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Using Low Cost Media for Astaxanthin Production by *Phaffia Rhodozyma*

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ABSTRACT

Cheap different carbon and nitrogen were used in the present study to examine their ability to support the growth of standard *Phaffia rhodozyma* strains ATTC-24202 & NRRL Y-10922 and their carotenoids production (astaxanthin forming 84.2 and 92.8 % of their carotenoids respectively). The ability of both *Phaffia* strains to utilize rice straw-hydrolysate and cotton seed meal as sole sources of carbon and nitrogen to produce astaxanthin pigment was investigated. The best concentration of rice straw-hydrolysate was 20g/L and the preferable carbon/nitrogen ratio was 20/1 obtained from rice straw-hydrolysate and cotton seed meal.

Optimized culture conditions for *Phaffia* strains proliferation and pigmentation revealed that both *Phaffia* strains reached the stationary phase after 120 hours of incubation. The carotenoids production reached the maximum after seven days. The carotenoid productivity of both *Phaffia* strains increased with raising the temperature up to 22°C and no growth was occurred at 27°C. The best hydrogen ion concentration of *Phaffia* growth was 5 while pH 6 was preferred for carotenoids production. Increasing sodium chloride concentrations in the medium gave negative results and agitation surplus static condition for carotenoids production

KEYWORDS: Radiation Grafting; Hydrophilic Monomers; Dyeing Properties

INTRODUCTION

Considering the natural astaxanthin is getting an increased volume in the market of carotenoids, *Phaffia rhodozyma* is one of the best source of this compound and this yeast can grow on a variety of carbon sources, including glucose, maltose, sucrose, cellobiose, xylose, arabinose and lactose ⁽¹⁾

Many investigations on less expensive and readily available 'carbon and nitrogen sources for *Phaffia* growth and astaxanthin production were done. The investigators ⁽²⁾ observed an improved carotenogenesis in *Phaffia* cells cultured on media made from peat hydrolysate, and suggested that the presence of cellobiose in the fermentation media responsible for this finding. Using of corn, potato and cassava starches as differential carbon source for *P. rhodozyma* growth were suggested ⁽³⁾. The best pigmentation resulted from a 'cassava starch hydrolysate. In addition, they found sugarcane molasses, the dark mother liquor after sucrose crystallization from concentrated sugarcane juice, supported *Phaffia* growth and astaxanthin production.

Fresh sugarcane juice has high sucrose content and some free glucose and fructose. It also contains smaller amount of several other ingredients, such as mineral and organic salts, pectin and wax ⁽⁴⁾. Cane bagasse, a byproduct from sugar mills, is another attractive source of inexpensive phytobiomass, since industrial hydrolyses are available for its depolymerization into syrups enriched in xylose, glucose and cellobiose. Both the sucrose rich juice (~15%) and less expensive lignohemicellulose, bagasse may be considered as low cost raw materials for *P. rhodozyma* growth and astaxanthin production ⁽⁵⁾.

Alkali treated wood hydrolysate based media has supported higher rates of *Phaffia rhodozyma* biomass and astaxanthin accumulation than synthetic media ⁽⁶⁾.

The aim of the present work is to replace the synthetic medium fermentation used for production of astaxanthin from *Phaffia rhodozyma* (ATCC-24202 & NRRL Y-10922) by cheap agro-industrial wastes available in Egypt and improvement carotenoid biosynthesis by optimization the culture conditions.

MATERIALS AND METHODS

Microorganisms

Two standard strains of pigmented yeast *Phaffia rhodozyma* ; ATCC-24202 (Symbol A) obtained from MERCEN-Faculty of Agriculture, Ain shams university, Cairo and NRRL-Y 10922 (Symbol Y) was kindly provided by Dr/Kurtzman, Microbial genomics and bioprocess research unit, National Center for Agricultural Utilization Research, USA.

Estimation of Carotenoid Production and Growth Yield

Preparation of inoculums and cultivation of yeast strains were determined⁽⁷⁾. Total carotenoids were extracted and measured by milligrams per liter while specific carotenoid concentration was measured by micrograms per one gram of yeast cell⁽⁸⁾.

HPLC Analysis

Astaxanthin was determined by HPLC (Perkin Elmer apparatus located in Micro-analytical center, Faculty of Science, Cairo University).

Carbon Sources

Choosing of Low-Cost Carbon Sources

Nine different carbon sources were chosen to estimate their ability to support the carotenoid production and the growth of *Phaffia rhodozyma*. They were sugar cane juice, red grape feculence, potato washing-water, berry washing-water, molasses, cantaloupe core, apple core and peel, rice straw- hydrolysate and peat moss-hydrolysate. Sugarcane juice was clarified by centrifugation in order to remove cell debris and solid impurities arising from milling⁽⁵⁾. Apple core and peel and cantaloupe core samples were cut to small pieces and dispersed into tap water. After that, these samples were autoclaved by steaming for five minutes. The previous autoclaved solutions were filtered and centrifuged to obtain clarified extracts of these samples. Red grape feculence obtained after squeezing out the red grape juice was resuspended in tap water and autoclaved as mentioned above. Berry washing water was obtained from Kaha factory for Food Industry. Molasses was obtained from Eldakahlia Sugar Factory for sugar refining. Potato washing-water was prepared from washing freshly cutting potato with tap water.

Rice straw was milled to pass a 1 mm screen, homogenized in a sterile lot, air dried and stored in polyethylene bags at room temperature. The actual composition of used rice straw was analyzed⁽⁹⁾. Acid hydrolysis for rice straw was performed by addition of milled rice straw to aqueous sulfuric acid (0.7%) in ratio of 24:1 (v/g), then autoclaved at 130°C for one hour.

The chemical composition analysis of rice straw cellulose, hemicellulose and lignin was made by standard procedure⁽¹⁰⁾. Rice straw-hydrolysate was neutralized with calcium carbonate to a final pH of six and the formed CaSO₄ was separated by filtration and centrifugation. Neutralized hydrolysate was contacted with charcoal for 90 minutes at room temperature in a stirred glass reactor using a hydrolysate: charcoal ratio of 50:1 v/g⁽¹¹⁾. The lignin-degradation products, which have inhibitory potential, were partially removed in the adsorption step (last step) leading to media suitable for yeast proliferation⁽¹²⁾.

Peat moss was obtained from Agricultural Ministry of Egypt. It was mixed with 1.5% sulfuric acid in ratio of 20-gram dry peat for 100 g water and autoclaved at 121°C for 2 hour. The extract was separated by pressing followed by filtration through Whatman filter paper.

The total carbohydrates of previous carbon sources were determined by phenol-sulfuric acid method ⁽¹³⁾ using glucose as a standard.

Selection of the Most Favorable Low-Cost Carbon Source

Thirty flasks containing 25 ml of yeast malt agar medium (YM) (Without glucose) were prepared. The previous low-cost carbon sources were aseptically added to media in replacement of glucose at the concentration (20g/L). Three flasks were represented each carbon source. Control flasks were supplemented with glucose. All flasks were received one milliliter of inoculum either *Phaffia rhodozyma* ATCC24202 or NRRL Y-10922. These cultures were incubated at 22°C for five days under shaking condition. After the end of incubation period, the total carotenoids and dry weight were estimated for each culture individually.

To investigate the effect of increasing carbon source concentration on the proliferation of *Phaffia rhodozyma* and carotenoid production, rice-straw hydrolysate was subjected to vacuum evaporation at 50°C to increase its sugar concentration. Different portions of rice-straw hydrolysate with increasing total carbohydrates concentration from 10g/L to 40g/L were received the same amount of nitrogen sources (peptone: yeast extract: malt extract in ratio of 5:3:3 g/L respectively). After inoculation of these media with either *Phaffia* strains, they were incubated under conditions mentioned previously. The most favorable carbon source concentration was estimated after the total carotenoids and yeast growth was determined.

Nitrogen Sources

Choosing of Low-Cost Nitrogen Sources

Three nitrogen sources were selected to determine their efficiency to support the carotenoid production and the growth of the yeast *Phaffia rhodozyma*. They were cottonseed meal, fermentol and soybean meal. In addition, ammonium nitrate and urea were used for comparison aim.

Cotton seed and soybean meals used in this study were obtained from El-Mahla Company for Soap and Oil. Fermentol was obtained from starch factory (Egyptian Company for Starch and Glucose). The total nitrogen for the previous sources was determined by Kjeldahl procedure (**Reddy et al., 1983**). The chemical composition of modified cotton seed meal: ash 9.1, cellulose and hemicellulose 29.15, moisture 13.0, total protein 43.75 and total lipids 5.0 (g/100g %).

Selection of the Most Favorable Low-Cost Nitrogen Source

The previous five nitrogen sources were used individually to prepare media suitable for *Phaffia rhodozyma* growth and carotenoid production either using rice-straw hydrolysate or glucose as sole carbon source. The concentration of nitrogen source added to the media was the same as that used in YM broth media.

After the choice of the best nitrogen source achieved the highest total carotenoid concentration, the best ratio between the concentration of carbon source (rice straw hydrolysate) and nitrogen source (cotton seed meal) [C/N ratio] was determined in individual experiment. The estimated C/N ratio were 2/1, 5/1, 10/1, 20/1 and 40/1. Six flasks were inoculated with *P. rhodozyma* either ATCC-24202 or NRRL Y-10922. Inoculated flasks were incubated at 22°C under shaking conditions for five days. The total carotenoids and *P. rhodozyma* growth were estimated for each ratio.

Determination of factors affecting carotenoid production

In order to obtain the highest level of carotenoid production by both *Phaffia rhodozyma* strains (ATCC--24202 and NRRL Y-10922) in the selected media composed of low cost carbon and nitrogen sources (rice straw hydrolysate and cotton seed meal respectively), the effect of incubation period (ranged from 6 hrs to 240 hrs), incubation temperature (15, 18, 20, 22, 24, 27, 30 and 35°C), hydrogen ion concentration (pH values ranging from three to nine), sodium chloride concentration (0, 0.5, 1, 2, 3, 4, 6, 8 and 10%) and aeration conditions (shaking or static) were studied.

Statistic Analysis

Statistic analysis was done ⁽¹⁴⁾.

RESULTS AND DISCUSSION

Using Low-Cost Media for Carotenoids Production by *Phaffia Rhodozyma*:

Carbon Source.

Agricultural wastes that frequently create serious environmental problems may be possibly used as low-cost carbohydrate sources for microbial fermentation. Since, the ever-increasing interest in the production of microbial carotenoids as an alternative for synthetic food colorants, several attempts have been carried out to use these cheap carbon sources for their production⁽¹⁵⁾.

In the present study for economic production of astaxanthin pigment by *Phaffia rhodozyma*, different cheap carbon sources were selected and their total carbohydrates were estimated. The total carbohydrates of tested carbon sources increased in the following order (g/l): peat moss-hydrolysate 15.4, rice straw-hydrolysate 26.25, apple core and peel extract 49.7, cantaloupe core extract 65.8, sugar beet molasses 82.6, berry-washing water 93.7, potato-washing water 100.0, red grape feculence extract 170.0 and sugarcane juice 252.3. It should be mentioned that sugarcane juice and sugar beet molasses containing sucrose as the main carbohydrate source whereas the polysaccharide starch was the sole carbon source in potato-washing water. Fructose and glucose were the sugar constituents of red grape feculence extract while arabinose and glucose were the only sugars present in berry-washing water. Regarding to rice straw and peat moss, lignocellulosic nature are composed of cellulose fiber with crystalline structure embedded in a matrix composed by hemicellulose and lignin. In order to improve these factors, the raw material can be pretreated to obtain their hydrolysate. The pretreatment steps may increase both operational costs and investment, but they improve the rate and yield of saccharification, enhancing the economic features of the process⁽¹⁶⁾.

The previous mentioned carbon sources plus glucose and glycerol as synthetic carbon sources with a concentration 20 g/l were used to determine their effect on the growth yield and carotenoids production of both *Phaffia rhodozyma* strains (A&Y). Nitrogen source was a mixture of peptone, yeast extract and malt extract in ratio (5:3:3) g/l. Table (1) reveals that in media containing glucose as sole carbon source (control), the growth yield of *Phaffia* A and Y strains were 11.35 and 10.54 g/l respectively.

Results (Table -1) indicated that *Phaffia rhodozyma* strains (A&Y) preferred sugar beet molasses and sugarcane juice over than the synthetic carbon sources either glucose or glycerol for growth. The increase in growth yield of *Phaffia* Y strain reached nearly 17 and 2.5% when sugarcane juice and sugar beet molasses were used respectively over than the glucose control medium.

Also table (1) clears that the cantaloupe core extract wasn't suitable for both *Phaffia* strains proliferation, apple core and peel extract propped a growth yield of *Phaffia rhodozyma* strains (A&Y) in the similar intensity of synthetic glucose and red grape feculence extract had a negative effect on the growth of *Phaffia* in comparison with glucose. The growth intensity of *Phaffia* dropped clearly when berry washing-water was assimilated as a sole carbon source while potato washing-water had a moderate decrease effect on the growth yield of *Phaffia rhodozyma* strains by 8% compared with glucose.

Table (1) also shows that the growth intensity of *Phaffia* decreased obviously when peat moss-hydrolysate was assimilated as a sole carbon source by more than 20%, whereas rice straw-hydrolysate supported the growth yield of *Phaffia* Y strain in similar intensity of control but it slightly decreased in case of *Phaffia* A strain.

Table (1): Screening of different carbon sources used for proliferation of *Phaffia rhodozyma* ATCC A-24202 & NRRL Y-10922 and their carotenoids production.

Carbon sources	ATCC A-24202			NRRL Y-10922		
	Growth (g/l)	Total carote. (mg/l)	Carote. concent. (µg/g)	Growth (g/l)	Total carote. (mg/l)	Carote. concent. (µg/g)
Glucose	11.35	3.40	299.56	10.54	379	359.58
Glycerol	12.02*	4.42	367.72*	10.19*	4.33	424.93*
Apple core and peel extract	11.18*	2.95	263.86*	10.28	1.81	176.07*
Berry-washing water	7.83*	0.65	83.01*	8.24*	0.25	3.34*
Cantaloupe core extract	4.20*	0.04	9.52*	4.50*	0.89	197.78*
Sugar beet molasses	12.43*	3.80	305.71*	10.80*	3.55	328.70*
Peat moss-hydrolysate	7.98*	1.42	177.94*	8.00*	1.85	231.25*
Potato-washing water	10.54*	0.19	18.03*	9.70*	0.28	28.87*
Red grape feculence extract	9.34*	2.23	238.76*	6.26*	0.79	126.20*
Rice straw-hydrolysate	10.49*	3.28	312.68*	10.32	3.77	365.31
Sugarcane juice	12.17*	3.50	287.59*	12.33*	3.68	298.46*

*The mean difference is significant at the 0.5 level

Regarding to carotenoids production by *Phaffia rhodozyma* (A&Y), it was clear that glycerol achieved the highest total carotenoids over all tested carbon sources (Table-1). As expected from growth results, sugarcane juice and sugar beet molasses also supported carotenoids production by both *Phaffia* strains.

It is proved that both *P. rhodozyma* strains could not utilize cantaloupe core extract for carotenoids production as same as another tested sources. Similarly, apple core, peel extract and red grape feculence extract had a negative effect on carotenoids production by *Phaffia* Y strain. Table-1 reveals that the decrease in total carotenoids reached 52.24 and 79.16% when *Phaffia* strains used apple core and peel extract or red grape feculence extract respectively.

Although potato washing-water supported a high growth yield for both *Phaffia* strains, it decreased the total carotenoids by 94.41 and 92.61% respectively. Further more the total carotenoids of both *Phaffia* strains decreased by more than 80% in media containing berry washing-water. At the same trend, peat moss-hydrolysate decreased the total carotenoids and carotenoid concentration of both *Phaffia* strains by nearly 50%. On the other hand, by using the rice straw-hydrolysate it supported both *Phaffia* strains to produce carotenoid pigment in nearly same amount that produced by glucose.

From table -1, it is clear that rice straw-hydrolysate still keeping the priority for carotenoid production by *Phaffia* strain Y (365.31 µg/g) followed by sugar beet molasses (328.70 µg/g) and sugarcane juice (298.46 µg/g). The lowest carotenoid concentration for both *Phaffia* strains (A&Y) were obtained by using potato washing-water (18.03 and 28.87 µg/g), berry washing-water (83.01 and 30.34 µg/g) and cantaloupe core extract (9.52 and 197.78 µg/g).

Several investigators used differential cheap carbon sources for carotenoids production by *P. rhodozyma* with different carbohydrates concentrations. Brown sugar, raw sugarcane juice and enzymatic hydrolysate of sugarcane bagasse were used to be minimal but satisfactory culture medium for carotenoids production by *Phaffia rhodozyma* while corn, potato and cassava starch for its growth⁽³⁾. Carotenoid-hyper producing mutant 2A2N of the yeast *Phaffia rhodozyma* was cultivated using a sugar beet molasses⁽¹⁷⁾. Pinewood-hydrolysate was also used as a substrate for *P. rhodozyma* proliferation and production of astaxanthin⁽¹⁶⁾.

From previous results, cantaloupe core extract, berry washing-water and potato washing-water were the worst results for *Phaffia* proliferation and pigmentation. Meanwhile red grape feculence extract, apple core and peel extract and peat moss hydrolysate weren't given the expected success. This may be due to the presence of fibers. Results proved that potato washing-water also had a negative effect on *Phaffia* pigmentation.

Using a Rice Straw-Hydrolysate as a Low Cost Carbon Source

Since the previous experiment revealed that rice straw-hydrolysate was the best carbon source for carotenoid production, It was used in the present study, as a low-cost carbon source. The actual chemical composition of used rice straw was: 23% cellulose, 29.4% hemicellulose, 14% lignin and 7.4% fiber and ash.

Figure (1) showed that the proliferation of *Phaffia rhodozyma* strains increasing gradually by increasing the total carbohydrate concentration of rice straw-hydrolysate up to 40 g/l. The growth yield increased by nearly 20% for both *Phaffia* strains in comparison with control. In the same time, figure (1) also indicated that the best carbohydrate source concentration of rice straw hydrolysate for *Phaffia rhodozyma* pigmentation was 20g/l. At this concentration, the carotenoid production recorded 300.17 and 373.05 $\mu\text{g/g}$ for *Phaffia* A and Y strains respectively. The decrease in total carotenoids was more obviously by increasing the total carbohydrate concentration in rice straw-hydrolysate to 40g/l, the decrease reached nearly 50% for both yeasts compared to control. The decrease in total pigment and astaxanthin content in *Phaffia* was accompanied with increasing sugar concentration and the decrease was more severe in culture containing grape juice as sole carbon source compared with media containing glucose; this could be attained to the presence of inhibitors⁽¹⁸⁾. The Decreasing of carotenoid production by increasing the total carbohydrate concentration up to a certain level is due to crabtree effect due to the increasing of ethanol production with an increase in the initial glucose concentration⁽¹⁹⁾.

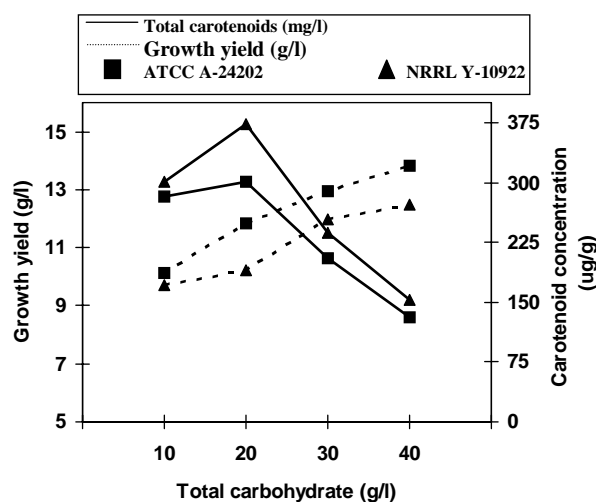


Figure (1): Effect of increasing total carbohydrates concentration of rice straw— hydrolysate on the proliferation of *Phaffia rhodozyma* and carotenoids production.

Nitrogen Source:

Unrefined nitrogen sources derived from agroindustrial wastes have been employed to make media for proliferation carotenoid-pigmented yeast (*Phaffia rhodozyma*). Nitrogen sources including cotton seed meal, fermentol and soybean meal, beside some another synthetic nitrogen sources for comparative purpose were studied using glucose or rice straw-hydrolysate as sole carbon source. The percentages of total nitrogen were determined for all studied nitrogen sources and were increased in the following order (%): fermentol 6.2, cotton seed meal 7.0, soybean meal 7.0, ammonium nitrate 35.0 and urea 46.6.

Regarding to table (2), ammonium nitrate supported the highest growth yield for both *Phaffia* strains either in media containing glucose or rice straw-hydrolysate as sole carbon source. On the other hand, ammonium nitrate decreased the total carotenoids and carotenoid concentration when glucose was used in the fermentation media, but it had a positive effect on carotenoids production by *Phaffia* in the media containing rice straw-hydrolysate. Ammonium nitrate achieved the maximum carotenoid productivity for *Phaffia* A strain (4.16 mg/l) comparing with 3.37 mg/l recorded by control. Ammonium nitrate resulted in distinctly higher β -carotene production than ammonium sulfate and ammonium chloride by investigated the wild strains

of *Rhodotorula glutinis* and *Rhodotorula rubra*(20). Yeast extract and peptone were utilized effectively for carotenoids production, while ammonium nitrate was a poor nitrogen source in case of gamma irradiated *P. rhodozyma* isolate 3A 4-8⁽²¹⁾.

For both *Phaffia* strains, dropping in carotenoids production was occurred by using urea and fermentol as sole nitrogen source (Table-2). The decrease in carotenoids production was more than 50% either in media containing synthetic glucose or in fermentation media based on rice straw-hydrolysate as sole carbon source. On the other hand the highest efficiency of yeast cell yield was obtained by one gram of urea whereas hydrolyzed soyabean resulted in the deepest colored *Phaffia* cells⁽⁵⁾. Urea 0.1% with 0.6% corn steep liquor were used to cultivate a mutant WSS-FF6 of *Phaffia rhodozyma*, the concentration of biomass and total carotenoids reached 6.58 mg/ml and 14.92 µg/ml after three days of incubation at 22°C⁽²²⁾. Since urea increased the production of astaxanthin as mentioned by previous review and decreased it in some others, this difference may be attained to the difference in used *Phaffia* strains or the used carbon source.

Table (2): Screening of different nitrogen sources for proliferation of *Phaffia rhodozyma* and carotenoids production using glucose or rice straw hydrolysate as sole carbon source.

carbon source	Nitrogen sources	ATCC A-24202			NRRL Y-10922		
		Growth yield (g/l)	Total carote. (mg/l)	Carote. Concen. (µg/g)	Growth yield (g/l)	Total carote. (mg/l)	Carote. Concen. (µg/g)
Glucose	Control	11.35	3.37	296.92	10.33	3.75	363.02
	Ammonium nitrate	15.53*	2.3	148.10*	13.27*	3.34	251.70*
	Cottonseed meal	11.38	3.49	306.68*	10.35	3.76	363.39
	Fermentol	10.35*	1.34	129.47*	6.58*	1.13	171.73*
	Soybean meal	13.73*	4.7	342.32*	10.02*	2.5	249.50*
	Urea	9.11*	1.68	184.41*	9.28*	1.37	147.63*
rice straw hydrolysate	Control	11.35	3.37	296.92	10.45	3.77	360.77
	Ammonium nitrate	14.22*	4.16	292.55*	14.71*	1.1	74.78*
	Cottonseed meal	11.24*	3.31	294.48*	11.08*	3.71	334.84*
	Fermentol	7.32*	0.97	132.51*	10.87*	1.5	137.99*
	Soybean meal	10.90*	1.99	182.57*	12.60*	3.34	265.08*
	Urea	9.57*	1.45	154.52*	9.93*	1.57	158.11*

Table (2) also cleared up that soybean meal had a positive effect on the growth yield of *Phaffia*. It enhanced the growth yield by nearly 20% for both tested strains where glucose was utilized as sole carbon source. When the fermentation media containing rice straw-hydrolysate and soybean meal, the growth yield was slightly decreased in comparison with control. Soybean meal also increased the carotenoid production by 39% for *Phaffia* A strain and decreased the carotenoid production by 11.4% in case of *Phaffia* Y strain (In glucose-based media). Data in table (2) revealed that replacing the nitrogen source in the fermentation media by the same amount of cotton seed meal, almost did not effect negatively on the growth of both *Phaffia* strains either in the medium containing glucose or rice straw-hydrolysate. The same pattern was occurred in case of carotenoids production since the production was almost similar to that produced by the control. It could be recommended from the previous results to use rice straw-hydrolysate and cotton seed meal as cheap sources of carbon and nitrogen respectively for industrial production of carotenoids by *P. rhodozyma*.

Using a cotton seed meal as a low-cost nitrogen source

The effect of different cotton seed meal concentrations as a nitrogen source on *Phaffia rhodozyma* growth and pigmentation in rice straw hydrolysate-based media was studied. Results (Fig-2) revealed that the high C/N ratios (2/1 & 5/1) or low one (40/1) had a negative effect on the proliferation of both *Phaffia* strains. The decrease at C/N equal to 2/1 was nearly 30% for both *Phaffia* strains compared to control while the growth of both *Phaffia* strains were decreased by 15.87 and 24.0% respectively at C/N ratio 40/1. The best carbon/nitrogen ratios for *Phaffia* proliferation was 10/1 while 20/1 ratio slightly decreased the growth; the percentage of decrease wasn't exceed 2%.

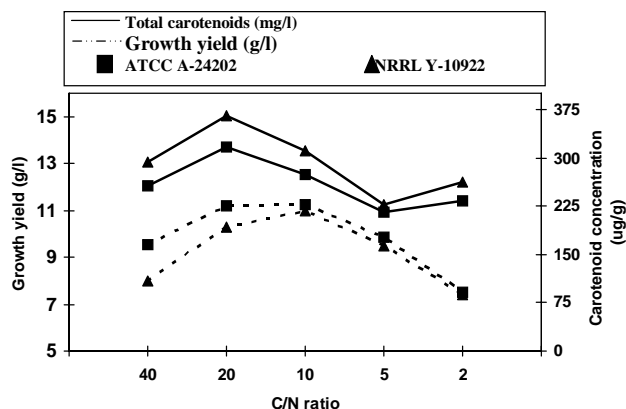


Figure (2): Effect of C/N ratio on the proliferation of *Phaffia rhodozyma* and carotenoids production using cottonseed meal as sole nitrogen source.

Figure (2) also showed that the total carotenoids recorded 2.45 mg/l for *Phaffia A* strain and 2.34 mg/l for *Phaffia Y* strain when C/N ratio was 40/1 and sharply increased by increasing the nitrogen source (cotton seed meal) concentration by 5.67% for *Phaffia A* strain and 2.74% for *Phaffia Y* strain at C/N ratio 20/1. Thereafter, the total carotenoids decreased by 7.76% and 6.58% for *Phaffia A* and *Y* strains respectively at C/N ratio 10/1. By increasing the nitrogen source concentration to C/N ratio 2/1, highly dropping in total carotenoids occurred and recorded 1.75 g/l and 1.94 mg/l for *Phaffia A* and *Y* strains in the same order, i.e. the decrease reached nearly 50% for both *Phaffia* strains.

All the results of *Phaffia* pigmentation showed that the reduction of nitrogen source concentration from C/N ratio equal to 2/1 to 20/1 leads to carotenoid synthesis stimulation. Thereafter the carotenoid production was decreased by increasing the C/N ratio to 40/1. This finding could be explained by the fact that when yeasts were grown in media containing high concentration of nitrogen source, quantity of carbon, ATP as energy and NADPH channeled into protein synthesis and that as nitrogen availability decreased the carbon demand for protein and nucleotide synthesis drops, as a result, ATP and NADPH are directed to fatty acids and carotenoid synthesis⁽²³⁾. Also, the astaxanthin synthesis in the yeast *P. rhodozyma* was shown to depend on the rate of the growth occurring in the first two days of cultivation and the growth rate was preset by the cultivation conditions, among which the C/N ratio was decisive. The intense anabolic processes coupled with active culture growth (High nitrogen concentration) during the first 24 hour significantly inhibited the synthesis of the key enzymes involved in astaxanthin synthesis, which lead to a marked decrease in the carotenoid production⁽²⁴⁾.

Briefly, rice straw-hydrolysate and cotton seed meal can be used for the commercial astaxanthin production by *Phaffia rhodozyma*.

Optimization of Culture Conditions:

Incubation Period

Figure (3) showed that the growth curves of both *Phaffia* strains (A&Y) were identical. The lag phase lasted nearly 14 hours and was followed by exponential phase that go on until 30 hours then another lag phase was continued to forty-eight hours. After 48 hours, the growth of both strains increased exponentially until 96 hour and reached the maximum at 120 hour. After 120 hr, the growth decreased for another forty- eight hours, a plateau reached afterward. The decrease in the growth could not attribute to cell death and lyses since no coloration of the supernatant was observed. The existence of two exponential phases was explained by several investigators. The presence of the first increase in *Phaffia rhodozyma* (PR 190) biomass reflect as a co-utilization of glycerol (Sole carbon source) and other nutrients present in the media such as yeast extract and

peptone by the yeast while the second increment was representative of the true yield of *Phaffia* biomass from the actual carbon source⁽²⁵⁾.

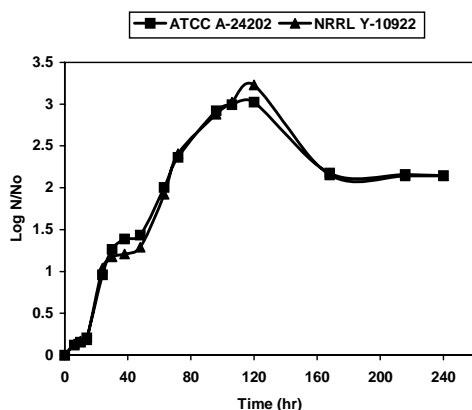


Figure (3): Effect of increasing times on the growth of both *Phaffia rhodozyma* strains (ATCC A-24202 and NRRL Y-10922).

Regarding to carotenoids production by both *Phaffia* strains (figure 4), the carotenoid pigment was not estimated until proximate 24 hours and then sharply increased with increasing incubation period. The carotenoid production reached the maximum level (5.359 mg/l) at 168 hour (stationary phase) for *Phaffia* Y strain whereas the utmost total carotenoids for *Phaffia* A strain achieved at 216 hour (4.319 mg/l). After these extreme levels, the total carotenoids for both *Phaffia* strains showed constancy. The increase in the astaxanthin content may a consequence of the decrease in the growth rate during the latter phase or possibly could be due to a shift in the biochemical equilibrium in the carotenoid biosynthetic pathway. *Phaffia rhodozyma* cells have to be maintained in the stationary phase for a period to allow the intracellular content of astaxanthin to reach high levels⁽²⁶⁾.

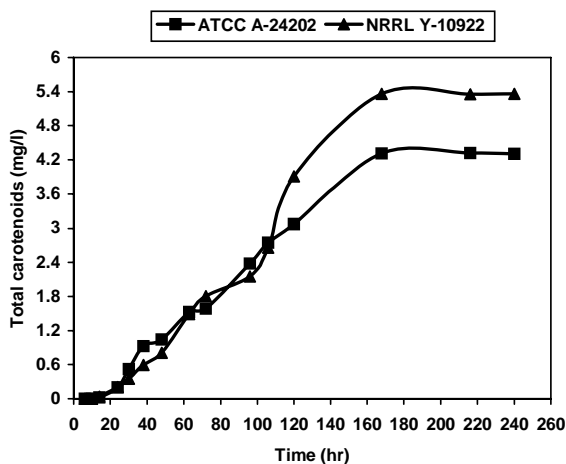


Figure (4): Effect of increasing times on the carotenoids production by both *Phaffia rhodozyma* strains (ATCC A-24202 and NRRL Y-10922).

Incubation temperature

To determine the optimal temperature for carotenoids production by *Phaffia rhodozyma* in rice straw hydrolysate-based media, inoculated media were incubated for seven days at different range of temperatures between (15-35) °C.

Figure (5) showed that the optimal temperatures for carotenoids production by both tested strains of *Phaffia rhodozyma* (A&Y) were between (18-22) °C. At 18°C and 22°C, the total carotenoids produced by *Phaffia* A strain were 4.45 and 4.82 mg/l respectively whereas it recorded 5.21 and 5.60 mg/l for *Phaffia* Y

strain in the same order. Up and down (18-22) °C range, the produced carotenoids by both *Phaffia* strains decreased sharply. Similar to carotenoid production, *Phaffia* can grow well at 18°C but the maximum growth yield was achieved at 22°C. The growth intensity of *Phaffia rhodozyma* decreased sharply at 15°C whereas the growth of *Phaffia* stopped completely at 27°C. The maximum specific growth rate of *Phaffia rhodozyma* (J4-3) was reached at 22°C, with a sharp decrease on either side of the optimal temperature and the highest temperature could be survived was 26°C with no growth occurring at 28°C ⁽²⁷⁾. The death of *Phaffia rhodozyma* at high tested temperatures may be attributed to the negative effect of high temperature on cell fluidity and ion exchange especially to psychrophilic microorganisms ⁽²⁸⁾.

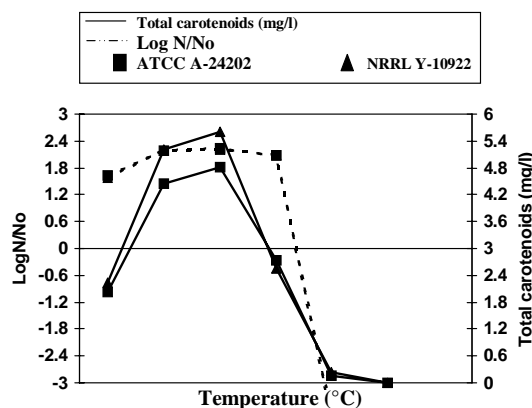


Figure (5): Effect of different temperatures on the proliferation of both *Phaffia rhodozyma* strains and carotenoids production.

Hydrogen ion concentration of the medium

Since hydrogen ion concentration (pH) is considered one of the most important factors, which not only affected the growth of microorganisms but also has a great influence on their physiological activity, the present experiment aimed to investigate the influence of pH on carotenoid production by tested *Phaffia rhodozyma* strains and their proliferation in the modified rice straw hydrolysate-based media.

Table (3) clearly indicated that the extreme acidic or alkaline pH had a negative effect on the growth of both *Phaffia* strains and carotenoid production. By increasing the acidity for one degree only from 5 to 4, decreased the total carotenoids by more than 55% for both *Phaffia* strains whereas raising the pH for one degree only from 6 to 7, decreased the total carotenoids by 40%. The optimum pH for *Phaffia* proliferation in modified rice straw hydrolysate-based media was five and it was six for carotenoids production. The pH 5.8 was found to be the optimal for yeast growth and astaxanthin production by *Phaffia rhodozyma* (PR 190) in media containing glycerol as sole carbon source ⁽²⁵⁾. Contrary, the best results for promoting the growth of *Phaffia rhodozyma* (ATCC-24202) was obtained at pH 3.5 in sugarcane juice modified media ⁽⁵⁾.

The change in *Phaffia rhodozyma* growth and pigmentation due to change in pH values could be explained by changing in pH may affected on the protein membrane charges with consequence nutrient uptake. It also affects on the degree of dissociation of mineral salts and the balance between dissolved carbon dioxide (CO₂) and bicarbonate ions ⁽²⁸⁾.

Table (3) showed the initial and final pH values during the proliferation of both *Phaffia rhodozyma* stains (A&Y), it was interesting to find the yeast strains have the ability to change the pH by lowering or rising to achieve the optimum pH for its proliferation. The change in pH values was slightly more or less than one degree for most cases. The change in initial and final pH of the medium may be attributed not only to yeast activity but also to carbon assimilation ⁽²⁷⁾. Also fungi alter the pH to some degree in order to create their suitable own environment by selective uptake and exchange of ions ⁽²⁸⁾.

Table (3): Effect of different hydrogen ion concentration on the proliferation of both *Phaffia rhodozyma* strains and carotenoids production.

Yeast strain	Initial pH	Growth		Total carotenoids (mg/l)	Change in total carotenoids (%)	Final pH
		Count (CFU/ml) x10 ⁵	Log N/No			
A	3.0	N.D**	-	-	-	3.8
	4.0	86.30	1.819	1.33	27.09	4.2
	5.0	245.00	2.273	4.75	96.74	5.5
	6.0	216.00	2.218	4.91	100.00	5.9
	7.0	130.00	1.997	2.90	59.06	6.4
	8.0	9.09	0.842	1.10	22.40	7.5
	9.0	3.00	0.361	0.77	15.68	9.4
Y	3.0	N.D**	-	-	-	3.8
	4.0	95.80	1.865	1.49	26.05	4.3
	5.0	234.00	2.253	4.99	87.24	5.7
	6.0	172.00	2.119	5.72	100.00	5.8
	7.0	139.00	2.026	3.49	61.01	6.3
	8.0	8.11	0.792	1.15	20.10	7.3
	9.0	4.09	0.459	0.88	15.38	9.3

*No = 1.308 x10⁵ (CFU/ml).

** N.D= Not Detected

Sodium chloride concentration

Carotenogenesis process is controlled by a cluster of genes. Each gene is responsible for production of certain enzyme that creates one step in carotenoids production process⁽²⁹⁾. To induce enzyme production by microbial cells, there must be an inducer. This inducer may either be a substrate present in the media or an added supplement. The present experiment was designed to elucidate the effect of different concentrations of NaCl on growth intensity of *Phaffia rhodozyma* and carotenoid production. Used media was adjusted at pH 6.0 and incubation period was seven days.

Figure (6) showed that the growth of both *Phaffia rhodozyma* stains (A&Y) keep its intensity until 1% NaCl concentration, afterward it decreased steadily. More than 30% of the growth yield of both *Phaffia* stains was decreased by raising the NaCl concentration to 3% and sharply decreased thereafter.

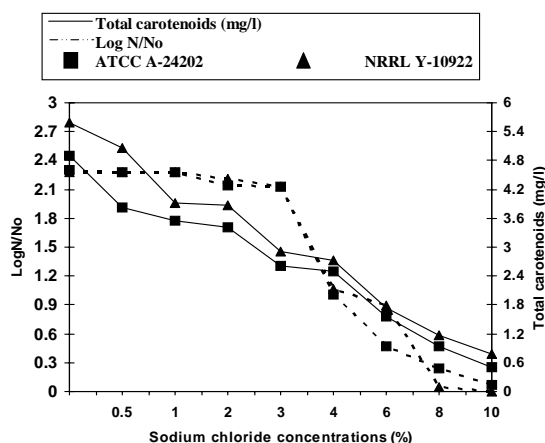


Figure (6): Effect of different NaCl concentrations on the proliferation of both *Phaffia rhodozyma* and carotenoids production.

Also, data pointed out the reverse relationship between the carotenoids production by tested *Phaffia* strains and increasing NaCl concentrations. At the same trend, investigating the pigmentation of unicellular

freshwater flagellated *Eglena gracilis* under different salt concentrations (5&10) g/l, revealed that the cells showed a complete loss of a carotenoid shoulder in the spectrum, which reappear again when the cells were brought back to standard media ⁽³⁰⁾. Also the effect of different salt concentrations (0.05-3.0) M NaCl on the total carotenoids accumulated in the microalgae *Dunaliella tetiolecta* DCCBC26, indicated that the highest amount of carotenoids detected (11.73 mg/l) was only in the salinity 0.5 M of NaCl during the stationary growth phase ⁽³¹⁾.

5. Aeration conditions

In the present study, it was interesting to establish the effect of agitation conditions on carotenoid production and proliferation of both *Phaffia rhodozyma* strains (A&Y). Inoculated flasks were incubated for the optimal time under shaking (150 rpm) or static conditions.

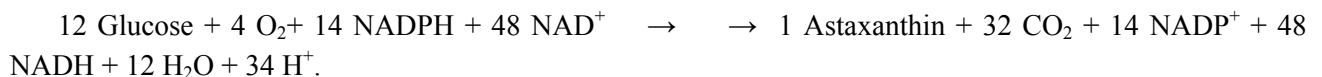
Table (4) clearly indicated that the growth intensity of both *Phaffia* strains was diminished by more than 50% under static conditions compared with shaking one. Under static conditions, the total carotenoids of *Phaffia* A strain was reduced from 4.82 mg/l to 1.19 mg/l whereas the total carotenoids of *Phaffia* Y strain decreased from 5.63 mg/l to 1.40 mg/l. i.e. shaking conditions favored carotenoid production by nearly four folds. The influence of oxygen on primary metabolism and carotenoid production by *Phaffia rhodozyma* (ATCC-24202) was performed in 100 ml-Erlenmeyer flasks with culture volumes (20, 40 & 60) ml. It was found that the final ethanol concentration increased and the maximum specific growth rate and the final astaxanthin decreased with increasing culture volume ⁽¹⁹⁾. This study suggested that to maintain high astaxanthin productivity, high levels of oxygen are necessary to repress crabtree effect (ethanol production). At the same trend, the effects of aeration rate on the growth and carotenoids production by soil yeast *Rhodotorula mucilaginosa* revealed that the total carotenoids were significantly increased with increasing aeration rate up to 2.4 vvm ⁽³²⁾.

Table (4) : Effect of agitation and static conditions on the proliferation of both *Phaffia rhodozyma* strains and carotenoids production.

Yeast strain	Agitation and static conditions	Growth		Total carotenoids (mg/l)	Change in total carotenoids (%)
		Count (CFU/ml)	Log N/No		
A	Shaking	2.40x10 ⁷	2.289	4.82	100.00
	Static	1.10x10 ⁷	1.949	1.19	24.69
Y	Shaking	2.30x10 ⁷	2.270	5.63	100.00
	Static	1.00x10 ⁷	1.908	1.40	24.87

*No = 1.236 x 10⁵ (CFU/ml).

The Reaction model for astaxanthin biosynthesis from glucose by *Phaffia rhodozyma* has described to explain the cause of astaxanthin enhancement by aeration respiration and astaxanthin repression by aerobic fermentation ⁽³³⁾.



The authors indicated that a large amount of NADH is produced as a side product of astaxanthin. Under O₂ limitation, NADH accumulates due to the decrease in the efficiency of oxidative phosphorylation that retarded the astaxanthin synthesis. When the oxygen is supplied to the yeast cell, NADH is reoxidized to NAD⁺. To keep NAD⁺ balance in *Phaffia* cells, the astaxanthin production might be stimulated. In addition, oxygen molecule itself is a starting material for astaxanthin biosynthesis ⁽¹⁹⁾.

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