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Production of a Melanin like Pigment by *Kluyveromyces marxianus* and *Streptomyces chibaensis*

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Abstract: The aim of the study was to determine the optimum conditions for brown pigment formation by *Kluyveromyces marxianus* and *Streptomyces chibaensis.* Pigment formation was followed during the time course experiment in medium contains 20 g/l D-xylose as carbon source, 12 g/l of a mixture of peptone and yeast extract as a nitrogen source at pH 5.0 when incubated at 32°C, the production of pigment reached approximately its maximum. From the FTIR spectrum analysis it could be concluded that the pigment might be melanin, and the particles of the present pigment used image analysis system, were similar to particles of the synthetic melanin.

Key words: *Kluyveromyces marxianus, Streptomyces chibaensis,* Brown pigment, Infrared, Image analysis.

INTRODUCTION

Melanins are multi-functional polymers of phenol or indolic compounds that are found throughout nature produce by various microorganisms by the fermentative oxidation, have the radio protective and antioxidant properties that can effectively protect the living organisms from UV radiation (Dastager *et al.*, 2006, Frases *et al.*, 2007). Actinomycetes can synthesize and excrete melanin or melanoid, which are considered to be a useful criterion for taxonomical studies (Dastager *et al.*, 2006) such as *S. aurofaciens* (Nakano *et al.*, 2000), also melanin can be manufactured by many yeasts like Yarrowia lipolytica (Carreira *et al.*, 2001).

Three principal types of melanin are recognised: *Eumelanins* which are black or brown, produced in the course of oxidation of tyrosine to o-dihydroxyphenylalanine (DOPA) and dopaquinone *pheomelanins*: yellow or red, generally contain sulphur and *allomelanins* do not contain nitrogen (Plonka, Grabacka 2006).

The best understood melanisation pathway is the classic Mason-Raper pathway (Mason 1948) in which tyrosinase (EC 1.14.18.1) hydroxylates tyrosine to dihydroxyphenyl-alanine (DOPA) and then to dopaquinone which then auto oxidizes and polymerizes known as eumelanins (Carreira *et al.*, 2001).

Infrared spectroscopy is a useful technique for analysing structures and structural changes of molecules and identification of unknown samples (Torben, Steffen 2002).

As a part of these studies on the formation and stability of pigments excreted from *Kluyveromyces marxianus* and *Streptomyces chibaensis*, the aim is to identify and optimize media composition to achieve more efficient production of pigment. The components of media such as carbon source, nitrogen source and their different concentrations, pH and incubation period are examined to improve the production of melanin. Finally, infrared spectroscopy and microscopy appearance are used as a trial to identify the obtained pigment from both microorganisms.

MATERIALS AND METHODS

Microbial Strains, Inoculum and Media:

Kluyveromyces marxianus was obtained from Microbial Culture Collection Centre, Faculty of Agriculture, Ain Shams University, Egypt and *Streptomyces chibaensis* was isolated from Toushka's soil, Egypt, clarified by Hewedy (2003). The media used for inoculums preparation for *K. marxianus* and *S. chibaensis* were glucose yeast extract peptone agar containing (g/l) 5 yeast extract, 5 peptone, 2 glucose, 20 agar and starch nitrate agar containing (g/l) 10 starch, 2 $NaNO_3$, 1K_2HPO_4, 0.5 KCl, 0.5 $MgSO_4 7H_2O$, 20 agar respectively.

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One ml inoculum suspension for an initial optical density (O.D.) at 640 nm. of 0.35 of both strains and a mixture of both was inoculated into 50 ml of the brown pigment production medium (YEPSuc) containing (g/l) 5 yeast extract, 5 peptone, 10 KCl, 20 sucrose (Petinate *et al.*, 1999) and were incubated for several days at 32°C (optimum temperature).

Quantitative Determination:

At the appropriate periods of culturing, pigment production was evaluated after filtering the sample (pore membrane size 0.22 µm) by measuring O.D. of the filtrate at 400 nm (Sanchez-Amat *et al.*, 1998). All determinations were performed in a Spectronic 21 (Bauch, Lomb, New York, USA) spectrophotometer.

Effect of Incubation Time on Pigment Formation:

Excretion of the pigment precursor were assessed in cells of both strains and a mixture of them grown in the broth YEPSuc medium inoculated as described above from 1 to 14 days of incubation at 32°C.

Effect of pH on Pigment Formation:

The effect of pH on pigment production was assessed in the broth YEPSuc medium, 0.1 M-phosphate buffer (pH 5.0-8.0).

Effect of Medium Composition on the Pigment Formation:

Effect of broth YEPSuc medium described replacing sucrose with glucose, galactose, fructose, mannose, lactose, arabinose, D-xylose, starch, mannitol, glycerol, succinic acid and/or malic acid as a carbon source on the browning activity was achieved, while peptone, yeast extract, aspartic acid, leucine, sodium glutamate, glutamine, urea, ammonium sulphate, amm. nitrate, amm. chloride, amm. dihydrogen phosphate and/or tryptone as a nitrogen source was studied in their effectiveness on the browning activity in broth YEPXyl medium (Chaskes *et al.*, 2008), all experiments were performed twice.

Extraction of Brown Pigment:

The pigment in the culture medium was extracted by the method of Wei, Chen (2005). The culture broth was mixed with a four volume of methanol and mixed vigorously using a vortex mixer. The resulting mixture was centrifuged at 10,000 rpm for 10 min. The organic portion was collected and filtered through a 0.22 µm filter paper. The filtrate was concentrated using a rotary evaporator and subsequently extracted with 3.0 M chloroform. The chloroform phase was collected and reconcentrated to obtain the resultant product.

Image Analysis:

Pigment particles derived from *K. marxianus*, *S. chibaensis* and mixture of them were observed and measured using image analysis system (Fukuii, 1988).

Fourier Transforms Infrared Analysis (FTIR):

FTIR (460 plus) used to detect the function groups of pigment structure were carried out by a Nicolet Nexus spectrophotometer coupled with a Nicolet Continuum Fourier transform infrared spectroscopy (FTIR) microscope equipped with a HgCdTe detector cooled with liquid N_2; spectra were recorded by a Graseby-Specac cell accessory in transmission mode between 4,000 and 700 cm^{-1} (Cappitelli *et al.*, 2007).

Statistical Analysis:

ANOVA test (one way) was calculated in all cases, significance was measured by LSD at $p<0.05$ using SPSS program version 8.0.

RESULTS AND DISCUSSION

Results:

The results confirmed that *K. marxianus* and *S. chibaensis* produced brown pigments.

Factors Affecting Pigment Production:

Incubation time. Pigment production was followed through 14 days at 32°C which was the optimum temperature for growth of both microorganisms.

Media started to develop reddish-brown colour after 48 h during the exponential phase of growth (O.D. 400±0.01) that slowly turned into a deep brown colour at the beginning of the stationary phase (11 days) of growth, the highest pigment production was from *S. chibaensis*+*K. marxianus* > *S. chibaensis* > *K. marxianus* respectively (Fig. 1a, b).

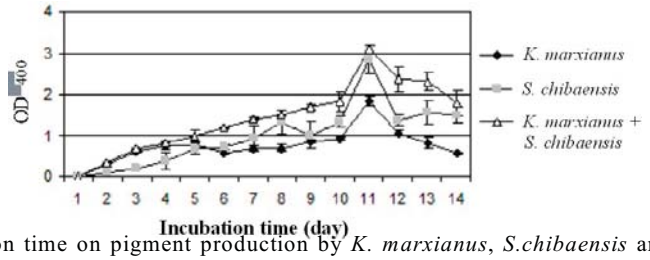

Fig. 1a: Effect of incubation time on pigment production by *K. marxianus*, *S.chibaensis* and mixture of both grown in broth YEPSuc medium at 32°C for 14 days.
LSD at 5%: *K. marxianus* → 0.15
S. chibaensis → 0.85
K. marxianus +*S. chibaensis* → 0.83

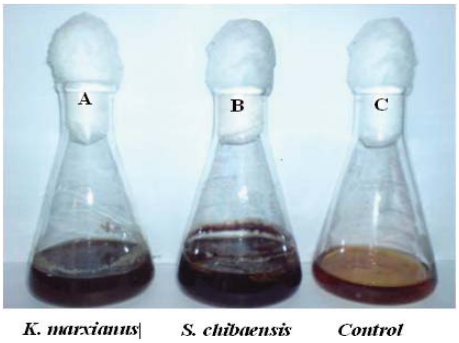

Fig. 1b: Brown pigment formation by *K. marxianus* (A) and *S. chibaensis* (B) after 12 days of incubation at 32°C.

Medium pH:

pH had a marked effect on the pigment production during the microorganism's growth. Fig. (2) indicated that the best production of pigment was at pH 5.0 (*S. chibaensis*+*K. marxianus*> *S. chibaensis* > *K. marxianus*). A decrease in the final brown colour intensity was observed when the pH increased.

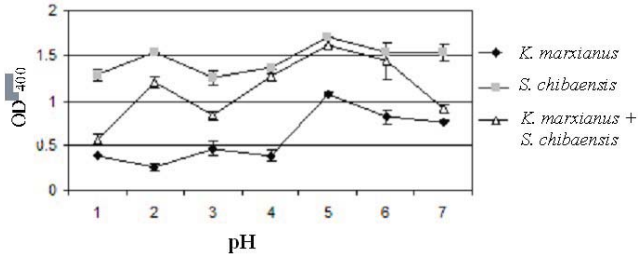

Fig. 2: Effect of pH on pigment production by *K. marxianus*, *S. chibaensis* and mixture of them grown in broth YEPSuc medium at 32°C for 11 days.
LSD at 5%: *K. marxianus* → 0.583
S. chibaensis → NS
K. marxianus + *S. chibaensis* → 0.419

Carbon Sources:

All different carbon sources used resulted in good growth and pigmentation production except succinic and malic acid. A significant increase of growth and pigmentation was noticeable with D-xylose (Table 1). Intense pigment production required D-xylose concentration of at least 20 g/l (Table 2) which was statistically the suitable concentration for the studied microorganisms (K. marxianus > S. chibaensis + K . marxianus > S. chibaensis).

Table 1: Effect of different carbon sources on pigment production by *Kluyveromyces marxianus*, *Streptomyces chibaensis* and mixture of them grown in broth YEP medium for 11 days of incubation at 32°C.

Carbon source	Browning activity (OD at 400 nm)			
	K. marxianus	S. *chibaensis*	*K. marxianus* + S. *chibaensis*	LSD at 5%
Glucose	0.408±0.107	3.090±0.042	1.283±0.138	0.438
Galactose	0.682±0.105	1.760±0	1.058±0.088	0.188
Fructose	0.485±0.086	3.095±1.223	1.170±0	1.610
Mannose	0.970±0.014	3.190±0.071	0.988±0.124	1.111
Sucrose	0.110±0.014	2.465±0.092	2.24±0.481	1.178
Lactose	0.206±0.064	1.194±0.256	1.013±0.0138	0.494
Arabinose	0.435±0.026	2.72±0.057	1.765±0.163	0.665
D-xylose	1.385±0.064	5.565±1.124	2.72±0.311	2.09
Starch	0.309±0.059	1.72±0.311127	1.63±0	0.706
Mannitol	0.282±0.062	2.058±0.336	0.809±0.018	0.888
Glycerol	1.155±0.071	1.642±0.427	1.815±0.177	NS
Succinic acid	0±0	0±0	0±0	NS
Malic acid	0±0	0±0	0±0	NS
LSD at 5 %	0.139	1.147	0.447	

Table 2: Effect of different concentrations of optimum carbon source (xylose) on pigment production by *Kluyveromyces marxianus*, *Streptomyces chibaensis* and mixture of them grown in broth YEP medium for 11 days of incubation at 32°C.

D-xylose concentration (g/l)	Browning activity (OD at 400 nm)			
	K. marxianus	S. *chibaensis*	*K. marxianus* + S. *chibaensis*	LSD at 5%
5	0.267±0.013	0.342±0.062	0.368±0	0.432
10.00	0.693±0.327	0.415±0.021	0.412±0.017	0.370
14.97	0.977±0.457	0.921±0.030	0.605±0.132	0.155
20.00	2.59±0.014	1.196±0.260	1.784±0.645	0.102
24.97	1.422±0.670	1.151±0.233	1.441±0.254	0.220
30.00	1.042±0.0	0.866±0.139	1.663±0.513	0.114
34.97	0.854±0.295	0.723±0.293	1.275±0.318	0.230
LSD at 5%	0.966	0.44	0.753	

Nitrogen Sources:

The comparative efficiency of various nitrogen sources was recorded in Table (3). Statistically, the favourable nitrogen source for *S. chibaensis* was yeast extract, while *K. marxianus* preferred peptone when each of them was used as a sole nitrogen source. The other different nitrogen sources resulted in excellent growth but produced light pigmentation in comparison with yeast extract and peptone at different concentrations (Tables 4&5). Mixture of both yeast extract and peptone, 6 g/l for each of them resulted in excellent growth and strong pigmentation for the three treatments (Table 6).

Table 3: Effect of different nitrogen sources on pigment production by *Kluyveromyces marxianus*, *Streptomyces chibaensis* and mixture of them grown in broth YEPXyl medium for 11 days of incubation at 32°C.

Nitrogen source	Browning activity (OD at 400 nm)			
	K. marxianus	S. *chibaensis*	*K. marxianus* + S. *chibaensis*	LSD at 5%
Amm. Sulphate	0 ± 0	0 ± 0	0 ± 0	NS
Peptone	0.925±0.01	0.239±0.152	0.56±0.190	0.526
Yeast extract	0.605±0.081	1.6±0	0.629±0.147	0.498
Aspartic acid	0±0	0±0	0±0	NS
Sod. Glutamate	0.202±0.040	0.147±0.015	0.181±0.007	NS
Leucine	0.156±0.009	0.085±0.026	0.144±0.052	NS
Glutamine	0±0	0.114±0.001	0.13±0.035	0.065
Amm. Nitrate	0±0	0±0	0±0	NS
Amm. Chloride	0±0	0±0	0±0	NS
Amm. Thiosulphate	0±0	0±0	0±0	NS
Urea	0.146±0.131	0.098±0.064	0.113±0.010	NS
Tryptone	0.281±0.004	0.142±0.004	0.238±0.027	0.070
LSD at 5%	0.458	0.108	0.163	

Table 4: Effect of different concentrations of yeast extract on pigment production by *Kluyveromyces marxianus, Streptomyces chibaensis* and mixture of them grown in broth YEPXyl medium for 11 days of incubation at 32°C.

Yeast extract concentration (g/l)	Browning activity (OD at 400 nm)			
	K. marxianus	S. *chibaensis*	*K. marxianus*+ S. *chibaensis*	LSDat 5%
9.61	0.336±0.042	0.223±0.030	0.306±0.043	NS
10.61	0.638±0.059	0.176±0.127	0.361±0.180	0.370
11.61	0.742±0.187	0.054±0.045	0.325±0.012	0.345
12.61	0.773±0.052	0.134±0.116	0.390±0.010	0.128
13.61	0.809±0.030	0.250±0.033	0.441±0.116	0.282
LSD at 5%	0.219	NS	NS	

Table 5: Effect of different concentrations of peptone on pigment production by *Kluyveromyces marxianus*, *Streptomyces chibaensis* and mixture of them grown in broth YEPXyl medium for 11 days of incubation at 32°C.

Peptone concentration (g/l)	Browning activity (OD at 400 nm)			
	K. marxianus	S. *chibaensis*	*K. marxianus* + S. *chibaensis*	LSDat 5%
6.71	1.750±0.127	0.611±0.395	0.511±0.071	0.620
7.71	1.925±0.035	0.276±0.022	0.710±0.099	0.218
8.71	2.385±0.219	0.843±0.448	1.630±0.071	1.165
9.71	2.199±0.214	0.434±0.055	1.596±0.079	0.302
10.71	2.590±0.014	1.183±0.279	1.784±0.645	1.107
LSD at 5%	0.333	0.829	0.503	

Table 6: Effect of different concentrations of yeast extract and peptone on pigment production by *Kluyveromyces marxianus, Streptomyces chibaensis* and mixture of both grown in broth YEPXyl medium for 11 days in incubation at 32°C.

Yeast extract and peptone concentration (g/l)	Browning activity (OD at 400 nm)			
	K. marxianus	S. chibaensis	K. marxianus +S. chibaensis	LSD at 5%
3+3	1.872±0.175	0.414±0.106	0.341±0.030	0.766
4+4	2.046±0.190	0.568±0.068	0.572±0.096	0.741
5+5	2.830±0.354	1.206±0.430	1.734±0.020	0.812
6+6	3.280±0.141	0.317±0.03	1.314±0.569	1.481
7+7	2.505±0.219	1.295±0.091	1.192±0.209	1.333
LSD at 5%	0.456	0.444	0.682	

Identification of Pigment:

One of the identification main tests for melanins is IR spectrum. Melanin exhibited bright spectral absorption lines, 2925-2938 cm^{-1} peak related to the hydroxy group (OH), 3344-3436 cm^{-1} peaks related to the amino second group (NH), 1243-1305 cm^{-1} peaks related to anhydride group (C-O), in synthetic melanin and all extracted microbial pigment. Also there were 2925-2938 cm^{-1} peaks related to methane group (CH), 1628 to 1651 cm^{-1} peaks related to amino group (NH_2) in pigment extracted from *K. marxianus*, *S. chibaensis* and synthetic melanin (Fig. 3).

Visualization and Measurement of Pigment Particles:

Under microscopic image, the pigment particles obtained from extracted pigment were dark, non refractile and granular with individual granules had the diameter of 3460 nm for *K. marxianus*, 2940 nm for *S. chibaensis* and 1630 nm for the particles obtained from mixture which notably were clustered (Fig. 4a, b, c).

Discussion:

Kluyveromyces marxianus can envisage a great biotechnological future because of some of its qualities such as a broad substrate spectrum, high growth rates and less tendency to ferment when exposed to sugar excess (Fonseca *et al.*, 2008), *S. chibaensis* as a soil organism has an important role as a plant growth promoting bacteria (PGPR), (Nafie 2003, Hewedy *et al.*, 2006) easy to culture in vitro and both can excrete pigment. To ensure a high brown pigment production, we culture *S. chibaensis* and *K. marxianus* and its mixture under different conditions. The technological conditions under which browning colour develops are not yet established and it remains unclear why this phenomenon had a sporadic occurrence (Carreira *et al.*, 2001).

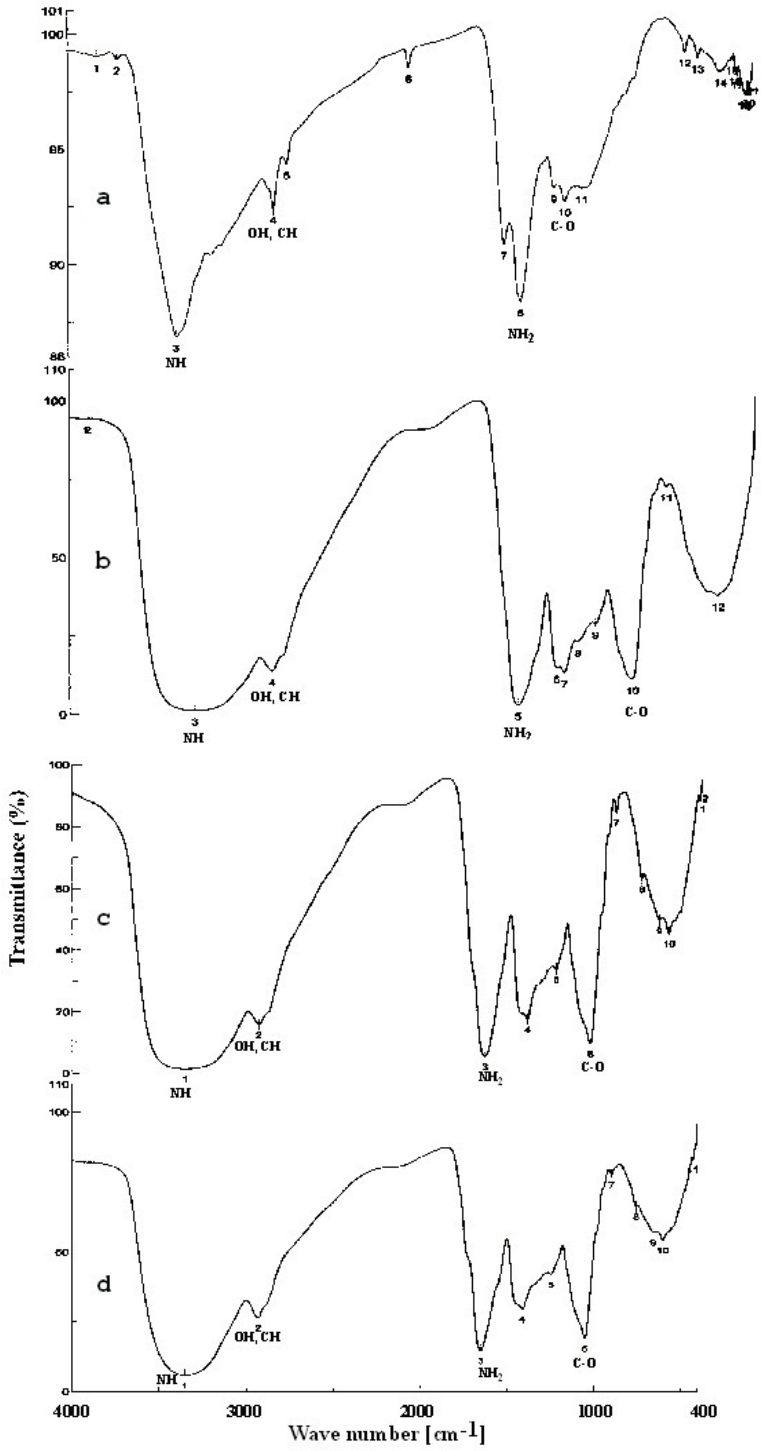

Fig. 3: FTIR spectrum of (a) synthetic melanin (b) pigment extracted from *K. marxianus;* (c) pigment extracted form S. chibaensis and (d) pigment extracted from a mixture of both strains.

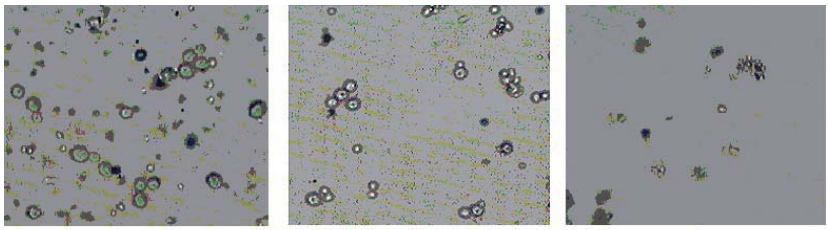

Fig. 4: Microscopic images showing brown pigment particles of (a) *K. marxianus* (b) *S.chibaensis* (c) mixture of *K. marxianus* +*S.chibaensis* (magnification×4000)

The appearance of pigment was noticed after 48 h, the highest pigment production was obtained after 11 days (the end of exponential phase). Carreira *et al.*, (2001) results indicated that the pigment was formed at the end of the exponential phase of growth, this finding was in accordance with the obtained results. They also demonstrated that a slight increase in colour was observed in the first 24 h of incubation for *Yarrowia lipolytica* while Coyne, Harthi (1992) found that the melanin production by *Vibrio cholerae* 569B grown at 37°C occurred after 3 days.

pH was the second factor used had marked effect. There were no clear differences in the growth or pigmentation of the studied three treatments. Consequently, pH 5.0 was selected as the working pH, in which the development of maximum brown colour was observed. Carreira *et al.*, (2001) found that the best pH for *Yarrowia lipolytica* was approximately 6.5. Coyne, Harthi (1992) reported that the initial pH ranged from 5.8 to 6.5 in case of *V. cholerae* 569B, the favourable pH for *Cryptococcus gattii* ranged from 5.35 to 7.35 (Chaskes *et al.*, 2008). Each *K. marxianus* or *S. chibaensis*, produced pigment in all carbon sources used in this work except succinic and malic acid. Statistically, the main effective carbon source for all treatments was D-xylose with concentration of 20 g/l. Carreira *et al.*, (2001) showed that glucose was the best carbon source for yeast while Dastager *et al.*, (2006) indicated that starch was the effective carbon source for *Streptomyces* spp. followed by glycerol and fructose, Chaskes *et al.*, (2008) had a different opinion that in case of *C. gattii*, the carbon source was fructose.

The current study suggested that it was feasible to combine yeast extract and peptone into a single medium to increase producing pigment from both strains, a good increase in growth and pigmentation at concentration of 6 g/l for each was obtained. In contrast, a weight of 4 to 1 for D-tryptophan/D-proline gave excellent growth and strong dark pigmentation for *C. gattii* (Chaskes *et al.*, 2008).

The chemical properties of the resulting dark pigment were determined including its elemental composition with infrared (IR) spectrum (Harki *et al.*, 1997). The results of this study were calibrated using data obtained with synthetic dihydroxyphenylalanine (DOPA) (eumelanin) and indicated that: (1) Purification with chloroform gave the best results (2) The synthetic and the present natural microbial melanins had the same chemical properties for each organism, the mixed culture differed in functional group (C-N), this may be a strain-related phenomenon, or C/N ratio might have resulted form impurities which were difficult to remove from melanin (Ito 1993, Willaimson *et al.*, 1998) or from the introduction of impurities during isolation procedures (Willaimson *et al.*, 1998). Generally, the chemical properties of melanin pigment produced by both microorganisms had the same percentage of nitrogen, the same function groups and the same degree of polymerisation, these agree with (Harki *et al.*, 1997). Based on this finding, we suspect that the resulted pigment may be eumelanin not allomelanin which is lacking nitrogen (Plonka, Grabacka 2006). Microscopic image revealed that the present black particle was composed of hollowed cell (Fig. 4a, b), similar results had also been reported with *Cryptococcus neoformans* (Wang *et al.*, 1996). The particles produced from the mixed culture were clustered (Fig. 4c) which was different from the two other microorganisms thus further investigations should be undertaken to confirm this finding.

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