Research article

Supplement comprising of laccase and citric acid as an alternative for antibiotics– *in vitro* triggers of melanin production

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Practical application

This study shows, for the first time, that extracellular laccase and citric acid induce intracellular production of melanin by yeast. It gives a potential green alternative to antibiotics used in poultry feed, which could counter the growing problem of antibiotic resistance.

Abstract

An indiscriminate use of antibiotics in humans and animals has led to the widespread selection of antibiotic-resistance, thus constricting the use of antibiotics. A possible solution to counter this problem could be to develop alternatives that can boost the host immunity, thus reducing the quantity and frequency of antibiotic use. In this work, for the first time, citric acid and laccase were used as extracellular inducers of melanin production in yeast cells and human cell lines. It is proposed that the formulation of laccase and citric acid together could further promote melatonin-stimulated, melanocyte-derived melanin production. Melanization as a probe of immunity described in this study, is an easy and a rapid test compared to other immunity tests and it allows performing statistical analyses. The results showed the synergistic effect of citric acid and laccase on melanin production by yeast cells, with significant statistical differences compared to all other tested conditions (p: 0.0005-0.005). Laccase and citric acid together boosted melanin production after 8 days of incubation. An increase in melanin production by two human colon cells lines (Cacao-2/15 and HT-29) was observed on supplementation with both laccase and citric acid in the cell growth medium. Produced melanin showed antimicrobial properties similar to antibiotics. Therefore, a formulation with citric acid and laccase may prove to be an excellent alternative to reduce the antibiotic use in human and animal subjects.

1. Introduction

The indiscriminate use of antibiotics at therapeutic and sub-therapeutic levels has led to the emergence of bacterial resistance to antibiotics, which has become a public concern. [1]. Thus, antibiotics no longer remain valid tools to counter the onslaught of deadly bacterial human diseases, and studies are being carried out to develop novel and efficient alternatives to antibiotics [2, 3]. Several studies have demonstrated organic acids and enzymes as biological alternatives to antibiotics [4, 5]. Citric acid is one of the most studied organic acids as an alternative to antibiotics due to its strong antimicrobial and immunomodulatory activity [6] [7]. Several enzymes have been known to improve gut performance, and therefore, overall health, by modifying the gut microflora [8]. Laccase, an oxidoreductase, is well known for its high ability to degrade phenolic compounds [9] and its antioxidant activity [10]. Additionally, laccase has also been reported to trigger the production of melanin [9, 11]. Melanin is a black pigment that serves as a cellular virulence factor against bacteria in form of melanophores [12]. Melanogenesis provides protection from environmental stress conditions to various groups of free living organisms [13]. In humans, melanin was shown to play an important role in intestinal inflammatory processes via melanin-concentrating hormone (MCH) [14, 15]. It was also reported that there is a link between melanin based pigmentary process and immune systems in the middle ear [16] and with inflammation accompanied obesity [17] in humans, while in birds, melanin based coloration variations are suggested to be indicators of environmental oxidative stress in a sexually selected manner [18]. The key enzyme for melanin synthesis is tyrosinase (EC 1.14.18.1) which is also responsible for biosynthesis of dopaquinone from tyrosine in sequential reactions. Melanin is synthesized by different organisms, such as bacteria, fungi, and plants [19]. However, laccase activity in yeast was reported for the first time by Roy et al [20]. In mammals, the production

of melanin by melanocytes is modulated by melatonin, a neurotransmitter. In this study, exogenous application of laccase and citric acid on yeast and human colon cells has been confirmed for increased production of melanin via a melatonin-independent pathway.

2. Material and methods

2.1 Production of laccase

Laccase was produced as optimized by Gassara et al. [21] and is briefly explained below.

a. Microorganism

Trametes versicolor (ATCC 2086) was grown in potato dextrose broth (PDB) medium in incubator shaker at 30±1°C, 150 rpm for 7 days before incubating it on potato dextrose agar (PDA) petri plate in static incubator at 30±1°C for 10 days [22]. **b. Solid state fermentation (SSF)**

Forty grams of apple pomace (AP) were used as substrate for the SSF. The moisture was adjusted to 75% (v/w) and Tween-80 (3 mmol/kg of dry substrate) was used as an inducer for laccase production. After sterilization at 121±1 °C, 15 psi for 20 min, the substrate was inoculated with fungal mats from the PDA petri plate. The fermentation was carried out in static incubator at 30±1 °C for 15 days. Sampling was done every 24 hours for profiling of laccase production [21].

c. Enzyme extraction and activity test

Sodium-phosphate buffer (50 mM) at pH 6.5 was added to sample (10/1, v/w), and incubated at 30±1°C, 150 rpm for 2 h. The mixture was centrifuged at 7000 x g for 20 min at 4^{o}C, the supernatant was collected and the enzyme activity was spectrophotometrically determined at 436 nm using 2,2-azino bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) in 0.1 M phosphate-citrate buffer at pH 4 and 45±1°C [21].

2.2 Production of citric acid

Citric acid production was carried out based on the protocol optimized by Dhillon et al. [23] as explained below.

a. Microorganism and spore solution

Aspergillus niger (NRRL 2001) was used to produce citric acid. The fungus was grown in potato dextrose broth (PDB) for 7 days in shaking incubator at 30°C and 150 rpm, followed by its incubation on PDA plate at 30 °C for 5 days in static incubator. The spores were collected from the PDA plate using a 0.1% (v/v) Tween-80 solution and counted using a haemocytometer [23, 24].**b. Solid state fermentation (SSF)**

Fifty grams of AP were used as substrate for SSF. The substrate moisture was adjusted to 75% (v/w) and autoclaved at 121±1°C, for 20 min. Methanol (3%v/w dry) was filter sterilized using Whatman paper and added to substrate as an inducer for citric acid production. 1×10^7 spores/g dry substrate were added to inoculate the medium. The fermentation was performed in static incubator at 30 °C ±1 °C for 5 days. The samples were withdrawn every 24 hours, for profiling studies [23, 24].

c. Citric acid extraction and analysis

Citric acid extraction was done by adding distilled water to the substrate (1/15, w/v). The mixture was incubated at 30°C ±1 °C, 150 rpm for 30 min followed by centrifugation for 30 min at 7000 × *g* at room temperature. The supernatant was used for citric acid estimation by the Marier and Boulet method [23-25].

2.3 Melanization test

Melanization was carried out using a modified protocol by Wang et al. and Sabiiti et al., [26, 27].

a. Melanization test with yeast cells

The yeast strain, *Saccharomyces cerevisiae,* was grown on yeast peptone dextrose (YPD) agar, and cultured in 3 ml YPD broth in shaker incubator at 30±1°C, 100 rpm, for 24 h. Cells were counted using a haemocytometer [26, 27].

The medium was prepared by adding to 75 mL distilled water: 15 mM glucose, 10 mM $MgSO_4$, 29.4 mM KH_2PO_4, and 13 mM glycine. The medium was sterilized at 121±1°C, 15 psi for 20 min, following which 3.0 mM vitamin B1 and 1.0 mM catechol was added under aseptic conditions [26, 27].

The test was performed under the following four conditions in triplicates with the number of the cells being the same (1.0×10^8 yeast cells): A: control with only yeast cells B: yeast cells and citric acid (15g/L), C: yeast cells and laccase (1400 U/L) and; D: yeast cells with laccase (1400 U/L) and citric acid (15g/L). The flasks were incubated at 30±1°C, 150 rpm, for 10 days [26, 27]. The samples were taken every 48 h for studying the kinetics of melanin production.

Yeast cells were centrifuged at 9000 × *g* for 20 min at room temperature. The pellet was washed with 1.0 M sorbitol in 0.1 M sodium citrate (pH 5.0), and re suspended in 5 mL of the same solution. The cells were counted using haemocytometer [26, 27], and used for melanin extraction and quantification.

b. Melanization test with human colon carcinoma cell lines

HT-29 (ATCC® HTB-38TM) human colon adenocarcinoma cell line was purchased from the American Type Culture Collection and the human colon carcinoma cell line Caco-2/15 was provided by J. F. Beaulieu, Université de Sherbrooke, Sherbrooke, Quebec, Canada. Both cell lines were maintained in monolayer cultures in DMEM/F-12 growth medium supplemented with Glutamax (2 mM), antibiotic-antimycotic solution

(1X), Hepes (25 mM), Normocin (used as an antimycoplasma reagent, 100 µg/mL), and 10% heat-inactivated fetal bovine serum at 37°C in a 95% air: 5% CO_2 atmosphere. Cells were regularly monitored for the presence of *Mycoplasma* spp. by means of a conventional PCR test [28] using 5 µg of extracted genomic DNA (PureLink genomic DNA mini kit) as a template. The cells from passages 2-3 were seeded (1.5×10^6/well) in 6-well plates containing 3 mL medium and 3 mL of melanization solution (30 mM glucose, 20 mM MgSO4, 58.8 mM KH2PO4, 26 mM glycine, 6.0 mM vitamin B1, 2.0 mM catechol, 2800 U/L laccase and 30g/L citric acid). After 3 days, cells and media were collected for melanin quantification.**c. Melanin extraction and quantification**

The cells were sonicated for 45 seconds. The cell debris was collected by centrifugation at 9000 × g for 20 min and resuspended in 4.0 M urea for 1h at room temperature. This was further centrifuged at 9000 x *g* for 20 min, and resuspended in 6.0 M HCl at 100°C for 1h. The solution was then filtered through Whatman paper (45µm) using a vacuum pump. The filter was dried completely in a desiccator before weighing to quantify melanin produced by the cells by adapting the method used by Wang et al. and Sabiiti et al. [26, 27].

d. Statistical analysis

Statistical analysis of cell survival, melanin production per total yeast cells and melanin production per cell was done using Variance (ANOVA) by Bonferroni's multiple comparisons test (Prism software) and. Also, the Student's *t*-test was used for analysis of melanin production per yeast cell in the presence of citric acid and laccase at different time periods (2-10 days). *P*-values below 0.05 were considered statistically significant.

e. Characteristics of melanization substrate

A physico-chemical characterization was done for substrate at T_0 and T_{12}, to observe the changes that occurred during the experiment.

- **pH:**

About 7 mL of sample was taken in 15 mL falcon tube and the pH was measured using a pH meter.

- **Total protein content:**

Total protein content was measured using the Bradford method [29]. About 1 mL of sample was centrifuged at 9000 x g for 10 minutes. The supernatant was used to measure the total protein content.

- **Total reducing sugars:**

About 1 mL of sample was taken and centrifuged at 9000 x g for 10 minutes. the supernatant was used to estimate the total reducing sugars using DNS method [30].

- **Total phenolic content:**

About 2 mL of sample was centrifuged at 9000 x g for 5 minutes. The supernatant was used to estimate total phenolic content using the Folin-Ciocalteu (F-C) reagent [31].

- **Total solids content and total volatile solids:**

For total suspended solids, 5 mL of the sample was taken on aluminum dish and weighed using a balance, and dried overnight at 60°C. Afterwards, the sample was weighed again to measure the total solids content. The dried sample from the total suspended solids content measurement was incubated at 500°C in a furnace for 15 min, and weighed again to calculate total volatile solids.

2.4 FT-IR analysis

The melanin produced by the cells was scraped from filters and dissolved in 2M NaOH solution. The same procedure was performed for the pure melanin standard, purchased from Sigma-Aldrich. Both solutions were analyzed using FTIR (Fourier Transform Infrared Spectroscopy).

2.5 Well diffusion test

The well diffusion test with *Escherichia coli* (NRRL 3707) was performed for qualitative estimation of the antimicrobial activity of the melanin produced during the melanization experiment following the protocol given by Fahey et al [32].

The test was performed in triplicates using nutrient broth supplemented with 2% (w/v) agar autoclaved at 121±1°C for 20 min. *E. coli* was cultured in nutrient broth at 37°C for 24h. About 50 ml nutrient agar was cooled to 45^{o}C, and inoculated with 1% (v/v) bacterial suspension. The inoculated agar was poured into the petri dishes and allowed to solidify. After this, the four wells were punched into the agar using sterile pipettes tips, and filled with 80 µL of 1 ppm chlortetracycline (positive control), 2 ppm of melanin extracted from the melanization experiment (sample), 2 ppm of pure melanin standard and sterile milli-Q water (negative control), respectively. The plates were incubated at 37±1^{o}C for 24 hours. The diameter of the clearance zone formed due to inhibition of bacterial growth was measured using a ruler.

3. Results

Trametes versicolor was selected for its high potential of production of laccase. The highest enzyme production of 35 U/g $_{\text{dry substrate}}$ was obtained in 13 days of incubation at 30° C (**Figure 1**). *Aspergillus niger* was chosen for its copious citric acid production capacity. The highest citric acid production averaged at 35.4±0.7 g/kg $_{\text{dry substrate}}$ after 50 hours incubation (**Figure 2**).

For the melanization tests, the yeast cells were counted for each condition after 10 days of incubation. Melanin from yeast cells was extracted using sonication and weighed. Though the cell count was similar for all the conditions, significant differences were observed in melanin harvested from yeast cells incubated with laccase (**Table1**). The highest production of melanin by the yeast cells was 1.04 x 10^{-9}g/cell, and was observed when both laccase and citric acid were present in the growth medium (**Figure 3A, B and C**).

Kinetic studies of melanin production by yeast cells is presented in **Figure 4**. During first 6 days, melanin production was slow, however the production increased significantly (p: 0.02-0.003) after 8 days of incubation (3.399 x10^{-9} g/cell) (**Table.2**).

Additionally, physicochemical properties of melanization process were characterized and are summarized in **Table 3**.

To observe melanin production induced by extracellular laccase and citric acid in human cells, qualitative production of melanin was observed with two human colon carcinoma cell lines - Caco-2/15 and HT-29 (**Figure 5**). In human cell lines, melanin production in laccase and citric acid supplemented media was higher than melanin produced by the yeast cells under similar condition just in 3 days (6.40x10^{-9}g/ Caco-2/15 cell, 1.08x10^{-8}g/ HT-29 cell and 6.25x10^{-10}g/yeast cell).

The FT-IR for yeast produced melanin and standard melanin showed similar spectra (**Figure 6**) confirming identity of the produced compound.

The well diffusion test was done to study the antimicrobial effect of the melanin produced during the experiments. The melanin produced by yeast cells during melanization experiment gave an inhibition zone diameter of 1.3± 0.1 cm (**Figure 7).** The inhibition zone diameters for chlortetracycline and melanin standard were observed to be 1.5± 0.1 cm 0.5± 0.1 cm, respectively.

4. Discussion

Trametes versicolor was selected for laccase production due to the high activity and oxidation-reduction potential of the enzyme. Also, it has been demonstrated that the laccase produced by *T. versicolor* exerts a highest catalytic activity on many types of aromatic compounds compared to laccases produced by other strains [33].

Aspergillus niger has remained the organism of choice for commercial production of citric acid due to higher production per unit time. The main advantages of using *A. niger* are its ease of handling, its ability to ferment a variety of cheap raw materials, and high yields [34].

Apple pomace was chosen as growth medium due to its rich carbon content, ease of availability, and high biodegradability [21]. It has been already reported that apple pomace can be used as substrate for the production of organic acids, protein-enriched feeds, edible mushrooms, ethanol, aromatic compounds, natural antioxidants and enzymes, such as pectinases, pectin methylesterase, and lignocellulolytic enzymes, among others. Furthermore, the potential of apple pomace as a substrate for production of ligninolytic enzymes and release of polyphenols, such as laccase, has also been already investigated at flask level [35]. According to Dhillon et al., *A. niger* grows efficiently on different fruit pomace substrates giving high citric acid yields (120–264 g/kg dry substrate), and apple pomace is particularly

suitable as a raw material and low-cost carbon substrate for the production of citrates by *A. niger* [23].

In this work, melanin production by yeast system and human cell lines was used to test the possible efficacy of a formulation comprising fungal laccase and citric acid. Solano reported that phenolases, such as laccases, play a key role in melanin production [18], as they induce synthesis of DHN-melanin and pyomelanin - the microbial melanins. In the fungal cells, laccase acts as an anti-oxidant via the production of eumelanin, a "good" form of melanin [36, 37].

The obtained results suggested that this formulation as a feed supplement could be an eco-friendly potential alternative of antibiotics. The decrease in the total phenolic content during the melanization experiment (from 1.49±0.41mg/mL to 0.45±0.03mg/mL) indicated the increasing antioxidant activity of the product. The fact that mammals do not produce laccase shows that this enzyme could be of potential interest for pharmacological studies. The melanization experiments showed that after incubation, the yeast cell count significantly decreased (p: 0.02-0.003), compared with the control group, only when laccase was present in the growth media (**Figure 3A and Table 1**)**.** These data demonstrated that laccase from innocuous yeasts could mimic the melatonin action of production of melanin, an antibacterial trigger. Well diffusion test indicated that the melanin produced during the melanization experiment possessed an antimicrobial activity **(Figure 7).** It has been already reported that mice infected with fungus having high laccase activity exhibited strong inflammatory protection [38].

Citric acid as a diet supplement in animal feed has shown to promote their growth by increasing gastric proteolysis [39]. It was also shown that organic acids were important contributors to human large intestinal function [40]. Bhatnagar et al. demonstrated the role of citrate in the regulation of melanin biosynthesis in B16/F10 murine melanoma cells [41].

Their results unequivocally showed that citrate induced melanin biosynthesis by stimulating the tyrosinase activity in B16/F10 murine melanoma cells.

The melanin extracted from each sample filtered on Whatman paper by vacuum filtration was examined (**Figure 3B):** panel A represents control, B the presence of citric acid, C presence of laccase and, D that of laccase and citric acid together. Compared with other conditions, citric acid and laccase together in yeast growth medium showed synergistic effect for 10 days. The quantity of melanin produced by the cells was significantly higher, with p values between 0.0005 and 0.005 (**Figure 3C; Table 1).**

The existing reports demonstrate that intracellular fungal laccase possess the ability to biosynthesize melanin and that mutant yeast cells with higher laccase activity produced increased quantity of melanin [26, 36]. To the best of our knowledge, this is the first study of its kind that showed that adding laccase exogenously induced melanin production in yeast cells and human intestinal cells (**Figure 5**) and that together with citric acid, the cells showed strong synergistic effect on melanin production in yeast (**Figure 3B and C; Table 1).** Thus, laccase and citric acid together as supplements, albeit after formulation, could mimic and/or supplement melatonin driven melanin production abating dual requirement of melatonin and antibiotics.

Concluding remarks

The extracellular presence of laccase and citric acid in the growth media for yeast and human colon cells induced intracellular production of melanin (statistically significant after 8 days in yeast cells, p-values: 0.02-0.003; Table 2). The observed phenomenon occurred in a melatonin-independent manner. The melanin exhibited antimicrobial properties similar to commonly used antibiotics. As a supplement, through their antioxidant and antibacterial properties, laccase and citric acid may mitigate the problem of bacterial antibiotic resistance that is coupled with adverse health and environmental impacts. This could have possible use

in humans as well as in animal feed and could lead to possible reduction in the use of antimicrobials.

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Conflicts of interest

The authors have declared no conflicts of interest.

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Table 1. Statistical analysis of cell survival (n=10), melanin production per total yeast cells (n=10) and melanin production per cell (n=10) in different conditions during melanin test: (A) cells only (control), (B) cells in the presence of citric acid, (C) cells in the presence of laccase, (D) cells in the presence of citric acid and laccase

Analysis of Variance (ANOVA) by Bonferroni's multiple comparisons test using Prism software.

p- values

Parameters tested	**A:B**	**A:C**	**A:D**	**B:C**	**B:D**	**C:D**
Cell survival	0.9999 (ns)*	0.009	0.0078	0.2904 (ns)*	0.2406 (ns)*	0.9999 (ns)*
Melanin production per total cell number	0.99 (ns)*	0.1965 (ns)*	0.1991 (ns)*	0.4131 (ns)*	0.4108 (ns)*	0.9999 (ns)*
Melanin production per cell	0.9999 (ns)*	0.0002	0.0001	0.0016	0.0001	0.4749 (ns)*

*ns: statistically non-significant

Table 2. Statistical analysis of melanin production per yeast cells (n=3) in the presence of citric acid and laccase in different time period (2-10 days)

Analysis of student's t- test **p values**									
2d*:4d	**2d:6d**	**2d:8d**	**2d:10d**	**4d:6d**	**4d:8d**	**4d:10d**	**6d:8d**	**6d:10d**	**8d:10d**
0.44 (ns)**	0.11 (ns)**	0.0003	0.0009	0.04	0.008	0.004	0.02	0.02	0.09 (ns)**

*d=days

**ns:statistically non-significant

Table 3. Physicochemical characterization of the melanization substrate at T_0 and T_{12}

Physico-chemical characteristics	**T=0**	**T=12 days**
pH	4.78±0.36	3.57±0.19
Total solids (mg/mL)	0.021±0.001	0.029±0.03
Total volatile solids (mg/mL)	0.005±0.001	0.006±0.000
Total reducing sugars (mg/mL)	0.21±0.05	0.08±0.01
Total protein (mg/mL)	0.75±0.04	1.24±0.31
Total phenolics(mg/mL)	1.49±0.41	0.45±0.03
O.D 600	0.21±0.01	1.63±0.11

Figure 1: Laccase production by *T. versicolor* in solid-state fermentation using apple pomace as substrate. The graph represents mean values ±SD (n=6).

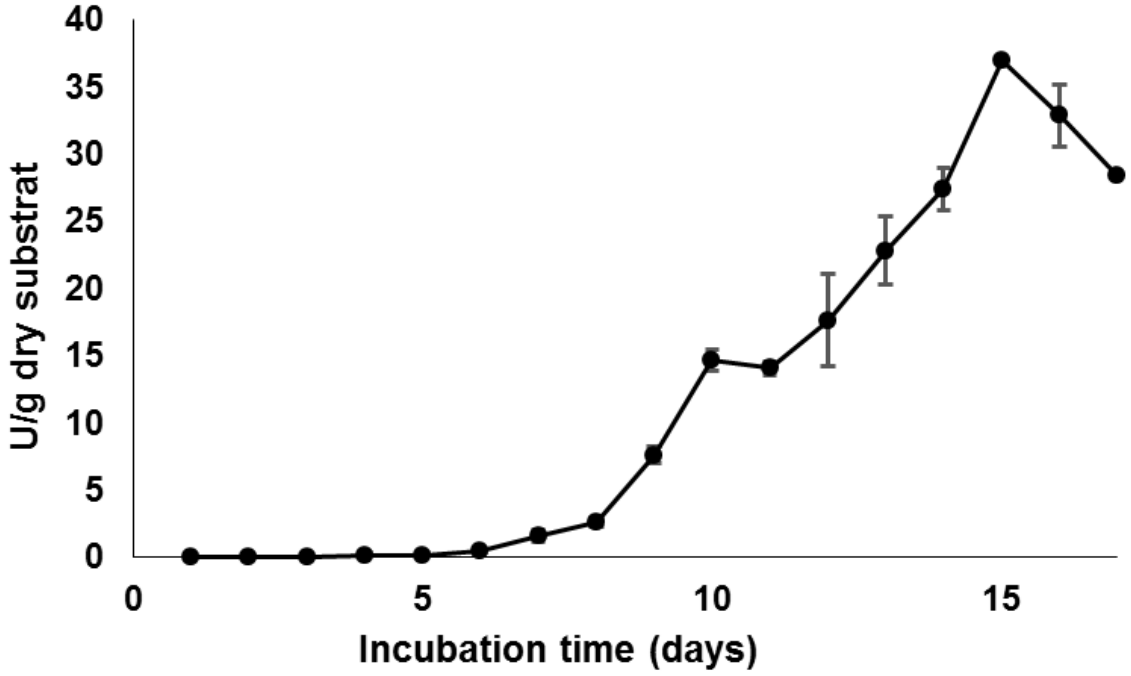

Figure 2: Citric acid production by *A. niger* in solid state fermentation using apple pomace as substrate. The graph represent mean values ±SD (n=6).

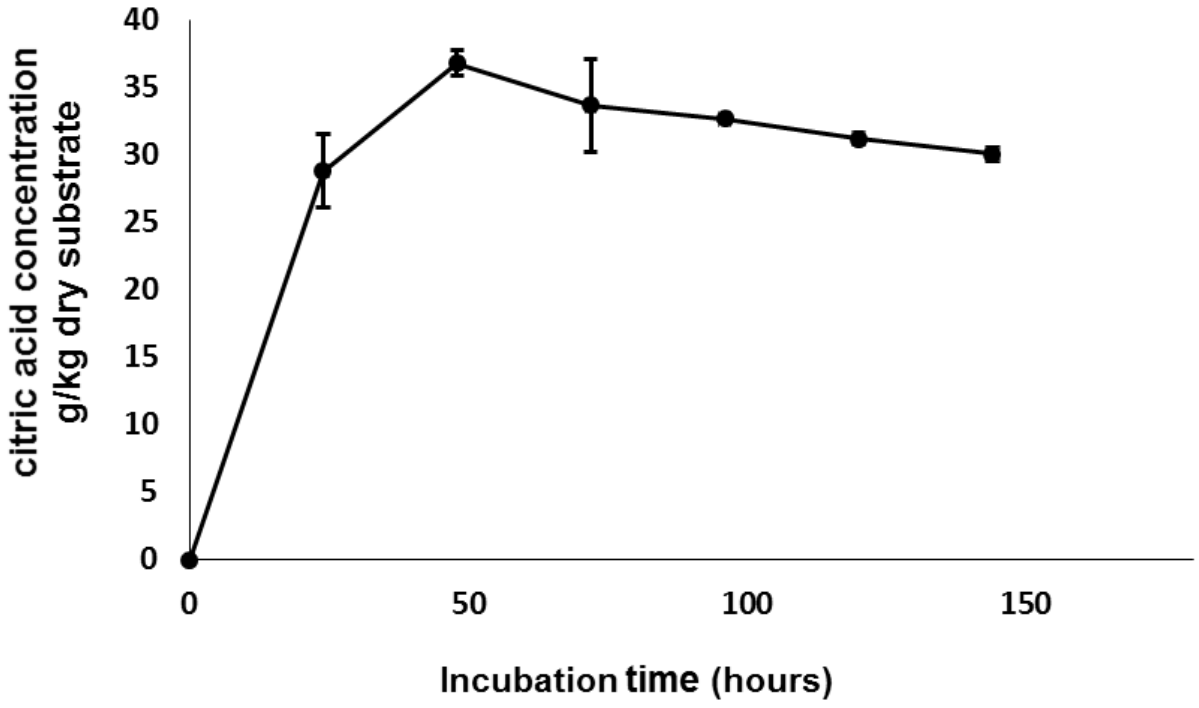

Figure 3A: Total yeast cell count after 10 days of incubation under different test conditions. The graph shows the mean values ±SD (n=20).

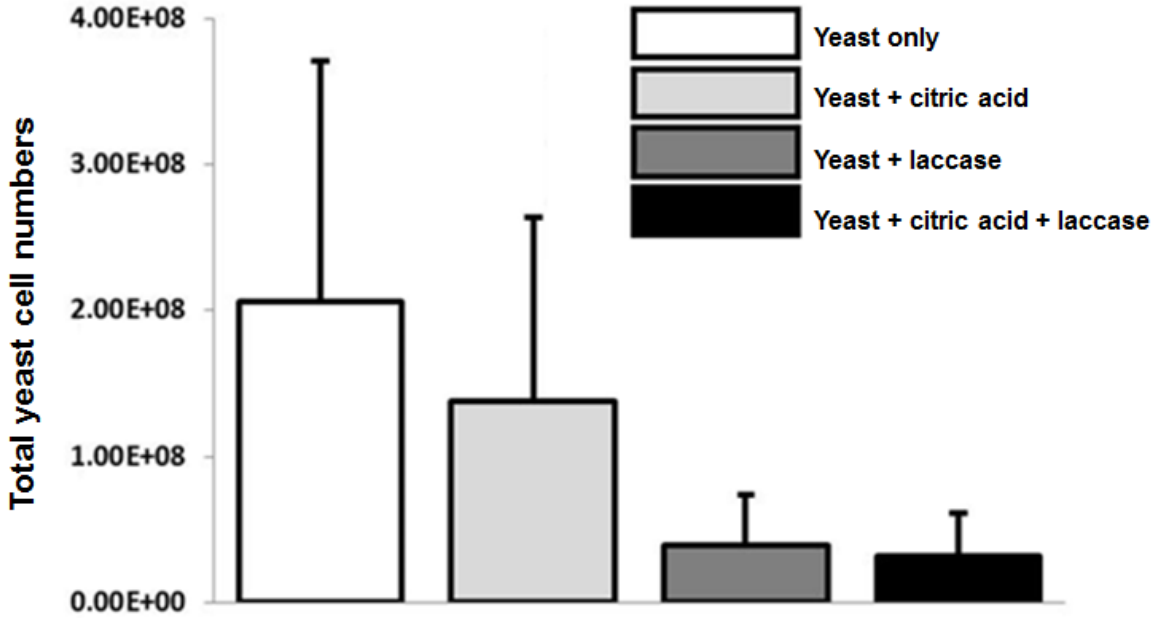

Figure 3B: Melanin production by: (A) cells only (control), (B) cells in presence of citric acid, (C) cells in presence of laccase, (D) cells in presence of citric acid and laccase. The photograph represents one out of 20 experiments.

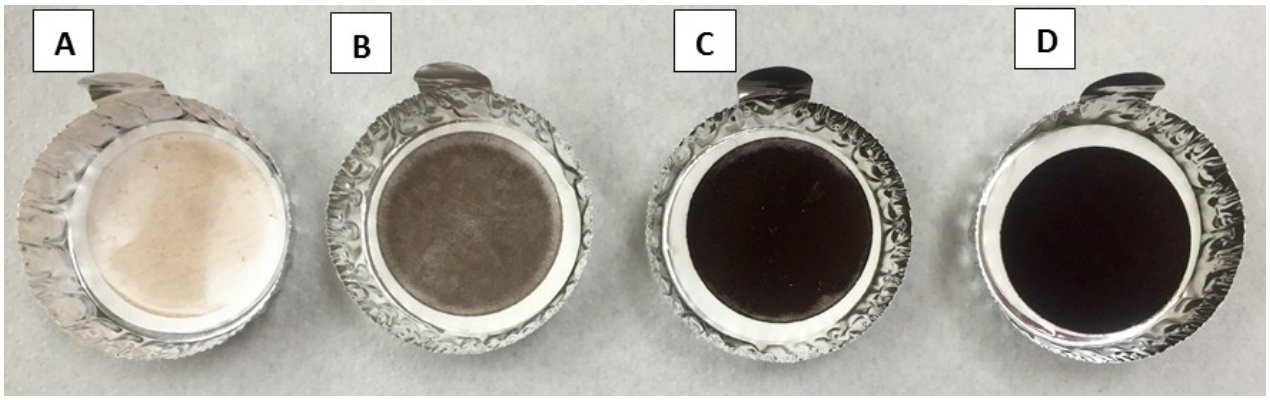

Figure 3C: Melanin production per yeast cell after 10 days of incubation under different test conditions. The graph shows the mean values ±SD (n=20).

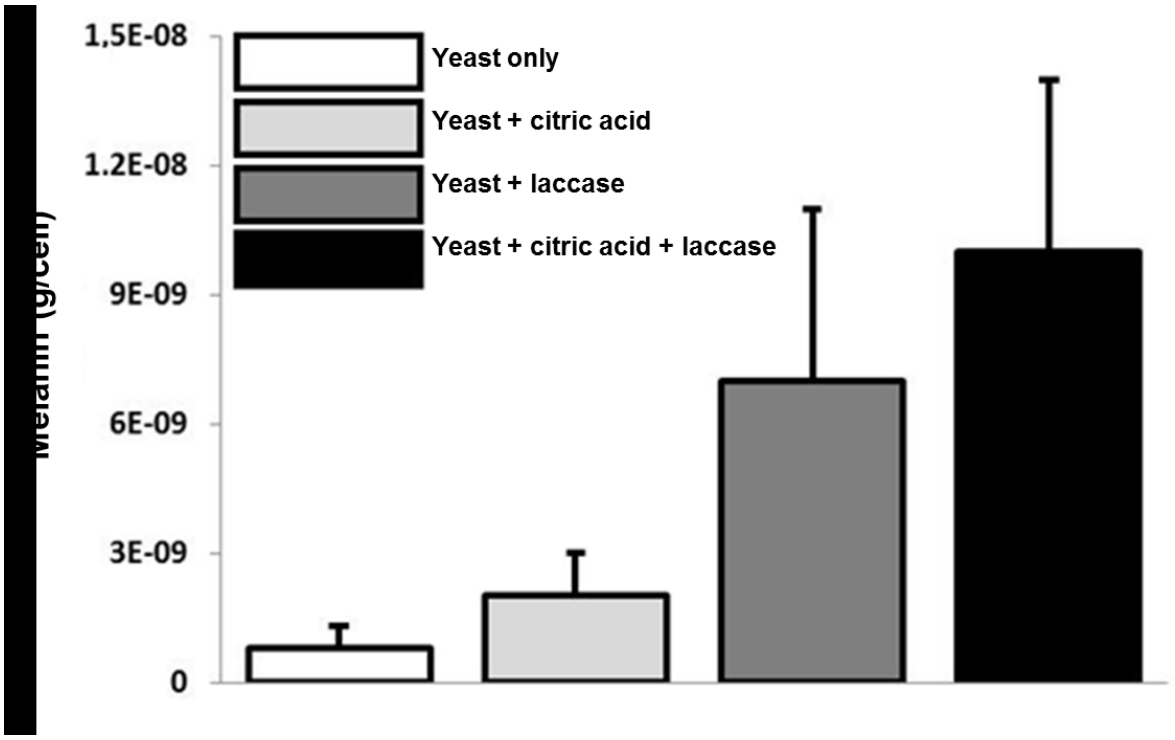

Figure 4: Melanin production kinetics by yeast cells in the presence of citric acid and laccase. The graph represents one out of 20 experiments

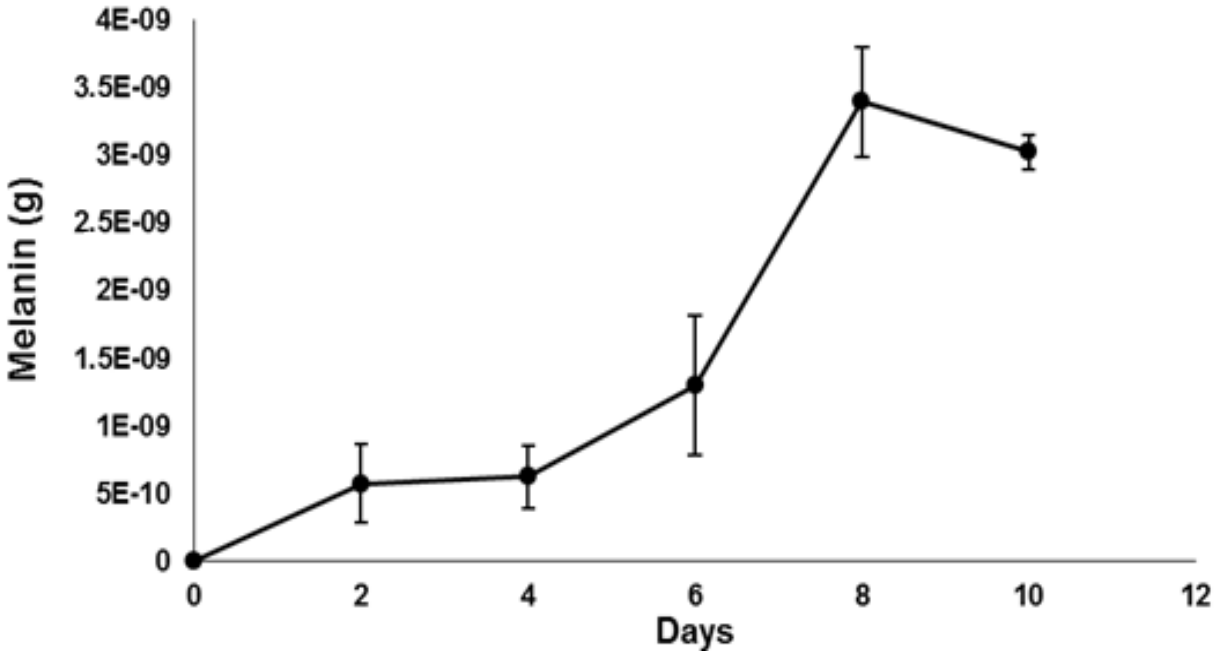

Figure 5: Melanin production by human colon carcinoma cells (Caco-2/15 and HT-29) after 3 days of incubation by addition of laccase and citric acid. The graph shows the mean values ±SD (n=3).

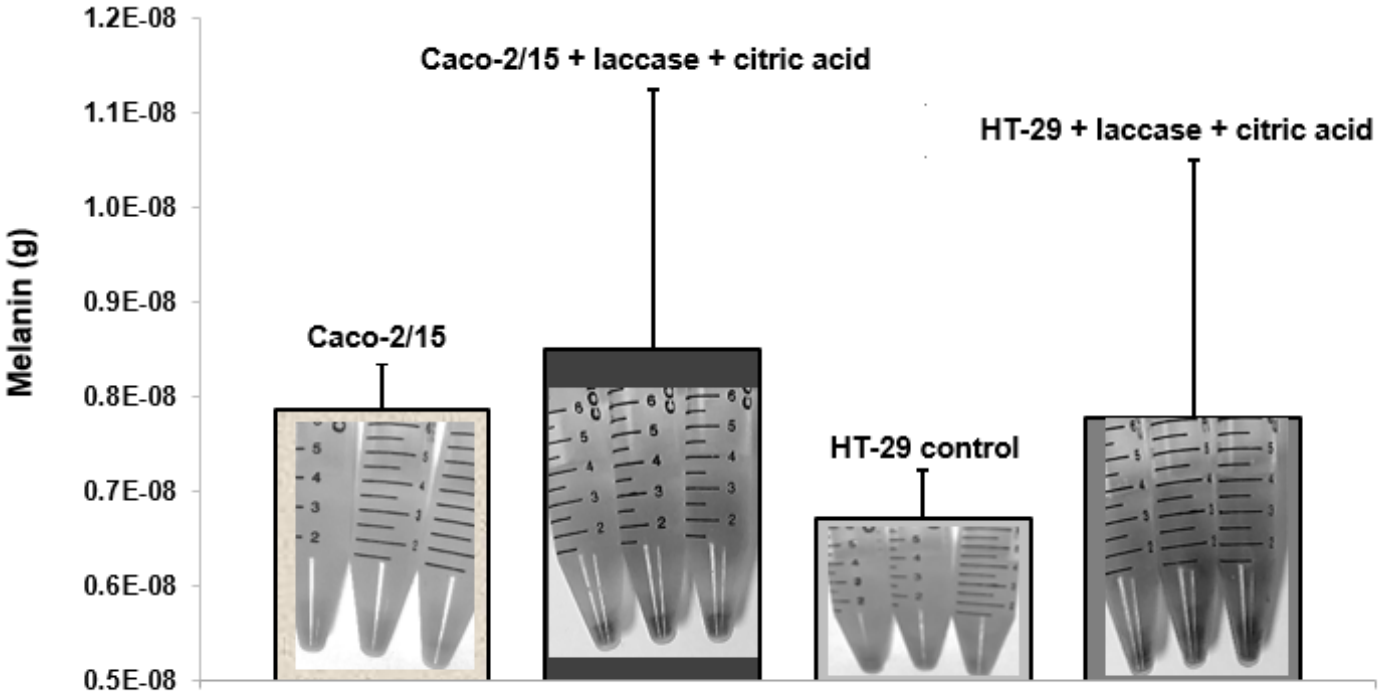

Figure 6: FTIR spectrum comparison between melanin standard and melanin produced by yeast cells.

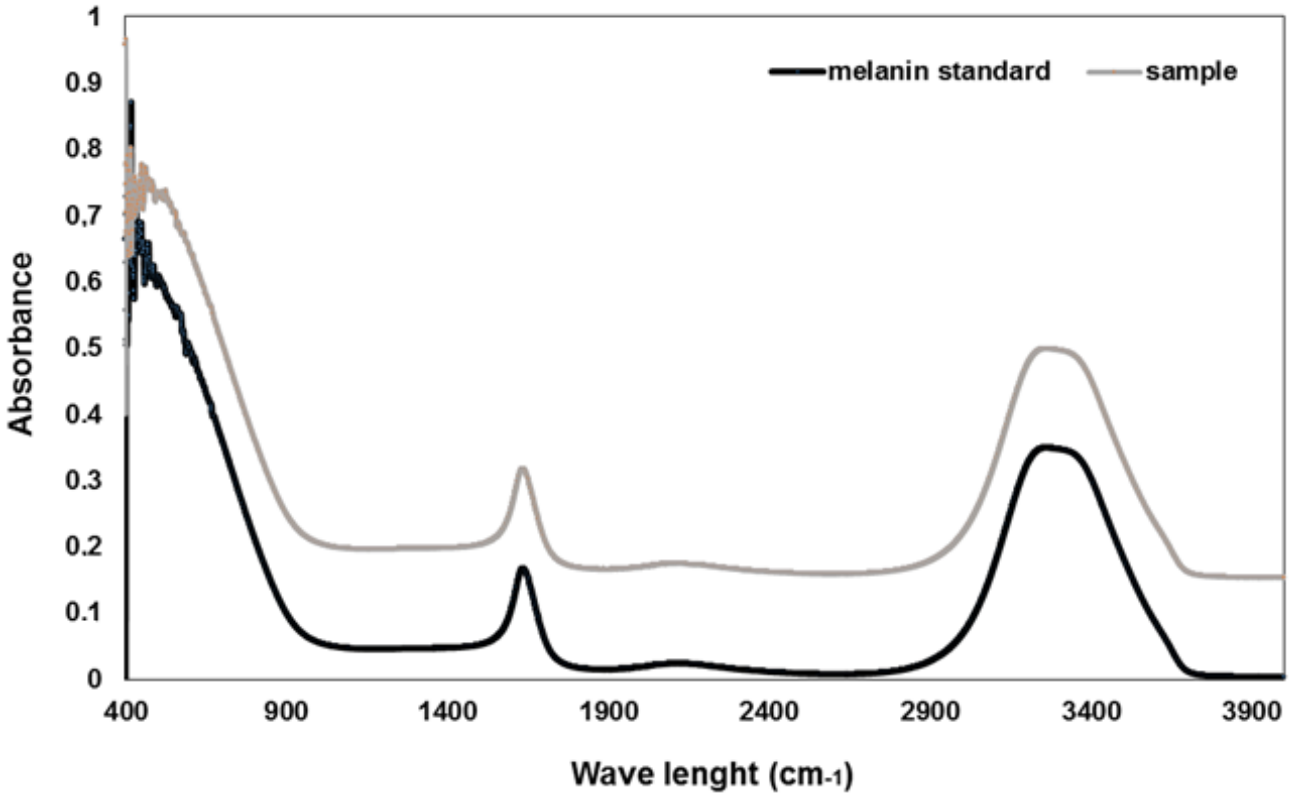

Figure 7: Well diffusion test for the melanin produced during the melanization experiment.

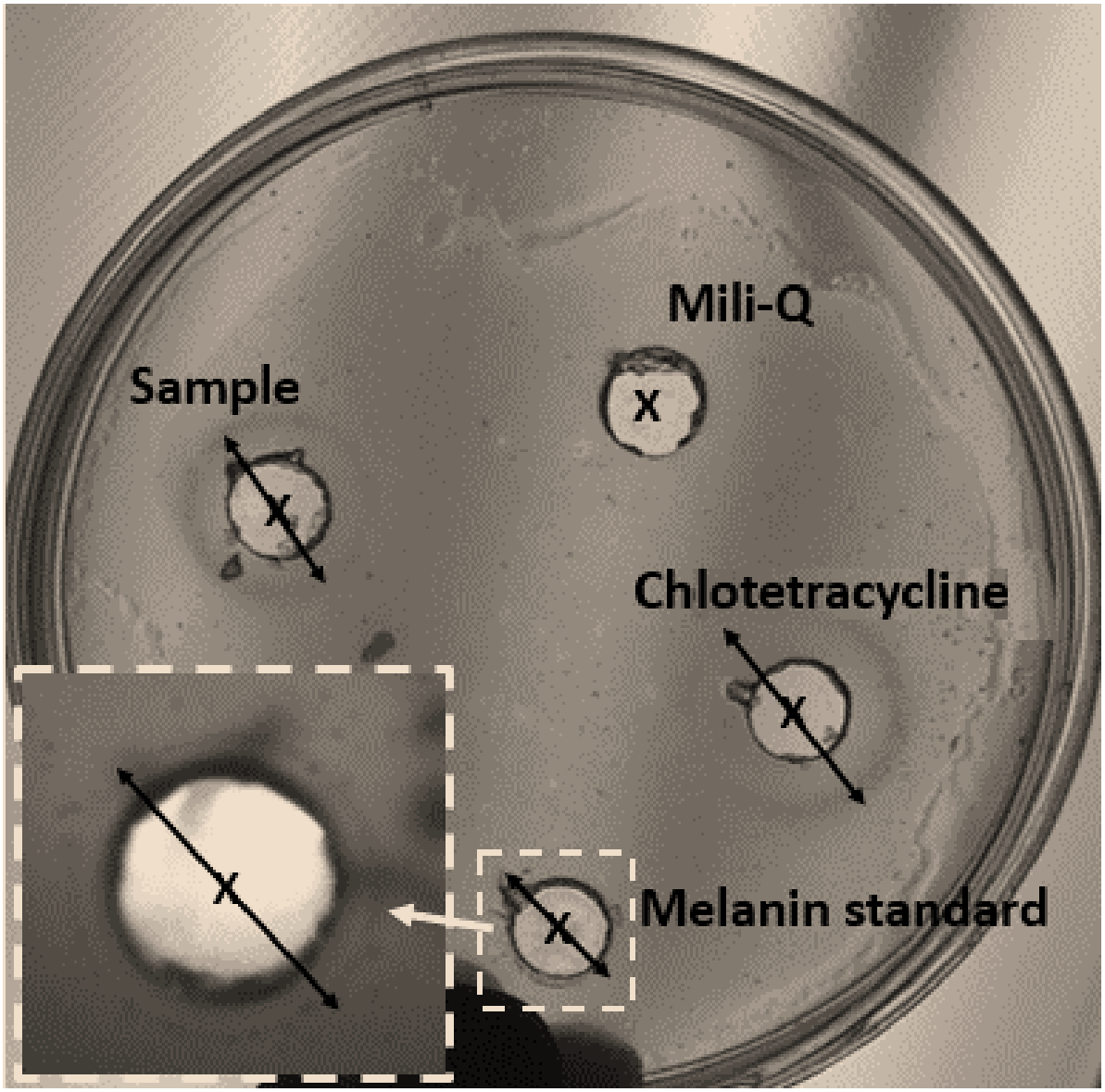

Figure 7