International Journal of Engineering Science Invention (IJESI)
ISSN (Online): 2319 – 6734, ISSN (Print): 2319 – 6726
www.ijesi.org ||Volume 7 Issue 7 Ver II || July 2018 || PP 78-83

A rapid and simple strategy for the formation and isolation of astaxanthin high-yielding Xanthophyllomyces dendrorhous mutant variants

Min Kong, Wen Xu, Hongpeng Wang, Hui Tang, Liping Zhang1
Key Laboratory of Microbial Diversity Research and Application of Hebei Province; Key discipline of biological engineering of Hebei province, College of Life Sciences, Hebei University, Baoding, 071002, China. Corresponding author: Liping Zhang.

Abstract:*To obtain the positive mutant Xanthophyllomyces dendrorhous strains producing the astaxanthin, a novel breeding strategy of atmospheric and room temperature plasma (ARTP) mutagenesis with 2-deoxyglucose resistance screening was developed. After three successful rounds of recursive breeding, one strain designated B-2m-8 was selected, which was elevated by 187.14 % compared to that of the parent strain 5-7. This work provided an appropriate strategy for obtaining high astaxanthin-yield strains by a novel random mutation method with an efficient screening assay. The results of mutation method and resistance screening indicated that ARTP combined with 2-deoxyglucose resistance was an efficient breeding approach for the rapid evolution of astaxanthin-producing Xanthophyllomyces dendrorhous strains. Finally, the prospect of ARTP mutagenesis is discussed.*

Key Word Index*: Antioxidant; Atmospheric and Room Temperature Plasma; 2-Deoxyglucose; Mutation; Xanthophyllomyces dendrorhous;*

Date of Submission: 21-06-2018 Date of acceptance: 09-07-2018

I. Introduction

Astaxanthin (3,3`-dihydroxy-diketo-β,β`-carotene-4,4`-dione), the most powerful antioxidant among carotenoids, is an important natural pigment with the specific applications such as strong anti-aging, anti-inflammatory, sun proofing, and immune system boosting effects on organisms[1-3] the worldwide market of astaxanthin is expected to increase dramatically.

Currently, astaxanthin is obtained by chemical synthesis or solvent-based extraction from natural microorganisms, such as the green alga Haematococcus pluvialis [4] and the yeast X.dendrorhous (formerly known as Phaffia rhodozyma) [5]. Microbial breeding has been widely used X.dendrorhous which can produce natural astaxanthin is the only yeast to be able to use a variety of carbon and nitrogen sources to accumulate astaxanthin as the main carotenoid by the year 2015. However, low yields have been a major factor limiting its industrial production. Therefore, many artificial mutagenesis strategies to improve the mutation rates and to screen the beneficial varieties have been studied and applied so far by using the chemical (e.g., alkylating agents, azides, etc.) or physical(e.g., γ-rays, X-rays, UV light, particle radiation, etc.) mutagens [6], which have been playing important roles in the modification of industrial strains for a long time. Among these conventional mutation methods, issues of the health and safety of operators and mutation efficiency are always major concerns [7]. Therefore, the development of an efficient breeding method for selecting highly productive astaxanthin mutants is highly desirable. In general, plasma is a partially or fully ionized gas and is sometimes known as the fourth and most energeticstate of matter. Plasmas are usually classified as thermal or non-thermal. Usually, non-thermal plasmas are characterized by a palpable non-equilibrium between very hot electrons and cold heavy particles. Among the different types of atmospheric pressure non-equilibrium discharge (APNED) plasma sources, atmospheric and room temperature plasma (ARTP), which is driven by a radio frequency (RF) power supply with water-cooled, bare-metallic electrodes, has shown promise in applications in biotechnology [8]. Here, we used a new ways to improve the astaxanthin content at the source due to its efficiency, safety, and environment-friendly nature, by selecting the type of yeast with high astaxanthin by ARTP. To obtain the target mutants, a rational and effective screening method, as well as an efficient breeding method is needed. Liu C. et al. [9] used a novel and effective screening method to isolate successfully a mutant of high astaxanthin-producing X.dendrorhous decreased by high concentrations of glucose [10] in pigment biosynthesis. The property of 2-deoxyglucose can influence natural metabolism of cell and synthesis of astaxanthin. It has been reported that

2-deoxyglucose can be used to isolate the promising mutant [11]. On this basis, we planned to screen for mutants of X.dendrorhous blocked in synthesis of astaxanthin, which adds the 2-deoxyglucose in the YM agar, If the addition of 2-deoxyglucose is more, the resistant colonies will decrease.

Previously, our laboratory had obtained the strain of X.dendrorhous, which is capable of producing a mixture of pigment. In this study, by using novel mutagenesis ARTP and screening methods, a 2-deoxyglucose -resistant X.dendrorhous mutant was isolated and the astaxanthin yield in the product mix was significantly increased. This work provides an appropriate strategy for obtaining high astaxanthin-yield mutant strains by a novel random mutation method with an efficient screening assay as well as an extension of the 2-deoxyglucose -resistant screening method.

II. Materials and methods

Strains, media, and growth

The experimental strain X.dendrorhous AS2.1557 were maintained on slants of yeast-malt medium (glucose, 10; yeast extract, 3; malt extract, 3; Bacto-Peptone, 5) with 2% agar in the refrigerator, and natural isolates and mutants were also stored in 40% glycerol-60% YM broth at -80°C. Strains were grown at 18 to 20°C on complex YM agar medium. X.dendrorhous was also grown in 30 ml of YM broth in 300 ml baffled flasks shaken at 220 rpm on an orbital shaker controlled at 18°C. For analysis of pigment production, yeasts were grown for 6 days in baffled shake flasks, usually in YM broth. The 2-deoxyglucose was purchased from Promega, USA.

Mutagenesis with the ARTP biological breeding system and mutant selection

The ARTP mutation breeding system (Si Qing Yuan Biotechnology Co.,Ltd, Beijing, China) consisted of a plasma generator, a helium gas supply and control subsystem, and a sample plate made of stainless steel which can be moved up and down. Pure helium was used as the plasma working gas in the ARTP mutation and the operating parameters were as follows: (i) the radio frequency power input was 100W; (ii) the distance between the plasma torch nozzle exit and the sample plate was 2 mm; (iii) the temperature of the plasma jet was below 30°C. Under these operating conditions, the ARTP mutagenesis dosage (Table 1) was only dependent on the treatment time. To determine the optimal treatment period, 10μl fresh spore suspension(OD_{600}, 0.6-0.8) was evenly spread on a sterilized steel plate and exposed to the ARTP jet for 30, 60, 90, 120, 150, 180, 210, 240s, respectively. After each treatment, the dry mycoderm was eluted with sterile 0.9% NaCl into a new tube and properly diluted, then grown on a YM medium with agar for 6 days at 20°C prior to determination of the lethality rate. The individual colonies on the control medium and each mutated medium were counted, respectively. The lethality rate was determined as follows:

$$\text{Lethality rate } (\%) = \frac{U - S}{U} \times 100\%$$

where U is the total colony count of the sample without treatment, and S is the total colony count after treatment by the plasma. All the colony numbers were obtained by the CFU (Colony-Forming Units) method on the solid medium. The mutation rate and the positive mutation rate were calculated using the following equations:

$$\text{Positive mutation rate } (\%) = \frac{S - P}{S} \times 100\%$$

where P is the CFU of the mutants with improved astaxanthin productivity than that of the wild strain. S is the total colony count after treatment by the plasma. 100 mutants were analyzed at each dose. Data were determined from a minimum of three replicates.

For the first round of screening after ARTP mutagenesis was supplemented with various 2-deoxyglucose concentrations (0.01, 0.02, 0.03, 0.04, 0.05, 0.075, 0.1, 0.125, 0.15, 0.25, 0.35, 0.45, 0.55 gl^{-1}). A suspension of the wild-type strain was incubated on these plates at 20°C for 4 days to achieve the proper concentration of 2-deoxyglucose for pre-selection. After determining the optimal concentration (Table 2), the irradiated strains were properly diluted and grown on a YM medium with 2-deoxyglucose for 4 days at 20°C. Relatively large colonies were selected and transferred to a freshmedium for re-screening in shake flasks. Using this screening method, astaxanthin high -yielding X.dendrorhous strain (5-7) obtained (Table 2) was used for the next ARTP mutation.

Extraction and determination of astaxanthin

Strains were grown in fermentation medium for 6 d on a 500 ml rotary shaker at 18 °C and 220 rpm. cells from the fermentation broth were harvested by filtration and washed twice with distilled water. The cells were lysed by incubating a mixture of 5ml biomass and 1 ml Dimethyl Sulphoxide (DMSO) in a water bath at 50°C for 1 min, followed by extraction of astaxanthin with 4ml methanol. The methanol extracts were pooled and centrifuged at 7000 rpm for 5 min. The clear supernatant (1 ml) was to test by the High Performance Liquid Chromatography (HPLC).

The carotenoid pigments of yeasts accumulated in lipid droplets and can thus be extracted with a variety of organic solvents. The efficient extraction of the intracellularly produced carotenoids of X.dendrorhous may require efficient disruption of the rigid cell wall of the yeast. In this content, astaxanthin was quantified by measuring the absorbance at 472 nm of the supernatants in DMSO and methanol and compared with a standard curve (Fig. 1) made with known concentrations of the astaxanthin standard (Aladdin, Seattle, WA, USA). The separation of the carotenoids of different polarity has been achieved using a common C18 stationary phase and methanol/acetonitrile (9:1) gradient as the mobile phase [12]. Growth and extraction of yeast strains were repeated at least twice in independent trials to accurately assess astaxanthin production.

Preservation of Xanthophyllomyces dendrorhous mutant strains

The methods corresponds to that described by Gorman et al. with few modifications. One is preservation of microbial strains in 80% glycerol at -80 °C. Colonies of strains were transferred to 500 µl YM broth to obtain a high density cellular suspension, and 500 µl of 80% glycerol (Scharlau Chemie) was added and mixed. 1 ml of this mixture were deposited at -80°C. The other is preservation of microbial strains in sterile skim milk at -80 °C. Colonies of strains were transferred to YM agar plates and added 1 ml sterile skim milk to obtain a high density cellular suspension. Cells suspension were vacuum freeze-drying at -49 °C. The tubes were sealed with flame and maintained at -20 °C.

III. Result

Mutation rate curve and choice of mutant dose

ARTP mutagenesis had been successfully used to improve diverse traits in bacteria [13], fungi [14] and microalgae [15]. However, it has not yet been used to generate high astaxanthin yield mutants in the basidiomycetous fungus X.dendrorhous. The effects of various plasma treatment times on the lethality rate and positive mutation rate of X.dendrorhous are shown in Fig. 2 [16]. In this content, the highest positive mutation was obtained and the lethality rate increased to 88.1% when the treating time was 120 s. The literature on mutagenesis breeding suggests that the highest positive mutation rate is obtained when the mortality rate of the microorganism is between 70% and 80%. Based on this theory, an exposure time of 120 s was used in this study to obtain a desirable positive mutation and lethality rate.

For the ARTP mutation breeding system, the RF power input, helium gas flow rate, the distance between the plasma torch nozzle exit and the sample plate, and the treating time are the key parameters affecting the mutation efficiency. The experimental protocols of mutation by the helium-based ARTP are shown in Table 1.

Mutagenesis and selection of mutants with improved astaxanthin productivity

To determine the sensitivity thresholds of wild-type X.dendrorhous to 2- deoxyglucose for pre-screening [17], a range of 2-deoxyglucose concentrations was added to YM plates. It was found that a YM medium with 0.05 gl^{-1} 2-deoxyglucose was suitable for pre-screening mutants with high astaxanthin activity after the every round of ARTP mutation. Strain 5-7 obtained via the pre-screening was used as starter for the first round of ARTP mutagenesis.

After ARTP mutagenesis as described in Table 1, the treated cell suspension was spread on YM agar plates containing 0.05 gl^{-1} 2-deoxyglucose and cultivated for 6 days at 20 °C. A library of mutants with rapid growth was constructed, and Single colonies with deep color intensity in screening agar plates were selected and transferred to a YM fluid medium. The level of astaxanthin production was determined by HPLC. Three successive rounds of ARTP mutagenesis were performed by the same method, and the astaxanthin activity of the mutant B-2m-8 (6.15 µg/ml) obtained has approximately exceeded that of the wild-type strain by about double.

Analysis of astaxanthin and long term storage for Xanthophyllomyces dendrorhous mutants

The selected strains of X.dendrorhous were cultured in the flasks to determine their astaxanthin-producing capacity by HPLC [18]. We obtained the highest astaxanthin yield strain B-2m-8 (Fig. 3).

Once a collection of mutants is obtained, it is necessary to store them for a long period of time in a viable form, maintaining their characteristics. In previous works we used standard protocols for yeast storage, as direct freezing at –80 °C of cell suspension in 20 - 25% glycerol without previous treatment [19]. However no positive results were obtained specially with mutant strains. Therefore, two kinds of preservation methods applicable at the laboratory scale were tested: (I) Incubation of cells with glycerol. In this methods, cells in 80% glycerol were submitted to different treatments before storage at - 70 ºC. Incubation at 4 ºC for 2 h showed the highest cell viability of the wild type and mutant strains of X. dendrorhous after 65 months storage. (II) Incubation of cells with skim milk. (III) Incubation of cells with liquid nitrogen. This method showed the worst results with cell viability and productive capacity of mutant strains. Hence, all the X. dendrorhous mutant strains were preserved by the method I or II.

IV. Discussion

Our results raise three important points: (i) ARTP mutagenesis method can be high efficiency for

improving mutation rates and astaxanthin-producing capacity of X. dendrorhous [14]. For example, three successive rounds of ARTP mutagenesis in this study appeared to result in increased the more astaxanthin accumulation. (ii) 2-deoxyglucose for pre-screening has saved the screening time, costs and amount of work [20]; (iii) Preservation of cells with glycerol provided convenience for X. dendrorhous mutant in later operations and long term storage [19]. These results suggest the ARTP mutagenesis method and 2-deoxyglucose for pre-screening could prove to be an effective microbial breeding method by altering the genomes for high astaxanthin-producing capacity studies and also for potential industrial applications such as engineering organisms for astaxanthin production.

Up to now, development of ARTP mutation breeding system and some applications of the helium ARTP mutagenesis for different kinds of microorganisms[13-15] have been successfully conducted by interdisciplinary collaboration. These results proved that the ARTP was a novel, powerful, and environmental friendly mutagenesis tool for generating microbial mutant library. However, this is just a start point of the ARTP mutagenesis in this study. Much more extensive and deep scientific research, as well as the technological development, has to be carried out in future work: (i) The discharge mechanisms and physical characteristics of the ARTP in terms of acting on the organisms under different operating parameters, especially with different plasma working gas or gas mixtures, are indispensable for generating more diverse plasma dosages. Indispensably, methodology for quantifying the ARTP dosage should be studied for the ARTP mutation instrument development and mutation operation. (ii) The synergy roles of various plasma agents interacting with the whole cells and the intracellular biomacromolecules need to be analyzed at both molecular and cellular levels. The ARTP mutagenesis mechanisms of microbes should be elucidated by combining the plasma physics with the genomics and other omics analyses. (iii) The multichannel ARTP generation system (or the RF APGD plasma jet arrays) capable of directly using in microplates needs to be developed with an exact control of the discharge features of each plasma generation unit for improving the microbial mutation efficiency in a high-throughput manner, and the development of appropriate high-throughput screening methods is also necessary to efficiently obtain the mutants with the desired phenotypes or functions after the ARTP mutagenesis. Based on these efforts, the ARTP mutation breeding system as a platform will powerfully contribute to the progress of a comprehensive study on the life evolution and industrial strain engineering for bioindustry.

Acknowledgements

The authors gratefully thank Min Kong, Hui Tang for fruitful discussions related to yeast storage aspects.

Reference

[1]. Higuera-Ciapara I., Felix-Valenzuela L., Goycoolea F. M. Astaxanthin: A review of its chemistry and applications. Crit Rev Food Sci., 2006, 46, 185.
[2]. Li J., Dai W., Xia Y., Chen K., Li S., Liu T., Yin Q. Astaxanthin Inhibits Proliferation and Induces Apoptosis of Human Hepatocellular Carcinoma Cells via Inhibition of Nf-Kb P65 and Wnt/B-Catenin in Vitro. Mar Drugs, 2015, 13, 6064.
[3]. Kuedo Z., Sangsuriyawong A., Klaypradit W., Tipmanee V., Chonpathompikunlert P. Effects of Astaxanthin from Litopenaeus Vannamei on Carrageenan-Induced Edema and Pain Behavior in Mice. Molecules, 2016, 21, 382.
[4]. Amado I. R., Vázquez J. A. Mussel processing wastewater: a low-cost substrate for the production of astaxanthin by Xanthophyllomyces dendrorhous. Microb Cell Fact, 2015, 14, 177.
[5]. Kwak H. S., Kim J. Y., Sim S. J. A microreactor system for cultivation of Haematococcus pluvialis and astaxanthin production. J Nanosci Nanotechnol, 2015, 15, 1618.
[6]. Andrea K., Rownak A. Physical and chemical mutagenesis. Methods Mol Biol Plant Func Genom, 2003, 236, 189.
[7]. Ye X., Guilmatre A., Reva B., Peter I., Heuzé Y., Richtsmeier J. T., Fox D. J., Goedken R. J., Jabs E. W., Romitti P. A. Mutation Screening of Candidate Genes in Patients with Nonsyndromic Sagittal Craniosynostosis. Plast Reconstr Surg, 2016, 137, 952.
[8]. Zhang X., Zhang X. F., Li H. P., Wang L. Y., Zhang C., Xing X. H., Bao C. Y. Atmospheric and room temperature plasma (ARTP) as a new powerful mutagenesis tool. Appl Microbiol Biotechnol, (2014, 98, 5387.
[9]. Liu C., Zhang X., Rao Z. M., Shao M. L., Zhang L. L., Wu D., Xu Z. H., Li H. Mutation breeding of high 4-androstene-3, 17-dione-producing Mycobacterium neoaurum ZADF-4 by atmospheric and room temperature plasma treatment. Journal of Zhejiang University Science B, 2015, 16, 286.
[10]. Alesci A., Salvo A., Lauriano E. R., Gervasi T., Palombieri D., Bruno M., Pergolizzi S., Cicero N. Production and extraction of astaxanthin from Phaffia rhodozyma and its biological effect on alcohol-induced renal hypoxia in Carassius auratus. Nat Prod Res, 2015, 29, 1122.
[11]. Fallahi F. Characterization of epitopes on the rabies virus glycoprotein by selection and analysis of escape mutants. Virus. Res, 2005, 220, 161.
[12]. Weber R. W., Anke H., Davoli P. Simple method for the extraction and reversed-phase high-performance liquid chromatographic analysis of carotenoid pigments from red yeasts (Basidiomycota, Fungi). J Chrom, 2007, 1145, 118.
[13]. Wang L. Y., Huang Z. L., Li G., Zhao H. X., Xing X. H., Sun W. T., Li H. P., Gou Z. X., Bao C. Y. Novel mutation breeding method for Streptomyces avermitilis using an atmospheric pressure glow discharge plasma. J Appl Microbiol, 2010, 108, 851.
[14]. Qi F., Kitahara Y. K., Wang Z. T., Zhao X. B., Du W., Liu D. H. Novel mutant strains of Rhodosporidium toruloides by plasma mutagenesis approach and their tolerance for inhibitors in lignocellulosic hydrolyzate. J Chem Technol Biotechnol, 2014, 89, 735.
[15]. Fang M., Jin L., Zhang C., Tan Y., Jiang P., Ge N., Li H. P., Xing X. Rapid mutation of Spirulina platensis by a new mutagenesis system of atmospheric and room temperature plasmas (ARTP) and generation of a mutant library with diverse phenotypes. PloS One, 2013, 8, e77046.
[16]. Wang Q., Feng L. R., Wei L., Li H. G., Wang L., Zhou Y., Yu X. B. Mutation breeding of lycopene-producing strain Blakeslea trispora by a novel atmospheric and room temperature plasma (ARTP). Appl Biochem Biotech, 2014, 174, 452.

[17]. Zong H., Zhan Y., Li X., Peng L., Feng F., Li D. A new mutation breeding method for Streptomyces albulus by an atmospheric and room temperature plasma. Afr J Microbiol Res, 2012, 6, 3154.
[18]. Álvarez V., Rodríguez-Sáiz M., de la Fuente J. L., Gudiña E. J., Godio R. P., Martín J. F., Barredo J. L. The crtS gene of Xanthophyllomyces dendrorhous encodes a novel cytochrome-P450 hydroxylase involved in the conversion of β-carotene into astaxanthin and other xanthophylls. Fungal Genet Biol, 2006, 43, 261.
[19]. Gorman R., Adley C. C. An evaluation of five preservation techniques and conventional freezing temperatures of -20 °C and -85° C for long-term preservation of Campylobacter jejuni. Letter Appl Microbiol, 2004, 38, 306.
[20]. Zhang D., Fei Q., Li J., Zhang C., Sun Y., Zhu C., Wang F., Sun Y. 2-Deoxyglucose Reverses the Promoting Effect of Insulin on Colorectal Cancer Cells In Vitro. Plos One, 2016, 11, e0151115.
[21]. Figure captions

Fig. 1-The standard curve of Astaxanthin. Measure sample concentration was determined as follows: y = 25512x + 5473.0 (R^2 = 1.0000). where y is the value of chromatographic peak area values . X is the sample concentration of mutant strains determined by HPLC.
Fig. 2-The lethality rate and positive mutation rate curve.
Fig. 3-HPLC analysis of the astaxanthin profiles in X.dendrorhous. a) Detection of astaxanthin standard through HPLC; b) Detection of high-yield astaxanthin mutant strain obtained in first successive round of ARTP mutagenesis through HPLC. c) Detection of high-yield astaxanthin mutant strain obtained in second successive round of ARTP mutagenesis through HPLC. d) Detection of high-yield astaxanthin mutant strain obtained in third successive round of ARTP mutagenesis through HPLC.

Table 1. The key plasma parameters under the typical operating conditions.

parameters	Symbols	Values	Units
Break voltage (rms)	V_b	220	V
Discharge voltage (rms)	V_d	150-300	V
Power	P	40-200	W
Gas temperature	T_g	310-330	K
Particle densities	n_i	10^{15}-10^{16}	m^{-3}
Cell densities	M	10^7	ml^{-1}
Treating time	T	30、60、90、120、150、180、210、240	S

Table 2. Results of these strains in the different ARTP mutagenic round.

Mutagenic Round	1	2	3
Strain	5-7	15-3-1	25-7-120S-8
Astaxanthin, μg/ml	3.34	4.27	5.42
2-deoxyglucose concentrations, g/l	0.05	0.05	0.05

Data were determined from a minimum of three replicates.

Fig. 1

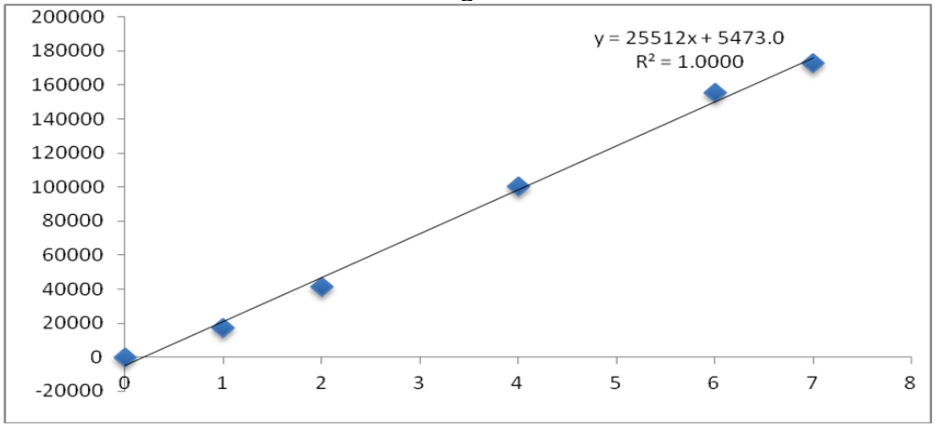


Fig. 2

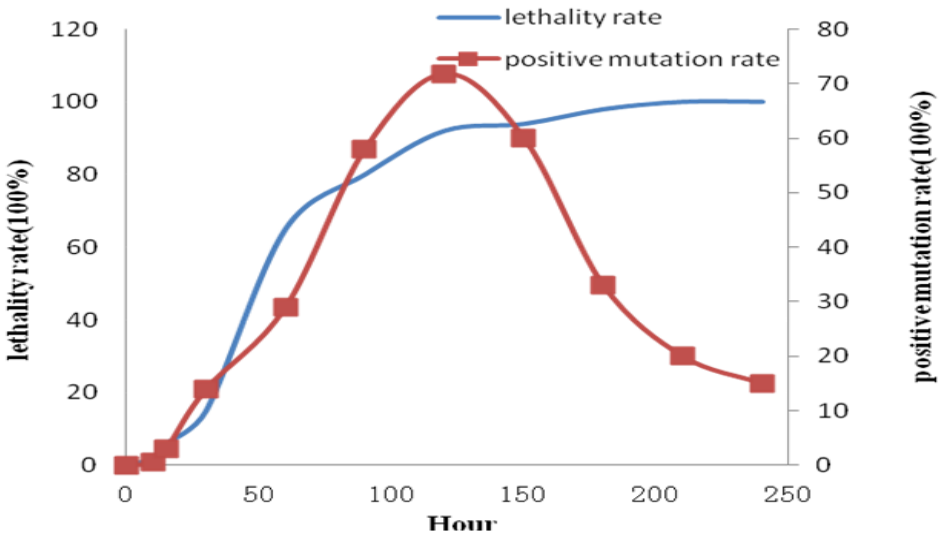
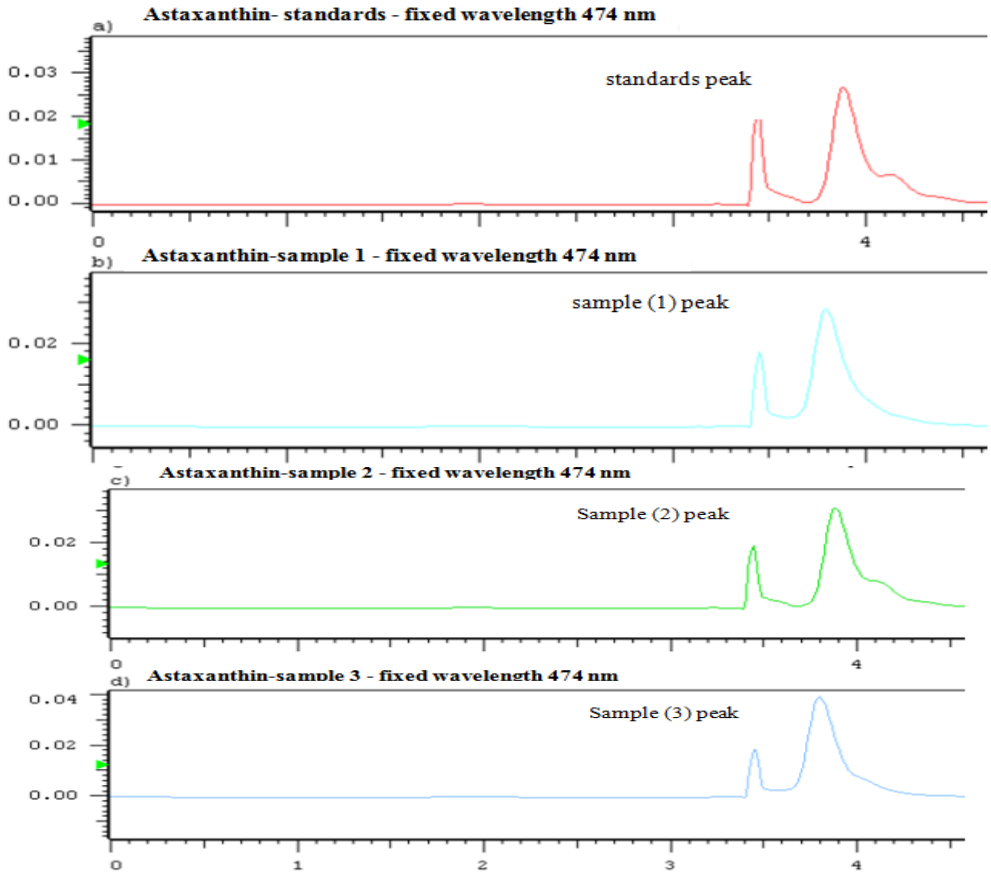


Fig. 3



Liping Zhang. "A rapid and simple strategy for the formation and isolation of astaxanthin high-yielding Xanthophyllomyces dendrorhous mutant variants "International Journal of Engineering Science Invention (IJESI), vol. 07, no. 07, 2018, pp 78-83