.L^{-1}$. The specific activity of amylase was found to be 396.35 IU/mg, while the same isolate in sagowaste water, produced a maximum biomass of 0.1246 $g.L^{-1}$, recording a maximum yield of 0.0366 $g.L^{-1}$ lipid, utilizing about 4.38$g.L^{-1}$ of starch. Similarly, the specific activity of amylase was observed to be 51.93 IU/mg. The lipid content of ASY1 was calculated as 48.96 % in the production medium and 29.41 % in sago waste water. Such comparative data suggest that the isolate ASY1 could perform better in sago waste water by utilizing the starch effectively, leading to comparable lipid content (Fig 3). In a similar study, 35 %of lipid content was observed in oleaginous yeast *Rhodotorula glutinis*, cultivated on corn starch waste water supplemented with waste syrup (Xue *et al*.,2010). Final pH in both production medium

and sago waste water varied only ± 0.2 points. A comparable observation was made, when sewage sludge was used to accumulate lipids by *Lipomyces starkeyi*, the highest lipid content in this case was observed at a pH of 5.0, while the the highest growth was at pH 6.5 (Angerbauer *et al*., 2008). Noticeably, in the present investigation, a good percentage of lipid was obtained from sago waste water, without any additional supplements, indicating the potential for conversion of biodiesel.

Conclusion

The lipid yields obtained in the present study gives an encouraging solution for economic production of lipids using sago processing waste water. Production of microbial lipids using these low cost raw materials would significantly reduce the lipid production cost. Thus, considering the suitability of sago waste water as a substrate for the cultivation of *Candida tropicalis* strain ASY1, studies related to biodiesel generation can be initiated.

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