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Original Research Article

Cultural conditions and antioxidant activity of astaxanthin produced by *Mucor circinelloides* f. *circinelloides*

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Astaxanthin is a xanthophyll, a very common red-colored pigment of marine organisms that are in increasing demand in the market because of their applications in the food, cosmetics, and pharmaceutical industries. Particularly, astaxanthin has been found to play protective roles against many chronic human diseases, and diseases associated with oxidative stresses caused by excess free radicals and other reactive oxygen species. *Mucor circinelloides* f. *circinelloides* is able to accumulate astaxanthin by stress conditions. The aim of this study was to produce astaxanthin by *M. circinelloides* f. *circinelloides* cultivated in the presence of exogenous stress conditions using a natural medium of cassava wastewater (CWW), continuous illumination with blue LED and fluorescent lamps in a bioreactor for 96 hours. The best condition for astaxanthin was under a fluorescent lamp (150 µg/g per dry biomass). The carotenoid fraction was extracted using acetone, followed by separation and purification by thin-layer chromatography. The astaxanthin was identified and quantified using spectrophotometry, based on the absorption coefficients. The antioxidant activity was evaluated using (2,2-Diphenyl-1-picrylhydrazyl) DPPH, and a higher antioxidant effect was observed by fluorescent lamp. This is one of the first reports on antioxidant activity in astaxanthin produced by *Mucor circinelloides* and the biotechnological potential for this fungus was demonstrated.

Key words: Astaxanthin; *Mucor circinelloides*; Agroindustrial waste; Antioxidant activity

INTRODUCTION

Astaxanthin pigment not only can act as vitamin A precursors, but it also has coloring and antioxidant properties, which have attracted the attention of industry and researchers. Its dietary consumption is also associated with a lower incidence of cancer, protection against cardiovascular diseases and its free radical scavenging activity. Astaxanthin has also been exploited for industrial use, principally as an agent for pigmenting cultured fish and shellfish (Gross and Lockwood 2004; Kim et al., 2006; Park et al., 2010; Romero et al., 2010).

A DPPH (2,2-Diphenyl-1-picrylhydrazyl) assay is routinely conducted to assess its potential for free radical scavenging of an antioxidant molecule which is considered to be one of the standard and easiest colorimetric methods for evaluating the antioxidant properties of pure compounds (Hsu et al., 2005; Cheng et al., 2006; Noipa et al., 2011; Tai et al., 2012).

The use of waste as a culture medium can reduce the cost of producing carotenoids. Agro-industrial wastes provide the carbon and nitrogen sources and other elements needed to carry out microbial metabolism (Mata-Gómez et al., 2014). Cassava (*Manihot esculenta*) residues, like cassava wastewater (manipueira), contain a small amounts of starch, protein, cellulose and other nutrients (Nitschke

and Pastore, 2004; Choubert et al., 2005; Kaewpintong et al., 2006; Vasconcellos et al., 2009; Casullo et al., 2010; Yang et al., 2011; Yimyoo et al., 2011, Saoudi and Fei, 2012). The liquid waste from industrial cassava processing contains a large number of pollutants and has a significant adverse environmental impact. However, cassava is widely used in human and animal nutrition, media for producing bioproducts and as raw material for several industrial products, the most important of which are cassava flour, cassava starch and sour cassava starch (Oboh, Akindahunsi, 2003a ; Oboh, Akindahunsi, 2003a; Oboh, 2005; Carvalho et al., 2005; Barros, et al.,2008; Araújo, 2010).

We report in this paper, a preliminary evaluation of *Mucor circinelloides* f. *circinelloides* for astaxanthin production using cassava wastewater medium as substrate in a Bioreactor, using two different sources of light (a blue LED and a fluorescent lamp), and an approach for evaluating the antioxidant potential of astaxanthin by the DPPH method.

MATERIALS AND METHODS

Microorganism and maintenation

The microorganism used was the filamentous fungus *Mucor circinelloides* f. *circinelloides* (UCP-0069) obtained from the Culture Collection of the Nucleus for Research in Environmental Sciences (NPCIAMB), UCP-(Universidade Católica de Pernambuco), deposited in 2000, and registered in the World Federation for Culture Collections (WFCC).The fungus was maintained on potato dextrose agar at 5°C.

Chemicals, Cassava wastewater (CWW) and lights

All reagents used were of an analytical grade. The tropical residue, cassava wastewater (CWW), was kindly provided by a local industry (PE, Brazil) and was used as the soluble substrate. This agro-industrial waste was used as the carbon source and the salt base from Helsseltine & Anderson medium (HA), according to factorial designs. The influence of exposure to light was evaluated using a blue LED and a fluorescent lamp (white lamp).

Cultural conditions for producing astaxanthin

The fungus was grown on Petri dishes containing potato dextrose agar for five days at 28°C. After this period the spores were removed and counted to 10^7/mL, and transferred to 250mL Erlenmeyer flasks containing 50mL of production medium (Cassava wastewater-CWW and the base of HA (Hesseltine & Anderson modified medium), incubated in an orbital shaker at 150 rpm, at 24°C for 24 h.

Bioreactor set-up

After this period the cultures were transferred to a mechanical bioreactor-Bioflo 2000 (New Brunswick Scientific CO., Inc., Box 4005 • 44 Talmadge Road • Edison, NJ 08818-4005) containing four liters of 4% CWW or HAM pH 5.5, aeration of 1 vvm, at 96h. The cultures were irradiated with blue LEDs (50 Lux) and fluorescent lamps. Cultures were maintained in the dark to control of astaxanthin production. The temperature was controlled at 28 ± 0.5°C until the end of the process. The biomass obtained from CWW or HA was collected by centrifugation at 5.000*g,* washed with distilled water to remove residual media, and then, the biomass was dried at 135°C for 2h, and transferred to a dissector until constant weight. All experiments were carried out in duplicate and the average values of the results.

Astaxanthin extraction

The astaxanthin content of the biomass was determined by the procedure described by Nghiem et al. (2010). The biomasses obtained from CWW or HA were homogenized in a Potter-Elvehjem homogenizer using cold acetone as the extraction solvent. The extracts were centrifuged at 2000*g* for 10 min at 5°C for the organic separation phase. The astaxathine production was detected in the organic extracts by Spectrophotometer (Biochrom Libra – S22 UV-Vis), at 470 nm, based on the absorption coefficient for determination.

Thin-Layer Chromatography (TLC)

The organic extracts obtained using a glass capillary were spotted on to a silica TLC plate (Silica gel 60 F_{254}, Merck) and, in addition, standard astaxanthin (Sigma-Aldrich) was used, and the assay run using the solvent toluene: acetone: ethyl acetate (85 : 8 : 7 v/v). The bands in the plate were detected under UV light. The bands were scraped and solubilized using acetone and then centrifuged at 2000*g* for 10 min at 5°C (Shokufeh et al., 2007).

DPPH Radical Scavenging Assay

The free radical scavenging activity was measured by the DPPH method as described by Loo et al. (2008). Different dilutions of the astaxanthin band extracts obtained (1ml) were added to 2 ml of DPPH (5.9 mg/100 ml methanol) and allowed to react at room temperature for 30 min. The absorbance of the mixtures was measured at 517 nm, after 30 min of incubation in the dark. A control was prepared without sample or standard (astaxanthin (Sigma-Aldrich) and measured immediately at 0 min – the lower the absorbance of the reaction mixture, the higher free radical scavenging activity. The percentage of the DPPH scavenging effect was calculated using the following equation: DPPH scavenging effect (%) = $(A_{control} - A_{sample}/A_{control}) \times 100$, where $A_{control}$ is the absorbance of the control reaction containing all reagents except the compound tested. A_{sample} is the absorbance of the test compound. The concentration of extract needed to scavenge 50% of DPPH free radicals was calculated from the graph-plotting inhibition

Table 1. Astaxanthin production by *Mucor circinelloides* in a bioreactor with Cassava wastewater and Hesseltine modified media, with exposure to blue LED, fluorescent lamp and control at 96 hours of cultivation

Medium	Blue light		Fluorescent lamp		Control (dark)	
	χ_1	χ_2	χ_1	χ_2	χ_1	χ_2
CWW	4.5	110	3.5	150	5.7	95
HM	2.7	55	3.4	29.8	3.0	20

CWW: 4% Cassava wastewater; HA: Hesseltine & Anderson Modified; χ_1: biomass yield (g/L); χ_2: astaxanthin yield (µg/g).

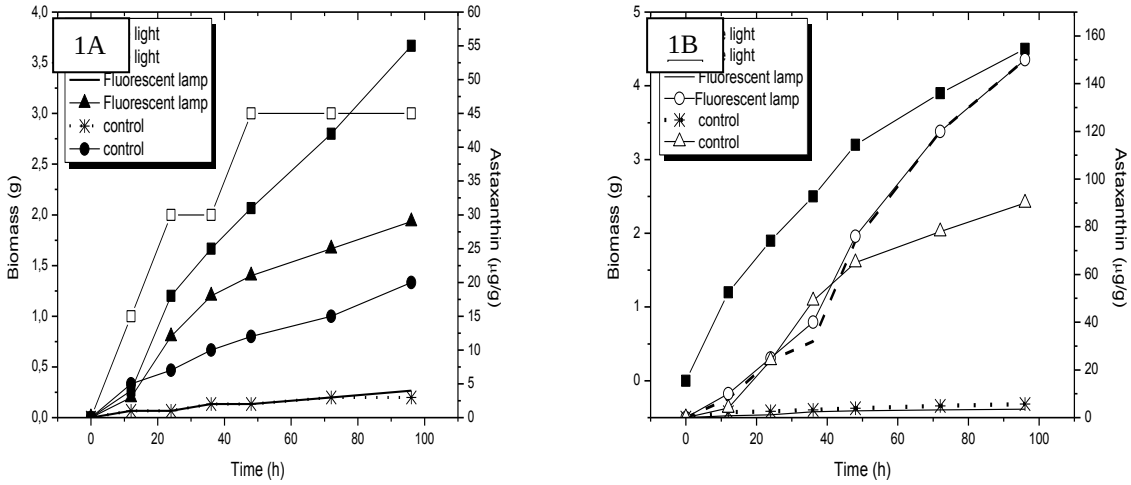

Figure1:Biomass and astaxanthin production in medium Hesseltine & Anderson modified (HM) and Cassava wastewater (CWW) by *Mucor circinelloides* (1A) HA medium with exposure to blue and white light and control; (1B) CWW Medium with exposure to blue and white light and control

percentage against extract concentration. Scavenging activities were expressed in mg/mL.

Statistical analysis

Statistical analysis was carried out using ANOVA and a *p* value <0.05 was accepted as statistically significant. The graphics were compiled and the statistics analysis conducted using Origin 8.0 software.

RESULTS

Influence of the medium of the biomass and of exposure to light on astaxanthin production

The results for astaxanthin production by *M. circinelloides* f. *circinelloides* using the optimized medium Cassava wastewater (CWW) and a Hesseltine & Anderson modified (HA) medium are shown in Table 1. The results showed the best condition of CWW using fluorescent light caused higher amounts of astaxanthin to be produced. This amounted to 150.0µg/g per biomass, and the yield of control HM medium to 29.8 µg/g. The date obtained corroborated those of Fontana et al. (2008) who demonstrated that Cassava wastewater is an excellent source for astaxanthin production.

Figure 1 shows the curve for biomass and astaxanthin production by *M. circinelloides* cultivated in all conditions tested in a fermentation time of 0-96h and the thin-layer chromatogram of astaxanthin isolation is shown in Figure 2. A small amount of astaxanthin is produced before the initial fermentation time (0-36h) after which there is an increment in the astaxanthin content. Yang et al. (2011) demonstrated the same parameter when producing astaxanthin with *P. rhodozyma* and showed that maximum production was after 36h of fermentation.

As shown in Table 1 and Figure 1, the use of a fluorescent lamp gave the best illumination conditions for producing astaxanthin, because this increased the content of astaxanthin (150 µg/g) compared with using blue light (110 µg/g) and control (95 µg/g) with a CWW medium. These results showed higher efficiency when compared with the HA medium using a Blue light (55 µg/g); a

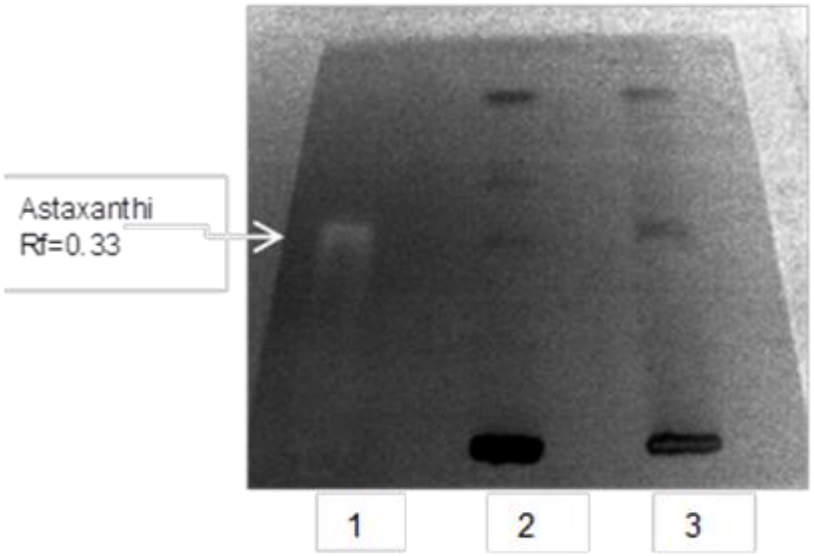

Figure 2: Thin-layer chromatogram of astaxanthin isolation. 1. Astaxanthin Standard; 2. Hesseltine & Anderson, exposure to white light; 3. Hesseltine & Anderson modified exposure to blue light.

Table 2. ANOVA for the dependent variable response to astaxanthin production in cassava wastewater medium (CWW)

Effect	Sum of squares	D.f	Mean Square	F – ratio	P value
Model	238741.0	1	238741.0	0.59	0.4492
Error	15549.02	4	5183.00667		
Total	16948.7	5			

R^2= 0.90; d.f: degrees of freedom

Table 3. ANOVA treatment for dependent variable response to astaxanthin production in Hesseltine & Anderson modified medium (HA)

Effect	Sum of squares	D.f	Mean Square	F - ratio	P value
Model	15020.00667	1	15020.00	31.15064	0.00505
Error	1928.69333	4	482.173		
Total	16948.7	5			

R^2= 0.85; d.f: degrees of freedom

Fluorescent lamp (29.8 µg/g) and control (20 µg/g). The light caused the suppression of cell growth, as shown in Table 1 and Figure 1, where there is no high production of biomass and there is an increment in the formation of astaxanthin. This is confirmed by Marova et al. (2012) in studies with the fungi *Sporobolomyces* sp. where they found a decrease in the biomass was accompanied by a very high carotene yield.

Table 2 shows the ANOVA results obtained from the model for astaxanthin in CWW medium in all light conditions. The model adjusts well to the experimental data as the variations in the total are only 1% and the confidence value was explained by the model R^2 = 0.90. The CWW medium and light proved to be very important factors in producing astaxanthin ($p<0.05$).

Table 3 shows the ANOVA of the model obtained for the astaxanthin in HM medium in all lighting conditions. The model adjusts well to the experimental data as the total variations are only 1.5%. The confidence limit was explained by the model R^2 = 0.85. The CWW medium and lighting proved to be very important factors in producing astaxanthin ($p<0.05$).

Thin-layer chormatograph

Thin layer chromatographic separation of carotenoid

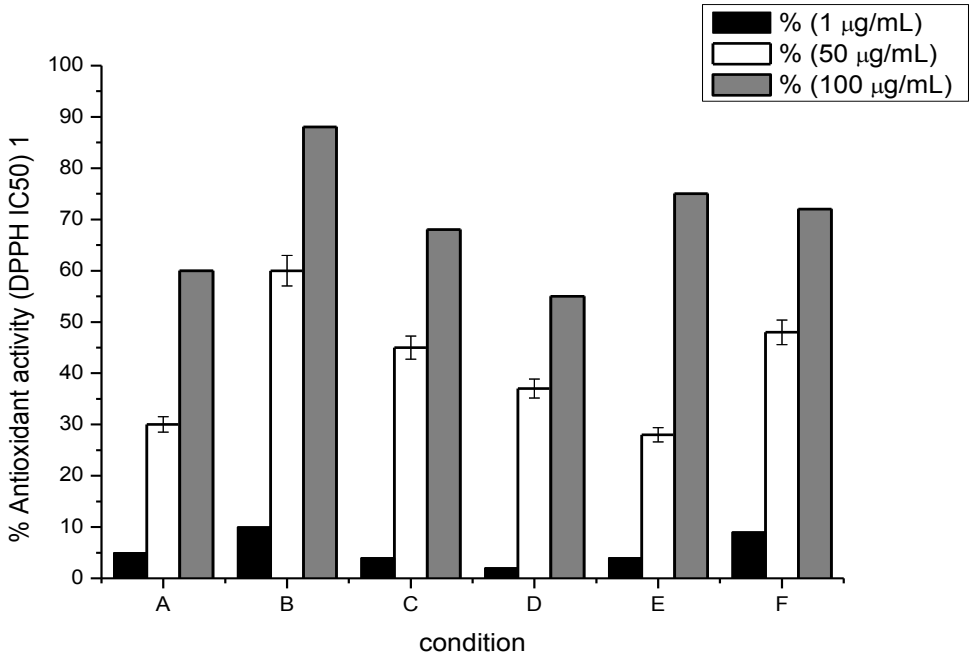

Figure 3: DPPH scavenging activity from astaxanthin produced by *Mucor circinelloides* in CWW medium (A-blue light; B- white light, and C- control) and HA medium (D- blue light; E- white light and F- control).

extracts from *M. circinelloides* cultivated in the medium HM (white fluorescent lamp and with blue Led) is shown in Figure 3. The R_f values for the bands were 0.33 which corresponded to astaxanthin and are in accordance with the standard astaxanthin R_f value (Figure 2).

Antioxidant activity (DPPH)

In these results, shown in Figure 4, the DPPH free radicals were decreased by approximately 10% at 1 mg/ml of astaxanthin; by 50% at 10 mg/ml and by about 80% at 100 mg/ml of astaxanthin in all conditions that were tested and compared to the control. This study clearly demonstrates the scavenging action of the DPPH free radical and this is very important information for astaxanthin studies.

DISCUSSION

The importance of this result is that it corroborates and builds on the studies of others. Golkhoo et al. (2007) produced astaxanthin with *Phaffia rhodozyma* (wild type and mutant forms) cultivated in a rich medium of YMB (yeast malt broth): 140 µg/g (wild type) and 230 µg/g of astaxanthin (mutant). This demonstrated that *M. circinelloides* is a promising source of astaxanthin.

Similar responses were found by Satoshi (2006) who demonstrated the influence of fluorescent lighting on the production of astaxanthin production and Katsuda, (2004) when he suggested that fluorescent lamps induce a high level of astaxanthin accumulation compared with blue LED lighting. In studies with *Haematococcus pluvialis* there are large accumulated amounts of astaxanthin in response to high irradiation with light and the condition; and to limiting the loss of nitrogen (Nagaraj et al., 2012).

Cassava wastewater has high toxicity (Barana and Cereda, 2000; Tung et al. 2003 and Arotupin 2007). Because of this, CWW acts as a stress factor for producing astaxanthin by *M. circinelloides*. This is in accordance with Marova et al. (2010) who described the increment in stress conditions when astaxanthin is produced by the fungi *Rhodotorula glutinis.*

Some articles reported the production of amylase by *M. circinelloides*, as perhaps being the responses to the growth of these fungi in a medium of cassava wastewater, which has a high content of starch (Alves et al. 2002; Bredenkamp et al. 2010).

As to methods the TLC technique is often used for separating and isolating individual classes of molecules, because it is rapid, effective, and relatively inexpensive (Rodriguez-Amaya, et al., 2004). It has also been shown that carotenoids can be oxidized or degraded if exposed to intense light or heat, or stored for a long time, so rapid analysis and appropriate storage conditions are required (Dutta et al., 2005;Carvalho et al., 2014).

The R_f values obtained for astaxanthin (R_f = 0,33) are in

agreement with the results reported by Khanafari, (2007) and Sánchez-Camargo et al. (2011).

DPPH is a purple stable organic radical with an absorption band in the range of 515-528 nm. When the radical accepts an electron or a free radical species, the result is a visually noticeable discoloration from purple to yellow. Because the DPPH radical can accommodate many samples in a short period of time and is sensitive enough to detect active molecules at low concentrations, it has been extensively used to screen antiradical activities in many organisms (Hsu et al., 2005).

Oxygen free radicals are produced in large amounts by various environmental factors, such as air pollution or smoke, ultraviolet rays, stress, and intensive exercise and the role that they play in damaging lipids, proteins, and nucleic acid is crucial. They cause various kinds of diseases and aging (Saoudi and Fei, 2012).This article gives the first results of antioxidant activities from astaxanthin by *M. circinelloides* and suggests it has the ability to remove free radicals.

CONCLUSION

This study shows that Cassava wastewater-CWW (manipueira), an inexpensive raw material for fermentation, is a promising substrate for producing *Mucor circinelloides* with increased astaxanthin. The optimum growth conditions, set out in this article, resulted in an astaxanthin yield of 150.0 µg/g biomass when exposed to fluorescent lighting, and 110 µg/ g of biomass when exposed to blue LED light for 96h. Microbial production has the advantage that it uses low-cost substrates, resulting in lower production costs. The influence of different lighting on astaxanthin production suggests that fluorescent light is an important factor for incrementing such production. We described the first report on the potential activity of astaxanthin from *M. circinelloides* to DPPH radical scavenging. Thus, microbial synthesis by Mucoralean fungus *Mucor circinelloides* offers a promising alternative for astaxanthin production.

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