Symposium

Strain improvement of *Phaffia rhodozyma* for astaxanthin production

Abstract

Objective: The aim of the present study is to evaluate and optimize different carbon sources at different temperatures in shake flask for cell growth and astaxanthin accumulation in ultraviolet (UV) mutant *Phaffia rhodozyma*. The focus is to make the process cost-effective and fruitful with higher productivity. **Materials and Methods:** Adaptive strain development was carried out so that the *P. rhodozyma* can give the best astaxanthin at a higher temperature (35°C), increasing the process economy. *P. rhodozyma* was cultivated with three broad spectrum antibacterial drugs - streptomycin, gentamycin, and ampicillin (200 μL) and with antifungal drug - fluconazole (200 μL) to determine the effect on yeast growth and astaxanthin production. Rice, cane juice, and sucrose were selected as carbon source and compared with dextrose at different fermentation temperatures - 22°C, 30°C, 33°C, and 35°C for astaxanthin production. **Results:** *P. rhodozyma* was resistant to antibiotic drugs inhibiting bacterial and fungal contaminants. Rice being more economical was selected as carbon source, but the strain was not able to utilize starch-rich substrate leading to its rejection. When *P. rhodozyma* was grown in cane juice, biomass is highest (OD 2.77) at 30°C and 610 nm wavelength, whereas astaxanthin productivity is highest (OD 2.64) in dextrose media at 30°C and 450 nm. Comparative metabolic and growth results for UV mutants showed significantly higher biomass and astaxanthin productivity in comparison to wild strain. **Conclusion:** The most efficient carbon source in terms of penny-pinching and astaxanthin productivity was found to be dextrose containing media at 30°C.

Key words:
Astaxanthin, carbon sources, fermentation, Phaffia rhodozyma

Introduction

Astaxanthin ($C_{40}H_{52}O_4$, molecular weight: 596.841 g/mol) is an orange-red carotenoid belonging to the family of xanthophyll.[1] It is a potent antioxidant molecule[2,3] because of its anomalous structure, which has long backbone of 40 carbon atoms with conjugated bonds (oil-loving) and two β-ionone rings (water-loving) present on either side of the long chain by virtue of which it can span to both hydrophobic and hydrophilic parts of cell membrane unlike other carotenoids.[4,5] In the present study, a nongenetic mutation process was used to obtain an enhanced astaxanthin-producing yeast strain. Major

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approach was to enhance the production of *Phaffia rhodozyma* wild strain by ultraviolet (UV) rays. Effective down streaming process was carried out by the enzyme β-glucanase to solve the extraction problem. Comparative metabolic and growth study for wild and UV mutant strain of *P. rhodozyma* and also adaptive strain development were carried out so that the *P. rhodozyma* can give its best at a higher temperature (35°C), increasing the process economy. Fermentation of *P. rhodozyma* was carried out with three broad spectrum antibacterial drugs - streptomycin, gentamycin, ampicillin, and with antifungal drug - fluconazole to determine the effect on yeast growth and astaxanthin production. Rice, cane juice, and sucrose were selected as carbon source and compared with dextrose at different fermentation temperatures for astaxanthin production.

Materials and Methods

Chemicals and media

Yeast malt (YM) media components were purchased from Merck and Central Drug House, Mumbai. Soluble starch and extra pure β-glucanase powder were bought from SRL (Sisco Research Laboratories Pvt. Ltd). Antibacterial drugs such as ampicillin sodium salt, gentamycin sulfate, streptomycin di-hydrochloride from HiMedia, and antifungal drug-fluconazole capsule I.P. (150 mg) from Torrent Pharmaceuticals Pvt., Ltd., were used. All other chemicals, reagents, and solvents are of analytical grade. Sugarcane juice for every batch was collected fresh from local vendors in New Delhi.

Strain, medium, and cultivation of *Phaffia rhodozyma*

P. rhodozyma MTCC 7536 was obtained from MTCC, IMTECH, Chandigarh, India. It was maintained on the slants of yeast-malt agar (YMA) at 4°C and sub-cultured for further research work.[6] Preparation of seed culture spore suspension of *P. Rhodozyma* MTCC 7536 was prepared from actively growing yeast in YM broth (YMB). Erlenmeyer flask (250 mL) containing 50 mL sterilized medium (10 g dextrose, 5.0 g peptone, 3.0 g malt extract, and 3 g yeast extract in 1000 mL distilled water; adjusted to pH 5.8) was inoculated with 5% spore suspension of *P. rhodozyma* and incubated at 22°C for 6 days at 200 rpm[6] with modification.

Physical mutagenesis using ultraviolet radiation

P. rhodozyma MTCC 7536 cells were grown in YM medium for 5 days in a rotator–incubator shaker at 200 rpm at 22°C. Cell density was determined in the culture and adjusted to 2×10^8 cells/mL. One mL of the culture was transferred to a sterile microcentrifuge tube. Cells were pelleted down by centrifugation for 10 min at 6500 rpm. Supernatant was discarded and cells were re-suspended in 1.0 mL sterile water. After the second wash, cells were re-suspended in 1.5 mL sterile, 0.1 M sodium phosphate buffer, with pH of 7.0. Cells were irradiated with UV radiation (254 nm) for different intervals of time (i.e. 0, 1, 5, 15, 30, and 60 min) and survived yeast was cultured in fresh media. Hyperpigment-producing cells were cultured in YMA media and stored at 4°C for further studies.

Enzyme-mediated astaxanthin extraction

Astaxanthin was extracted as per the method described by Michelon *et al*.[7] with modification. After fermentation, 10 mL of the culture was pelleted down for 10 min at 3000 rpm. After centrifugation, β-glucanase (10 mg/mL) in 0.1 M sodium phosphate buffer (pH 7.0) was added to the pellet while the supernatant was discarded and kept for shaking at 100 rpm for 8 h at 35°C in dark. Then, it was probe sonicated for 5 min and centrifuged. The supernatant collected was analyzed for astaxanthin content by UV-vis spectrophotometer at 450 nm.[8]

Comparative growth and metabolic study

Fermentation process was done for the two strains of *P. rhodozyma* in YMB at 22°C (pH 5.8) with 200 rpm shaker speed. Change in pH after fermentation, sugar analysis by di-nitro salicylate method, wet weight, astaxanthin content, and biomass (610 nm) were analyzed for wild and UV mutant strains of *P. rhodozyma*.

Adaptive stain development

Fresh UV mutant Petri plates were prepared in YMA media at 22°C with pH 5.8. These were then sub-cultured after 6 days in fresh YMA media and kept at 30°C. The course of adaptive strain development was repeated until a temperature of 37°C.

Antibiotic resistance screening

UV mutant strains of *P. rhodozyma* MTCC 7536 were screened for different antifungal and antibacterial drugs in

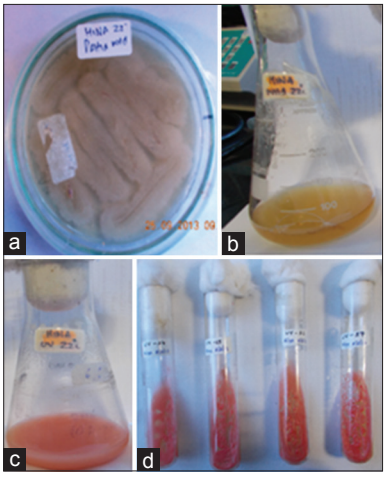

Figure 1: Strain improvement by physical mutagen (ultraviolet) (a) Wild *Phaffia rhodozyma* (un-mutated) in yeast-malt agar at 22°C for 6 days, (b) shake flask fermentation of un-mutated strains of *Phaffia rhodozyma* in yeast malt broth at 22°C for 6 days, (c) shake flask fermentation of mutated strains of *Phaffia rhodozyma* in yeast malt broth at 22°C for 6 days, (d) ultraviolet-mutated strain of *Phaffia rhodozyma* in yeast-malt agar at 22°C for 6 days

YMA media at an optimum temperature of 33°C for 6 days. Three broad spectrum antibacterial drugs - streptomycin, gentamycin, and ampicillin separately and an antifungal drug - fluconazole (200 µL each) were selected to determine the effect on yeast growth and astaxanthin production.

Sub-merged fermentation with different carbon sources at different temperatures

Different carbon sources: Rice, sucrose, and cane juice were selected for astaxanthin productivity in allegory to dextrose. Dextrose was compared with sucrose (10 g) and cane juice (20%), and shake flask fermentation was carried out in YMB at four different temperatures (22°C, 30°C, 33°C, and 35°C) for 6 days at 200 rpm, keeping inoculum volume (5%) and pH constant. After fermentation, change in pH, residual sugar determination, wet weight, biomass, and astaxanthin analysis were observed.

Results and Discussion

Comparative metabolic and growth study

Comparative metabolic and growth results for UV mutants showed significantly higher biomass and astaxanthin productivity in comparison to wild strain [Figure 1]. There was no much significant change in pH after fermentation; sugar by wild strain was utilized for biomass or for other metabolic process, but not for astaxanthin production as there was no color produced in the broth even after 6 days [Figure 2].

Adaptive strain development

Astaxanthin is a host-defense mechanism for *P. rhodozyma* to cushion itself from the stress. In this study, UV mutant strain of *P. rhodozyma* was able to handle stress only up to 35°C due to which orange-red color was produced. The color was slightly faded out at a temperature of 35°C in comparison to 33°C and ruled out at a temperature of 37°C. It may be due to the elevated stress, but yeast growth was full-fledged at all temperatures. Till temperature 33°C, results were promising on the basis of color [Figure 3].

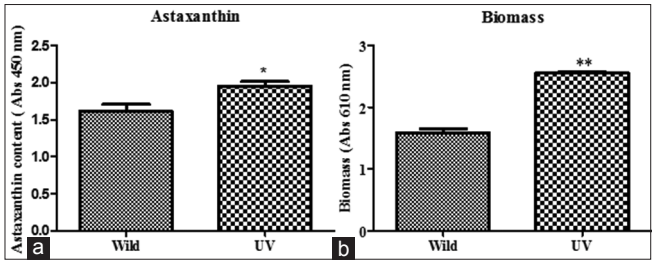

Figure 2: (a) Astaxanthin estimation (450 nm) and (b) biomass content (610 nm) of mutated and unmutated strain of *Phaffia rhodozyma* by ultraviolet spectrophotometer after 6 days of incubation in yeast-malt agar media. Data are means of two independent experiments. Bars are mean ± standard error of the mean. Student's *t*-test: $^{*}P < 0.05$; $^{**}P < 0.001$

Antibiotic resistance screening

The adapted mutant strain of *P. rhodozyma* when cultivated with three broad spectrum antibacterial drugs and antifungal drug was positive in either case. *P. rhodozyma* was able to resist antibiotic drugs as yeast growth was not hampered, inhibiting other undesired bacterial and fungal contaminants. Antibiotic drugs do not have any inhibitory effect on yeast growth and astaxanthin production [Figures 4 and 5].

Effect of carbon source on ultraviolet-mutant *Phaffia rhodozyma*

UV mutant *P. rhodozyma* was not able to utilize starch-rich substrate as carbon source (rice). Starch was not digested by *P. rhodozyma* after 6 days incubation at any temperature (22°C, 30°C, and 33°C) [Figure 5]. When *P. rhodozyma* was grown in cane juice, biomass is highest (OD 2.77) at 30°C and 610 nm [Figure 6], whereas astaxanthin productivity is highest (OD 2.64) in dextrose media at 30°C and 450 nm [Figure 7].

Adaptive strain development was done, as the best temperature optimized till date for the growth of *P. rhodozyma* is between 18°C and 27°C.[9] The main purpose for this work is to reduce the process cost since fermentation at low temperature, i.e. 22°C is less economical [Figure 8]. Since contamination is a problem during fermentation, the development of antibiotic-resistant yeast strain will reduce the process

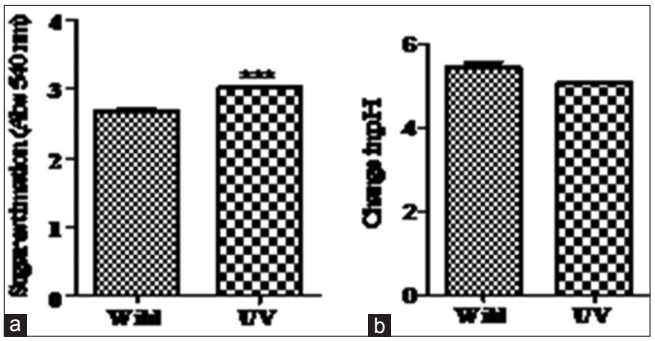

Figure 3: (a) Sugar estimation at absorbance 540 nm by ultraviolet spectrophotometer: (b) Change in pH after sub-merged fermentation. Residual sugar left in broth and change in pH after sub-merged fermentation by mutated and nonmutated strains of *Phaffia rhodozyma*; initial pH 5.8; initial sugar concentration: 10 mg/mL. Data are means of two independent experiments. Bars are mean ± standard error of the mean. Student's *t*-test: $P = 0.05$ (nonsignificant) for pH change and $^{***}P < 0.0001$ for sugar consumption

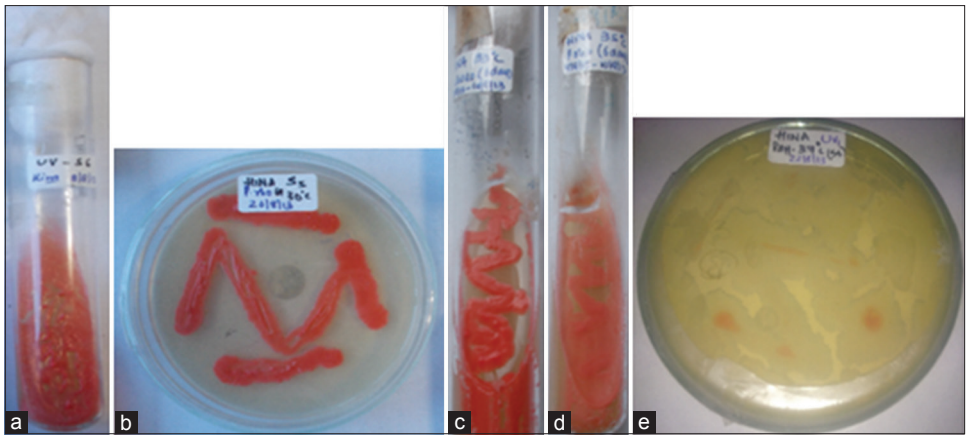

Figure 4: Adaptive strain development of ultraviolet mutant *Phaffia rhodozyma* in yeast-malt agar media at different temperatures (a) 22°C, (b) 30°C, (c) 33°C, (d) 35°C, (e) 37°C

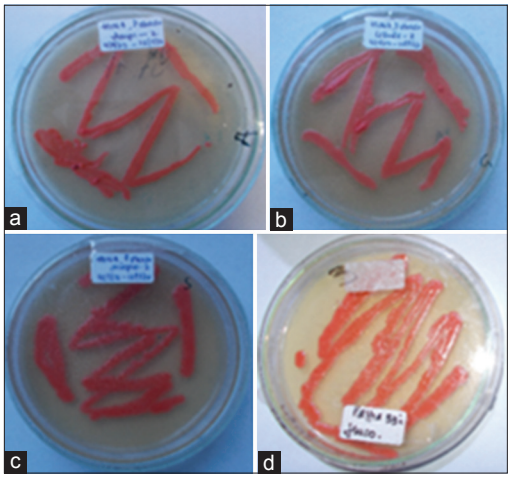

Figure 5: Antibiotic resistance screening of ultraviolet mutant *Phaffia rhodozyma* in yeast-malt agar media at 33°C. (a) Ampicillin sodium salt, (b) gentamycin sulfate, (c) streptomycin di-hydrochloride, (d) fluconazole

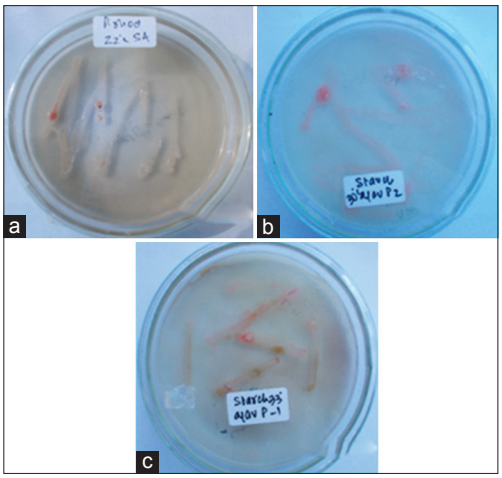

Figure 6: Starch hydrolysis test of ultraviolet mutant *Phaffia rhodozyma* in starch media at different incubation temperatures (a) 22°C, (b) 30°C, and (c) 33°C

cost. The significantly high concentration of astaxanthin by UV mutagenesis and effective down streaming by β-glucanase indicate the possibility of commercial production of astaxanthin by *P. rhodozyma*.

Previous studies showed that *P. rhodozyma* is able to utilize different kinds of sugars including dextrose, sucrose, and arabinose.[10] To select a suitable carbon source, astaxanthin yield and biomass content were evaluated under sub-merged fermentation in YM medium supplemented with various carbon sources (sucrose and cane juice). Initial sugar concentration of cane juice was found to be 5.49 mg/mL. The red yeast *P. rhodozyma* is the only yeast, which is able to ferment sugars because of which different carbon sources can be easily assimilated by this species.[11] The low cost media like cane juice may contain unknown carotenogenesis inhibitors because of which astaxanthin synthesis was significantly decreased.[12] Highest content of astaxanthin and biomass was found to be in dextrose-supplemented media at 30°C and in the second study, astaxanthin content was compared in YM media supplemented with sucrose and cane juice at 30°C. All the sources induced significantly lower astaxanthin content as compared to dextrose. Sucrose- and cane juice-supplemented media showed slightly similar growth pattern with lowest biomass and astaxanthin yield at 35°C. The lower astaxanthin content in dextrose at a temperature of 35°C can be related to glucose repression.[13] Johnson in 1984 concluded that the dextrose-supplemented media is an efficient carbon source for pigment production, but other carbon sources such as sucrose resulted in intermediate levels of astaxanthin content.[14] In our study, we found that the mutated strain of *P. Rhodozyma* MTCC 7536 was not able to utilize polysaccharide starch and to lesser extent disaccharide sucrose. Cane juice produced the highest biomass and lowest pigment production at all temperatures in allegory to dextrose. Sucrose being an

undefined media may contain other nutrients factors such as urea and biotin, which are responsible for biomass, yielding low levels of astaxanthin. It was found that there is correlation between the biomass and astaxanthin content irrespective of carbon source, signifying that there may be no significant influence of carbon source. Astaxanthin content was also varied in mutated strain in comparison to wild. This can be correlated to the anti-oxidant activity of astaxanthin, which protects the host *P. rhodozyma* from the oxidative stress induced by physical or chemical mutagens.[5,15] Cane juice contains three sugars, namely glucose, fructose, and sucrose. If any particular sugar is not utilized, growth pattern of yeast is disturbed due to osmotic pressure.[16] Sugar utilization pattern by *P. rhodozyma* was same for all three sources at a temperature of 33°C.

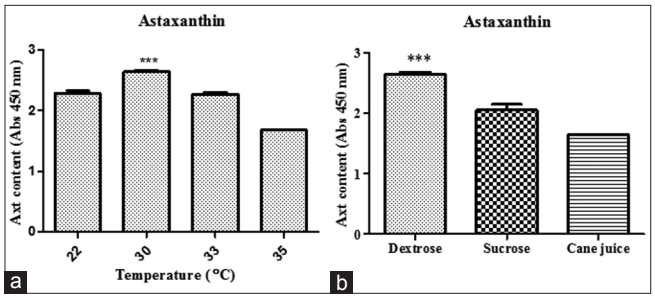

Figure 7: Carotenoid content of ultraviolet-mutated *Phaffia rhodozyma* grown in different fermentable carbon sources. (a) The yeast grown in yeast malt media supplemented with dextrose at different fermentation temperatures - 22°C, 30°C, 33°C, and 35°C (***$P < 0.0001$). (b) The yeast grown in yeast malt media supplemented with dextrose, sucrose, and cane juice at a temperature of 30°C (P value with respect to cane juice). Carotenoid content of mutated strain of *Phaffia rhodozyma* under sub-merged fermentation conditions was measured after 6 days of incubation in yeast malt media. Data are given in terms of mean of two independent experiments. Bars are mean ± standard error of the mean. ANOVA: (***$P < 0.0001$)

Conclusion

In the present research work, high market value of astaxanthin can be effectually produced by strain improvement technique and efficient enzymatic down streaming method. *P. rhodozyma* is moderately psychrophilic yeast and can be grown within the temperature range of 0–27°C, but the yeast was successfully grown with highest astaxanthin content at 30°C. The most efficient carbon source in terms of penny-pinching and astaxanthin productivity was found to be in dextrose, containing media at 30°C. The UV mutant strain developed by the strain improvement technique proved to be very beneficial as it can be used as parenteral strain for further investigations.

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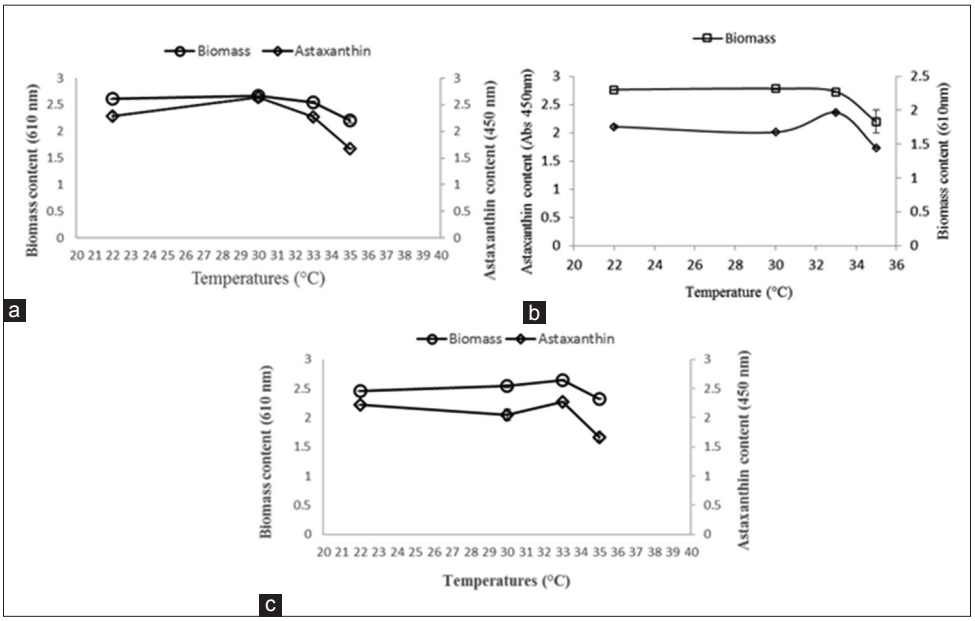

Figure 8: Astaxanthin content and biomass of ultraviolet mutant *Phaffia rhodozyma* grown in different fermentable carbon sources (a) dextrose (b) sucrose (c) cane juice at different incubation periods (22°C, 30°C, 33°C, and 35°C)

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Nil.

Conflicts of interest
There are no conflicts of interest.

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