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Orange processing waste valorisation for the production of bio-based pigments using the fungal strains *Monascus purpureus* and *Penicillium purpurogenum*

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ABSTRACT

Orange processing wastes have been evaluated for the production of pigments by the fungal strains *Monascus purpureus* ATCC 16365 and *Penicillium purpurogenum* CBS 113139. Solid state fermentations were initially conducted on waste orange peels with the fungal strain *M. purpureus* aiming to assess various pigment extraction methods, the effect of particle size as well as the effect of nitrogen addition and incubation time. Under the optimum conditions, solid state fermentations were also performed with the strain *P. purpuronegum* to evaluate pigment production on waste orange peels. *M. purpureus* was more efficient than *P. purpurogenum* for the production of pigments during solid state fermentation, yielding 9 absorbance units (AU) per g of dry fermented substrate. Semi-solid state fermentations were subsequently conducted by suspending waste orange peels in liquid media leading to pigment production of up to 0.95 AU mL^{-1}. Submerged fermentations were carried out with both fungal strains using aqueous extracts from either boiled or hydrodistilled orange peel residues as the sole fermentation medium showing that up to 0.58 AU mL^{-1} of pigment production could be achieved. Each fermentation feedstock and fermentation mode influenced significantly the production of pigments by each fungal strain used.

Keywords: pigment, fungi, food waste, orange peel residues, bioconversion

1. INTRODUCTION

The market demand for natural pigments is gradually increasing due to increasing consumer demand reflecting their concerns over artificial pigments. The production of natural pigments can be achieved either via extraction from plants (e.g. carotenoids, anthocyanins and chlorophylls) or by fermentative synthesis through the cultivation of fungi, yeast and algae (e.g. phycocyanins, xanthophylls) (Mapari et al., 2010; Dufosse et al., 2014). However, the high production cost and the limited stability of natural pigments compared to the synthetic ones hinder the industrial implementation of bio-based pigment production via fermentation (Dufosse et al., 2014; Panesar et al. 2015).

Filamentous fungi produce natural pigments as secondary metabolites with a wide range of colours and diversified chemical structures (Mapari et al., 2010). On top of that, the utilisation of fungal strains for pigment production provides significant flexibility as the seasonal cultivation of plants for natural pigment production is avoided. Industrial production of bio-based pigments in Asian countries is dominated by strains belonging to the *Monascus* genus. *Monascus* pigments are secondary metabolites including yellow (monascin and ankaflavin), orange (rubropunctatin and monascorubin) and red (rubropunctamine and monascorubramine) constituents, the production of which is dependent mainly on fermentation process parameters. Besides pigments, other major metabolites are also synthesized by *Monascus* strains, including lovastatin (monakolin K), γ-aminobutyric acid, dimerumic acid and citrinin (a hepato-neuro-toxic mycotoxin) (Zhang et al., 2015; Chen et al., 2015). Red and yellow pigments from *Monascus sp*. have been widely implemented in food applications in the form of fermented mould rice, red koji and Anka among others (Feng et al., 2012; Panesar et al., 2015). However, the potential production of citrinin by *Monascus* strains prevents the commercialisation of *Monascus* pigments in the European Union and the USA. Genetic manipulation and chemotaxonomic selection of non-citrinin producing fungal strains could minimise toxin synthesis. In recent years, it has been reported that *Penicillium* strains can synthesize *Monascus*-like azaphilone pigments, in particular red and yellow pigment, in the

absence of mycotoxins (Mapari et al., 2010; Dufosse et al., 2014). Frisvad et al. (2013) presented the capability of red pigment production and the classification of various *Penicillium* strains, previously classified as *Talaromyces*, without toxin production.

Worldwide citrus cultivation amounted to more than 110 million t, including orange, lemon/lime and grapefruit, while specifically fresh orange production was estimated at 45.8 million t (www.fas.usda.gov). Approximately 27% of the overall citrus production is used for juice production (Choi et al., 2015). The orange processing industry generates considerable amounts of by-product streams that can reach up to 50% of the initial weight, composed of orange peels, pulp and seeds (Balu et al., 2012). Thereof, an approximate production of 13.7 million t of waste orange peels (WOP) could be estimated. Orange processing waste is composed of significant quantities of soluble sugars, cellulose, hemicellulose, pectin and essential oils that could be extracted as value-added products (Rezzadori et al., 2012). Essential oils are predominantly volatile compounds (85-90%), while limonene, a cyclic monoterpene, constitutes the main component (Negro et al., 2016). The sugars, polysaccharides and protein contained in the orange processing waste could provide nutrient sources for microbial fermentations (Choi et al., 2015).

The current application of solid residues derived from citrus processing is directed as cattle feed or for bioenergy production (Rezzadori et al., 2012). Driven by the directive EU 1998/2008/EC that inaugurates treatment and recovery processes from waste residues prior to disposal in landfills, recent research is focussed on the chemical or biotechnological valorisation of orange processing waste to generate diversified end-products (Negro et al., 2016). These include D-limonene, pectin, hydrolytic enzymes, dietary fibers, bio-oil and bio-ethanol among others, targeting different market outlets such as the food and feed industry, cosmetics and pharmaceuticals among others (Rezzadori et al., 2012; Choi et al., 2015). For instance, ethanol production from citrus waste begins with enzymatic hydrolysis using commercial enzymes to generate C5 and C6 sugars that are subsequently fermented by yeast for the production of ethanol (Choi et al., 2015). The hydrolysis and fermentation stages can be carried out separately or simultaneously (Widmer et al., 2010).

The aim of the present study was the valorisation of industrial orange processing waste as the sole nutrient source in the fermentative production of natural pigments by *Monascus purpureus* ATCC 16365 and the chemo-taxonomically selected non-toxigenic strain *Penicillium purpurogenum* CBS 113139 (formerly known as *Talaromyces atroroseus*) implementing different fermentation strategies, including solid state fermentation, semi-solid state fermentation and submerged cultivation. The ability of pigment production by *P. purpurogenum* was compared with *M. purpureus*. Furthermore, various pigment extraction methods were assessed. The total phenolic content (expressed as mg of ellagic acid equivalents) and the antioxidant activity of the produced pigments were evaluated. This study demonstrates that the non-toxigenic strain *P. purpurogenum* can produce *Monascus*-like pigments using agri-industrial side streams, such as orange peel residues, as the sole fermentation feedstock.

2. MATERIALS AND METHODS

2.1. Microorganisms

Microbial bioconversions for pigment production were performed using two different fungal strains. The strain *Monsacus purpureus* ATCC 16365 was maintained at 4 °C in petri dishes containing potato dextrose agar (PDA), whereas the strain *Penicillium purpurogenum* CBS 11339 was maintained at 4 °C in petri dishes containing Czapek-Dox medium (in g L^{-1}: agar 15.0; sucrose 30.0; $NaNO_3$ 3.0; KCl 0.5; $FeSO_4 \cdot 7H_2O$ 0.01; $MgSO_4 \cdot 7H_2O$ 0.5; K_2HPO_4 1.0). Fermentation inocula preparation was carried out in Erlenmeyer flasks (250 mL) in order to increase the fungal spore concentration. Briefly, 5 mL of sterilized tap water and Tween 80 (0.01%, v v^{-1}) were added in each petri dish and the surface was scratched with a wire loop. Subsequently, 1 mL of spore suspension was transferred from the petri dishes on the solid medium of the Erlenmeyer flasks that were incubated for 5 days. The fungal spore suspension that was used as fermentation inocula was prepared by adding 50 mL of distilled and sterilized water with Tween 80 (0.01%, v/v) on the surface of the solid medium of each Erlenmeyer flask followed by addition of sterilized glass beads

(4 mm diameter) and shaking of the flask. The spore concentration in the inoculum suspension was 2.4×10^7 spores mL^{-1} and 12×10^7 spores mL^{-1} for *M. purpureus* and *P. purpurogenum*, respectively.

2.2. Raw material

WOP was collected from a local bakery shop. Orange albedo was manually removed and the peels were cut in large pieces (0.5 cm) and further dried at 60 °C. After drying, orange peels were macerated in smaller pieces using a conventional kitchen blender and separated in two different sizes (2-4 mm and <2 mm) using sieves. Wheat milling by-products (WMB) were employed as substrate in solid state fermentation (SSF) for the optimisation of pigment extraction method. The composition of WMB has been previously described by Tsakona et al. (2014). Sunflower meal (SFM) was a by-product of biodiesel production, kindly provided by P.N. Pettas S.A. industry (Patras, Greece). The composition of SFM has been previously reported by Kachrimanidou et al. (2013).

2.3. Solid state fermentation

SSF was initially carried out with the fungal strain *M. purpureus* ATCC 16365 on WOP to evaluate the effect of pigment extraction method, particle size and nitrogen source. A known quantity of WOP (5 g) was weighed and transferred in 250 mL Erlenmeyer flasks that were subsequently autoclaved at 121 °C for 20 min. Each flask was inoculated with a fungal spore suspension of the designated fungal strain, also used to adjust the initial moisture content of the substrate at 65% (w/w, on a dry basis) unless otherwise stated. Under the optimum conditions of *M. purpureus* growth, the strain *P. purpurogenum* was also evaluated in SSF for pigment production. All flasks were incubated at 25 °C and 30 °C in an orbital shaker (ZHWY-211C Series Floor Model Incubator, PR China) for each fungal strain, respectively. The content of a complete flask was used at regular intervals to estimate pigment production.

2.4. Semi-solid state fermentation

Semi-solid state fermentations (semi-SSF) were performed by dispersing WOP pieces (< 2 mm) in sterilised distilled water in order to provide a carrier substrate for fungal growth. Two different initial solid concentrations were evaluated, namely 20 and 50 g L^{-1} (on a wet basis, wb). A specific quantity of WOP was weighed and dispersed in distilled water in Erlenmeyer flasks (250 mL) followed by autoclaving at 121 °C for 20 min. Subsequently, the flasks were inoculated with 2 mL of freshly prepared fungal spore suspension, achieving 50 mL of final working volume. Incubation of the flasks was performed for 16 days in rotary shaker under agitation (180 rpm) at 30 °C and 25 °C for *M. purpureus* and *P. purpurogenum*, respectively. Flasks were periodically used to measure substrate consumption, total dry weight (TDW) and pigment production. In the case of semi-SSF, the TDW corresponds to the total dry solids that are present in the flask. The pH value was manually adjusted at the beginning of fermentation in the range of 5.6 – 6.2 for both fungal strains.

2.5. Submerged fermentation

Hydrodistillation and conventional boiling were implemented as thermal processes to remove essential oils contained in the orange peel, comprised mainly of D-limonene. The liquid stillage obtained after each thermal treatment was also evaluated as the sole nutrient supplement in submerged fungal fermentations, performed in 250 mL Erlenmeyer flasks. The stillage provided the sole nutrient supplement in each experiment without the addition of conventional inorganic nutrient sources. Each flask was inoculated with 2 mL of fungal spore suspension, while the final fermentation volume was 50 mL. Boiling of WOP was carried out by mixing 100 g of WOP with 600 mL of distilled water for 1 h at 100 °C in 1 L Duran bottles. Hydrodistillation of WOP was performed by mixing 50 g of WOP and 300 mL of distilled water in a Clevenger apparatus for 3 h based on a slightly modified method reported by Gardeli et al. (2008). The experimental setup followed during this study is illustrated in Figure 1.

2.6. Pigment extraction and estimation

A variety of pigment extraction protocols were applied aiming to optimise the extraction of pigments. WMB were initially employed as a substrate in SSF to evaluate the effect of organic solvent (70% and 95% v v^{-1} aqueous ethanol solutions and isopropanol) along with the utilisation of wet or dry fermented solids. Drying of SSF solids was carried out at 60°C. Subsequently, these methods were evaluated for pigment extraction during SSF conducted with WOP of different particle sizes.

During the following experiments, pigment extraction was based on the method previously reported by Nimnoi and Lumyong (2011) with slight modifications. In particular, a known quantity of fermented solid material (5 g on wet basis) was mixed with 50 mL of 70% (v/v) aqueous ethanol solution and the mixture was placed in an ultrasonic bath for 30 min at 25 °C. Subsequently, pigments were extracted in a rotary shaker at 30 °C and 180 rpm for 1 h. The ethanolic extracts were centrifuged (9000 rpm, 20 min) and the supernatant was used for pigment estimation by measuring the absorbance at three different wavelengths (400, 475 and 500 nm). For each parameter tested, a flask with unfermented substrate was used as the blank for pigment assessment. The unfermented substrate was subjected to sterilisation, but inoculation was not carried out. Pigment production was expressed as absorbance units per g of dry fermented substrate (AU $gdfs^{-1}$).

During semi-SSF and submerged fermentation extracellular pigment production was evaluated in the liquid phase after centrifugation of each fermentation sample. Intracellular pigment production was assessed in the fungal biomass obtained after sieving and extraction with 50 mL of 70% (v/v) aqueous ethanol solution. Similarly, the mixture was placed in an ultrasonic bath for 30 min followed by extraction in a rotary shaker at 30 °C and 180 rpm for 1 h. Pigment production during semi-SSF and submerged cultures was expressed as absorbance units per mL (AU mL^{-1}) including both intra- and extracellular pigment production.

2.7. Analytical techniques

Total dry weight (TDW) of fungal biomass and other fermentations containing solids with fungal biomass was determined by drying the samples at 60 °C and cooling down in a desiccator until a constant weight was obtained.

Yellow, orange and red pigments were determined by measuring the absorbance in three different wavelengths, namely 400 nm, 475 nm and 500 nm, respectively. Blank samples were used in every case depending on the experiment carried out. The total pigment production was calculated using the pigment production measured in the three wavelengths and was expressed as absorbance units (AU).

Glucose, fructose and sucrose were quantified by High Performance Liquid Chromatography (HPLC, Waters 600E) equipped with an Aminex HPX-87H (300 mm x 7.8 mm, Bio Rad, CA) column, coupled to a differential refractometer (RI Waters 410). Operating conditions were as follows: sample volume 20 µl; mobile phase 10 mM H_2SO_4; flow rate 0.6 ml min^{-1}; column temperature 65 °C. Prior to analysis, samples were diluted to appropriate concentration with deionised water and filtered through a 0.2 µm membrane filter.

Consumption of free amino nitrogen (FAN) during fermentation was determined according to the ninhydrin colorimetric method (Lie et al., 1973) promulgated in the European Brewery Convention.

The total phenolic content (TPC) was estimated by the Folin-Ciocalteu colorimetric method based on the protocol reported by Singh et al. (2002) using ellagic acid (EA) as the standard phenolic compound. TPC was expressed as EA equivalents (mg EAE per mL of extracts). The free radical-scavenging activity of the extracted pigments was assayed using the modified DPPH˙ (1,1-diphenyl-2picrylhydrazil radical) method based on the estimation of the reducing ability of antioxidants against the DPPH˙ radical, according to the method described by Moon et al. (1998). Briefly, 1 mL ethanol solution (97% v/v) was mixed with 950 µL of Tris-HCl (0.05 M, pH=7.4), 1 mL DPPH˙ (0.01 mM) and 50 µL of sample. The radical scavenging activities of the extracts were expressed as percentage inhibition of DPPH˙ using the following equation:

$$\% \, Inhibition = \left[\frac{A_B - A_A}{A_B}\right] * 100$$

where A_B and A_A correspond to the absorbance values of the control and the sample, respectively (Gardeli et al., 2008).

3. RESULTS AND DISCUSSION

3.1. Optimisation of pigment extraction protocol

Fungal polyketide pigments are secreted usually as secondary or late stationary phase metabolites (Mapari et al., 2010; Kim et al., 2006). Their chemical structure is a key parameter related to the pigment extraction method, affecting also their solubility in water or organic solvents. Previous literature cited publications have reported various methodologies for pigment extraction based predominantly on the utilisation of organic solvents (e.g. ethanol, methanol). Therefore, preliminary experiments focused on the optimisation of pigment extraction from both wet and dry fermented solids. Three different solutions, specifically 70% and 95% (v v^{-1}) aqueous ethanol solutions and isopropanol, were assessed for pigment extraction using WMB as the sole nutrient substrate in SSF. Treatment in an ultrasonic bath was applied followed by extraction in a rotary shaker. Table 1 presents the effect of organic solvent on pigment extraction from wet and dry fermented solids after SSF using *M. purpureus* cultivated on WMB. WMB were utilised because they have been widely evaluated as substrate in SSF processes, providing all the essential nutrients for the proliferation of filamentous fungi (Tsakona et al., 2014; Domínguez-Espinosa and Webb, 2003). Application of aqueous ethanol solutions enhanced pigment extraction as compared to isopropanol. Dry fermented solids resulted in significantly lower extraction yields than wet fermented solids. This could be attributed to the thermal instability of natural pigments, especially at temperatures higher than 60 °C (Silveira et al., 2013). The increased pigment extraction achieved with ethanol as compared to isopropanol could be attributed to its highly polar nature (Carvalho et

al., 2005). Carvalho et al. (2005) reported that ethanol performs almost equally to methanol in terms of pigment extraction efficiency.

The optimum pigment extraction method based on the use of aqueous ethanol suspensions was subsequently evaluated for pigment extraction during SSF carried out with WOP using two different solid particle sizes (Table 1). During this experiment, isopropanol was not assayed based on the previous low pigment extraction performance. Pigment extraction with 70% and 95% aqueous ethanol suspension was evaluated in parallel with incubation time although the results demonstrated in Table 1 correspond to the maximum pigment extraction achieved. The same procedure followed for pigment extraction was also applied in non-fermented substrate in order to ensure that the extracted pigment was produced entirely during fungal fermentation. The 70% (v v^{-1}) aqueous ethanol suspension led to the highest pigment extraction efficiency. The pigment extraction was measured in three different wavelengths corresponding to yellow (400 nm), orange (475 nm) and red (500 nm) pigments using wet or dry fermented WOP.

3.2. Solid state fermentation of WOP

Pigment production by the fungal strains *M. puprureus* ATCC 16365 and *P. purpurogenum* CBS 113139 was evaluated via SSF, semi-SSF and submerged cultures in order to elucidate the mode of operation that leads to high pigment production efficiency. Pigment production by *M. purpureus* cultivated in SSF on renewable resources has been successfully demonstrated in literature-cited publications (Velmurugan et al., 2011; Babitha et al., 2007; Nimnoi and Lumyong 2011). In most cases, the solid substrates were supplemented with minerals to support fungal growth. The fungal strain *M. purpureus* secretes citrinin and for this reason *P. purpurogenum* was also evaluated in this study for the production of *Monascus*-like pigments without the production of toxins that hinder further commercialisation.

During the first experimental setup, SSF of *M. purpureus* using WOP was carried out. Based on preliminary experiments (data not shown) implementing a range of initial moisture contents (60-80

%, w w^{-1}) maximum pigment production was observed after 10 days, whereas an initial moisture content of 65% (w w^{-1}) exhibited optimum pigment production. The effect of WOP particle size and the supplementation with nitrogen source on pigment synthesis was subsequently evaluated. Figure 2 illustrates the effect of two WOP particle sizes (2 - 4 mm and < 2 mm) on pigment production during SSF with *M. purpureus* ATCC 16365. The smallest particle size (< 2 mm) exhibited higher pigment synthesis, particularly in the case of the yellow pigment (9 AU $gdfs^{-1}$). Velmurugan et al. (2011) achieved a pigment production of 25.42 AU $gdfs^{-1}$ when the fungal strain *M. purpureus* KACC 42430 was cultivated in SSF of corn cob powder under the optimum cultivation conditions. SSF cultures of *M. purpureus* cultivated on corn and sugar cane bagasse showed higher pigment production (0.59 AU $gdfs^{-1}$) than submerged cultures (Rajeswari et al., 2014). The same trend was also observed when SSF of *P. purpurogenum* CBS 113139 was carried out using different particle sizes of WOP (data not shown). In the case of pigment synthesis by SSF of *P. purpurogenum* cultivated on WOP, both red and orange pigment production was significantly lower (0.05 AU $gdfs^{-1}$) than *M. purpureus* cultures. Figure 3 presents the orange and red pigment production during SSF of *P. purpurogenum* CBS 113139 carried out on WOP with particle sizes of less than 2 mm. The production of yellow pigment was low in the case of SSF of *P. purpurogenum*.

Supplementation of the fermentation substrate with nitrogen sources influences pigment production efficiency (Subhasree et al., 2011; Feng et al., 2012). It has been demonstrated that organic nitrogen shifts the metabolism to biomass production, while inorganic sources of nitrogen are beneficial for pigment synthesis (Carels and Shepherd, 1977; Babitha et al., 2006). This could be attributed to the fact that the final steps in the metabolic pathway of *Monascus* sp. for pigment production involve a condensation or amination reaction that converts monascorubrin and rubropunctatin (orange pigment) to monascorubramine and rubropunctamine (red pigment), respectively (Chen et al., 2015).

In this study, experiments were carried out combining SFM in various ratios with WOP (0%, 20% and 40% w w^{-1}). SFM was selected because it is rich in protein and it is readily available by

biodiesel producers in several EU countries (Kachrimanidou et al., 2013). Maximum red pigment production by *M. purpureus* was observed after 10 days of fermentation when WOP was used as the sole substrate, whereas supplementation with 40% (w w^{-1}) SFM led to enhanced red pigment synthesis after 14 days of cultivation. The enhanced pigment production observed after the addition of SFM could be attributed to the absence of nitrogen and other nutrients from WOP that seem essential for efficient fungal growth and pigment production. Similar results were obtained during the cultivation of *P. purpurogenum*, where red pigment production increased with SFM addition. Padmavathi et al. (2013) evaluated the effect of the addition of different carbon and nitrogen sources on pigment production by *M. purpureus* during SSF of orange peels, showing that the addition of fructose led to higher pigment synthesis (1.47 AU $gdfs^{-1}$) than the case that only orange peels (0.65 AU $gdfs^{-1}$) were used. Babitha et al. (2006) reported that the addition of monosodium glutamate in jackfruit seed SSF cultures of *M. purpureus* improved the production of red (30.8 AU $gdfs^{-1}$) and yellow (25.5 AU $gdfs^{-1}$) pigments, followed by the addition of soybean meal, peptone and chitin powder.

3.3. Semi-solid state fermentation on WOP

Semi-solid state fermentation was carried out in order to combine the advantages of SSF with enhanced accessibility of nutrients provided by the liquid phase (excess water) of submerged cultivations (Machado et al., 2013; Economou et al., 2010). In semi-solid state fermentations, pigment production was evaluated using two initial solid concentrations of WOP (20 and 50 g L^{-1} on a wet basis). Figure 4 shows the consumption of total sugars and FAN during semi-SSF performed with *M. purpureus* at an initial solid concentration of 20 g L^{-1} along with the production of pigments measured at three different wavelengths. The TDW corresponds to the total solids that were measured at any fermentation time in the flasks. Total sugar concentration at the beginning of fermentation was 5.5 g L^{-1}, while the initial FAN concentration was 13 mg L^{-1}. It should be stressed that the fungal strain may hydrolyse proteins and polysaccharides during fermentation generating

additional carbon and nitrogen sources. Pigment production was triggered within the first 2 days and it was increased at 12 days to 0.027, 0.023 and 0.02 AU mL^{-1} for yellow, orange and red pigments, respectively. After the depletion of nutrients in the fermentation broth at 8 days, the extracellular pigment production increased from 20% to 80% of the total pigments until 16 days.

Figure 5 presents the consumption of total sugars and FAN as well as the production of TDW (including WOP solids and fungal biomass) and pigments during semi-SSF of *P. purpurogenum* cultivated on 20 g L^{-1} of initial WOP concentration (on a wet basis). The initial total sugar and FAN concentrations were 5.32 g L^{-1} and 26 mg L^{-1}, respectively. The maximum red pigment production including both intra- and extracellular pigments reached 0.42 AU mL^{-1} at 12 days, which was observed when the TDW reached a plateau (around 15.3 g L^{-1}). The total pigment production achieved was around 0.95 AU mL^{-1}. The correlation of complete sugar and FAN consumption with pigment synthesis was more evident in the fermentation of *P. purpurogenum*. In a similar manner, intracellular pigment synthesis was higher than extracellular pigment production until the 10^{th} day followed by a decreasing trend that coincided with increased extracellular production of pigments.

3.4. Utilisation of WOP liquid stillages

3.4.1. Submerged fermentation with *M. purpureus* ATCC 16365

Submerged fermentations for pigment production were carried out using remaining aqueous extracts produced by boiling or hydrodistillation of WOP. Both processes removed the essential oils contained in orange peels. The extraction of D-limonene would result in the generation of a value-added co-product. Boiling of orange peels was initially selected on the basis of treating larger quantities of WOP based on the methodology reported by Li et al. (2010) that led to the removal of more than 90 % of essential oils and other volatile compounds. The remaining aqueous stillage contained mainly soluble free sugars extracted from the WOP, including glucose, fructose and sucrose. The application of hydrodistillation or boiling influenced the concentration of total sugars in the liquid stillage (data not shown). For instance, boiling of WOP led to a total sugar

concentration in the stillage of 8.7 g L^{-1}, while the application of WOP hydrodistillation increased the total sugar concentration to more than 20 g L^{-1}. The pH value of the WOP derived stillages at the beginning of submerged fermentations varied in the range of 5.7 - 6.2 for both fungal strains and remained almost stable throughout all fermentations. Figure 6 presents the profile change of total sugars and FAN consumption as well as TDW and pigment production during submerged cultures of *M. purpureus* on stillages derived via hydrodistillation of WOP. The initial total sugar and FAN concentrations were 21.1 g L^{-1} and 58.7 mg L^{-1}, respectively. The concentration of FAN in the fermentation medium was low that could be attributed to the low protein content of the peels. The red pigment production achieved was 0.19 AU mL^{-1} with a total pigment production of 0.58 AU mL^{-1} at 16 days. The fungal strain *M. purpureus* consumed initially glucose followed by fructose, while sucrose concentration remained relatively stable until the fermentation was terminated.

Monascus strains have been previously employed in fermentation processes using renewable resources as substrates for pigment production. Zhou et al. (2014) cultivated the strain *M. purpureus* CICC5041 in corncob hydrolysate under dark conditions in the presence of caprylic acid leading to the production 25.8 absorbance units measured at 500 nm per mL of broth with reduced citrinin production. Haque et al. (2016) employed bakery waste derived hydrolysates as nutrient-rich media for the production of bio-based pigments by *M. purpureus* where the highest pigment yield was achieved at low initial glucose concentration of 5 g L^{-1}.

3.4.2. Submerged fermentation with *P. purpurogenum* CBS 113139

Submerged cultivations of *P. purpurogenum* CBS 113139 were also carried out in order to evaluate the potential of this fungal strain for pigment production when liquid stillages produced via hydrodistillation or boiling of WOP were used. The stillage obtained after hydrodistillation for three hours contained 25 g L^{-1} of total sugars and approximately 40 mg L^{-1} FAN. Pigment production started within the fourth day of fermentation. The pigment production achieved was relatively low in all wavelengths, not exceeding 0.012 AU mL^{-1} even after 16 days, whereas the maximum total

dry weight reached 7.84 g L^{-1}. Thus, hydrodistillation of WOP led to a medium that did not stimulate pigment production.

The nutrient medium obtained after boiling of WOP was also assessed in submerged cultivations of *P. purpurogenum* CBS 113139 (Figure 7). Initial FAN concentration remained in the same range as the previous experiments (41.5 mg L^{-1}), while the concentration of total sugars achieved was 11.2 g L^{-1}. Optimum pigment production (0.34 AU mL^{-1}) was observed at the 8th day with the red pigment (0.14 AU mL^{-1}) being the predominant one, while the highest TDW (6.35 g L^{-1}) was also observed at the 8th day.

Fermentation processes employing *Penicillium purpurogenum* (previously classified as *Talaromyces atroroseus*) are emerging as an alternative for *Monascus*-like pigment generation due to the absence of mycotoxin based on chemotaxonomic analysis (Mapari et al., 2010; Frisvad et al., 2013). The optimisation of culture conditions is essential in order to maximise pigment production by *P. purpurogenum*. Méndez et al. (2011) used a conventional medium (Czapek-Dox) and xylose as carbon source to elucidate the coupled effect of pH and temperature on pigment production by *P. purpurogenum* GH2, with the highest production of red pigment (2.46 g L^{-1}) achieved at a pH value of 5 and 24°C. The effect of light during the cultivation of five fungal strains including *P. purpurogenum* has been assessed using conventional nutrient supplements, with red light leading to the highest extracellular and intracellular pigment production by *P. purpurogenum* (Velmurugan et al., 2010). Diversified carbon and nitrogen sources were implemented in the cultivation of *P. purpurogenum* DPUA 1275 to assess pigment production with sucrose and yeast extract being the most efficient carbon and nitrogen sources (Santos-Ebinuma et al., 2013). Santos-Ebinuma et al. (2013) reported that yellow pigment production was more profound at the beginning of fermentation followed by the synthesis of red and orange pigments, assuming thereof that the chemical structure of yellow pigments could be modified leading to the production of red and orange pigments, similar to *Monascus* biosynthetic pathway.

The present study demonstrates that WOP could be used as raw material for the production of *Monascus*-like pigments by *P. purpurogenum.* However, further process optimisation is required in order to identify the process parameters that lead to high pigment production and therefore facilitate industrial implementation of this process. Furthermore, the development of sustainable processes using food waste as feedstock should rely on the principles of biomass refining leading to the production of diversified end-products with various market outlets.

3.5. Total phenolic content and antioxidant activity of the extracts

Monascus fermented products contain a variety of bioactive compounds that contribute beneficially in human health, including monakolin K (lovastatin), γ -aminobutyric acid, dimerumic acid and antioxidants (Yang et al., 2006; Lee et al., 2008). Table 2 presents the TPC and the antioxidant activity of the extracts obtained during selected fermentations carried out in this study when the highest pigment production was achieved. The highest TPC in each fermentation was observed at the highest pigment production. Solid state fermentation and semi-SSF of *M. purpureus* ATCC 16365 resulted in the highest values of TPC (123.7 and 114.7 mg EAE mL^{-1}, respectively). The highest TPC (74.7 mg EAE mL^{-1}) in the case of *P. purpurogenum* CBS 113139 was observed when submerged fermentation with liquid stillage from boiling of WOP was carried out. Free radical scavenging activity of the extracts was evaluated by $DPPH^{\cdot}$ method and expressed as % Inhibition (%I) (Table 2). All the extracts exhibited significant antioxidant activity whereas the highest antioxidant activity was obtained from the extracts deriving from semi-SSF of *M. purpureus* on 50 g L^{-1} of orange peel, thereof providing the potential of possible implementation as functional ingredients.

Literature-cited publications focus on the evaluation of the TPC and antioxidant activity of *Monascus* fermented products. Razak et al. (2015) reported that the TPC of fermented rice bran employing solid state fermentation of *M. purpureus* was increased, contrary to the DPPH radical-scavenging activity of the fermented samples that was insignificantly affected. Water extracts from

SSF of soybean with *Monascus* fungal strains showed that cold water extracts exhibited higher reducing power and lower scavenging ability on DPPH radicals than hot water extracts (Lee et al., 2008). Smith et al. (2015) demonstrated the antioxidant activity and total phenolic components of ten different fungal strains including *M. purpureus*, indicating that submerged fermentation exhibits a valuable approach for the production of antioxidants.

4. CONCLUSIONS

Orange processing wastes have been evaluated as nutrient-rich media for the production of fungal pigments by *M. purpureus* and *P. purpurogenum*. Pigment production was carried out via solid state fermentation, semi-solid state fermentation and submerged fermentation. The fungal strain *P. purpurogenum* successfully produced *Monascus*-like pigments from waste orange peels, in the absence of mycotoxins, indicating the potential development of an integrated bioprocessing strategy for valorisation of orange processing waste. However, further optimisation studies are needed in order to maximise pigment production by *P. purpurogenum*.

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Figure Captions

Figure 1 Flow chart of the experimental setup implementing different fungal bioconversion processes for the valorisation of waste orange peel (WOP)

Figure 2 Evaluation of substrate pore size on the production of pigments during SSF of WOP with *M. purpureus* ATCC 16365

Figure 3 Profile change of orange (■, 475 nm) and red (▲, 500 nm) pigment during SSF of *P. purpurogenum* CBS 113139 cultivated on WOP with particles sizes of < 2 mm

Figure 4 Profile change of FAN (□), total sugars (○) and TDW (∆) along with pigment production (●, yellow at 400 nm; ■, orange at 475 nm; ▲, red at 500 nm) during semi-SSF of *M. purpureus* ATCC 16365 cultivated on initial WOP concentration of 20 g L^{-1}

Figure 5 Profile change of FAN (□), total sugars (○) and TDW (∆) along with pigment production (●, yellow at 400 nm; ■, orange at 475 nm; ▲, red at 500 nm) during semi-SSF of *P. purpurogenum* CBS 113139 cultivated on initial WOP concentration of 20 g L^{-1}

Figure 6 Profile change of FAN (□), total sugars (○) and TDW (∆) along with pigment production (●, yellow at 400 nm; ■, orange at 475 nm; ▲, red at 500 nm) during submerged cultivation of *M. purpureus* ATCC 16365 employing the liquid stillage derived from hydrodistillation of WOP

Figure 7 Profile change of FAN (□), total sugars (○) and TDW (∆) along with pigment production (●, yellow at 400 nm; ■, orange at 475 nm; ▲, red at 500 nm) during submerged cultivation of *P. purpurogenum* CBS 113139 employing the liquid stillage derived from boiling of WOP

Table 1 Evaluation of different pigment extraction methods during solid state fermentation at 65% (w w^{-1}) initial moisture content using *M. puprureus* ATCC 16365 cultivated on WMB or WOP

	Pigment production (AU/gdfs)								
Substrate used for pigment extraction	**70% (v v^{-1}) Ethanol**			**95% (v v^{-1}) Ethanol**			**Isopropanol**		
	400 nm	**475 nm**	**500 nm**	**400 nm**	**475 nm**	**500 nm**	**400 nm**	**475 nm**	**500 nm**
WMB (dry fermented solids, db)	0.05	0.07	0.21	0.1	0.04	0.03	0.21	0.3	0.16
WMB (wet fermented solids, wb)	1.57	1.53	1.24	0.85	0.48	0.47	0.12	0.06	0.05
WOP (dry fermented solids, 2-4 mm)	4.16	2.6	1.55	0.58	0.18	0.05	ND*		
WOP (wet fermented solids, 2-4 mm)	6.31	3.07	1.77	9.4	7.91	1.37	ND		
WOP (dry fermented solids, < 2 mm)	2.65	0.14	0.06	1.48	0.89	0.36	ND		
WOP (wet fermented solids, < 2 mm)	9.02	3.45	2.46	4.13	0.71	0.9	ND		

* ND: not determined

Table 2 Total Phenolic Content (TPC) and antioxidant activity (%I) of the ethanolic extracts obtained from different fermentations at maximum pigment production

	TPC (mg EAE* mL^{-1} extract)		**$DPPH^{\cdot}$ (%I)**	
Bioconversion process	*M. purpureus* ATCC 16365	*P. purpurogenum* CBS 113139	*M. purpureus* ATCC 16365	*P. purpurogenum* CBS 113139
Solid state fermentation	123.7	60.7	78.8	78.4
Semi-SSF (20 g L^{-1})	73.4	63.6	56.5	40.1
Semi-SSF (50 g L^{-1})	114.7	34.5	83.6	73.9
Submerged fermentation using liquid stillage from boiling of WOP	73.2	74.7	73.2	73.5
Submerged fermentation using liquid stillage from hydrodistillation of WOP	47.1	56.6	76.5	55.2

* EAE: Ellagic acid equivalents

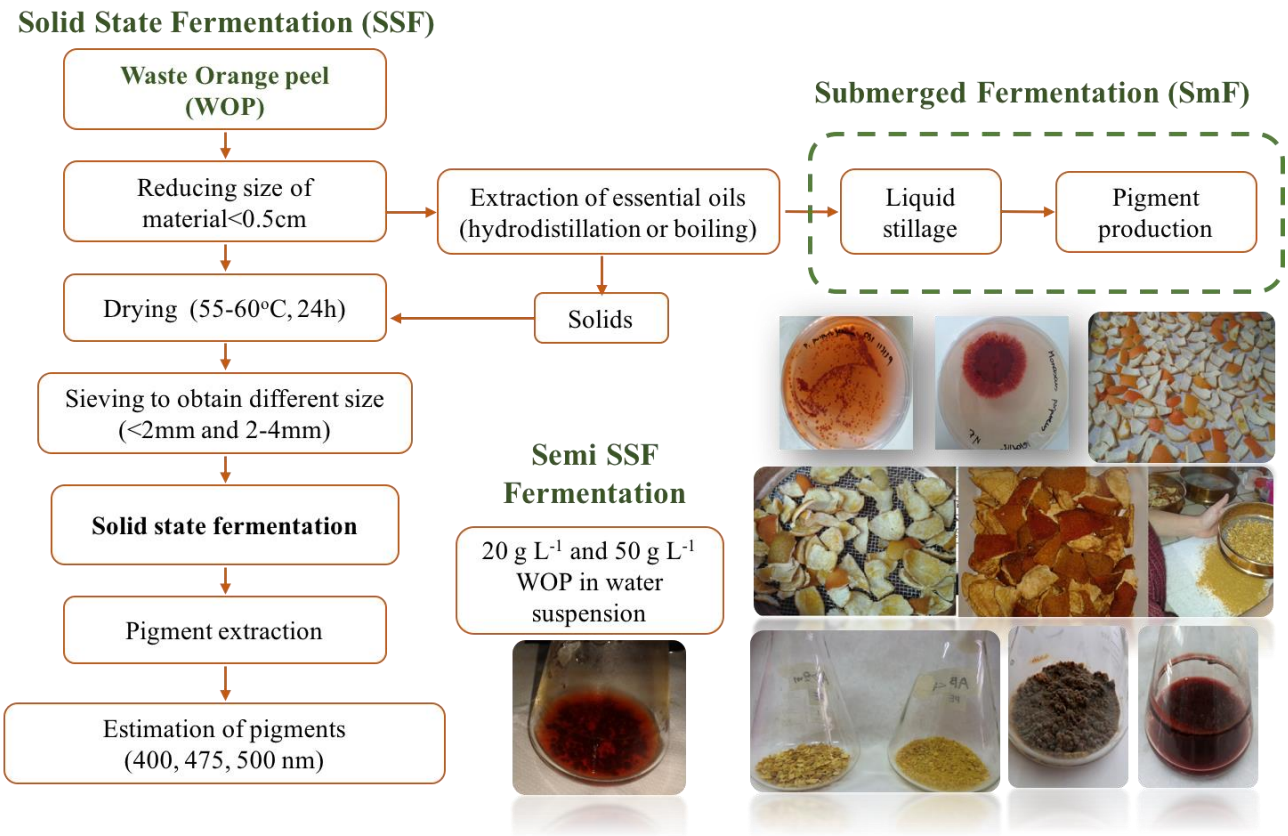

Solid State Fermentation (SSF)
Waste Orange peel (WOP)
Reducing size of material<0.5cm
Drying (55-60°C, 24h)
Sieving to obtain different size (<2mm and 2-4mm)
Solid state fermentation
Pigment extraction
Estimation of pigments (400, 475, 500 nm)
Extraction of essential oils (hydrodistillation or boiling)
Solids
Submerged Fermentation (SmF)
Liquid stillage
Pigment production
Semi SSF Fermentation
20 g L-1 and 50 g L-1 WOP in water suspension

Figure 1

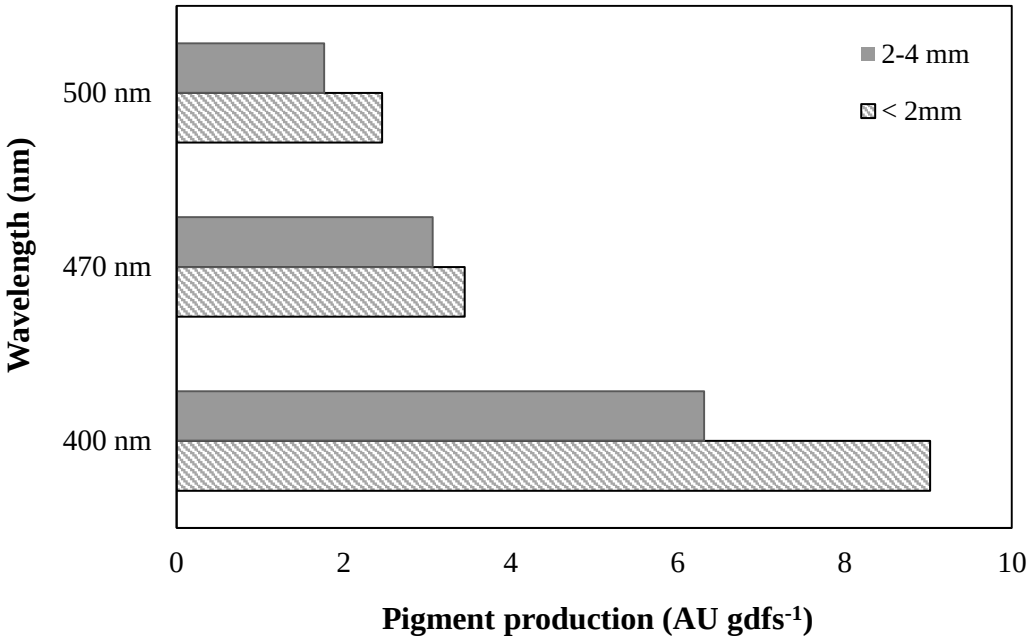

2-4 mm
< 2mm
500 nm
470 nm
400 nm
Wavelength (nm)
0
2
4
6
8
10
Pigment production (AU gdfs-1)

Figure 2

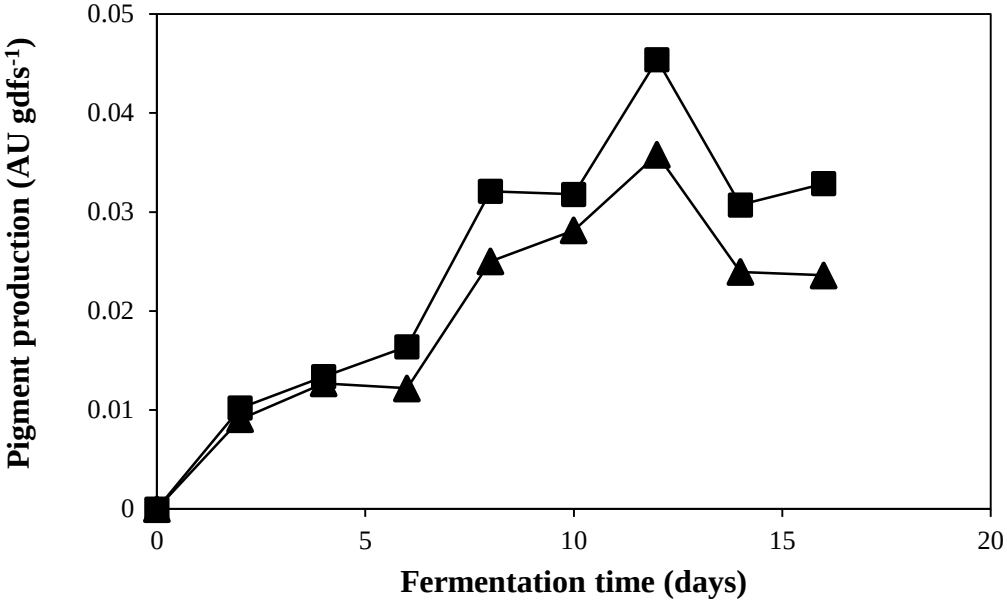

Pigment production (AU gdfs-1)
0.05
0.04
0.03
0.02
0.01
0
0
5
10
15
20
Fermentation time (days)

Figure 3

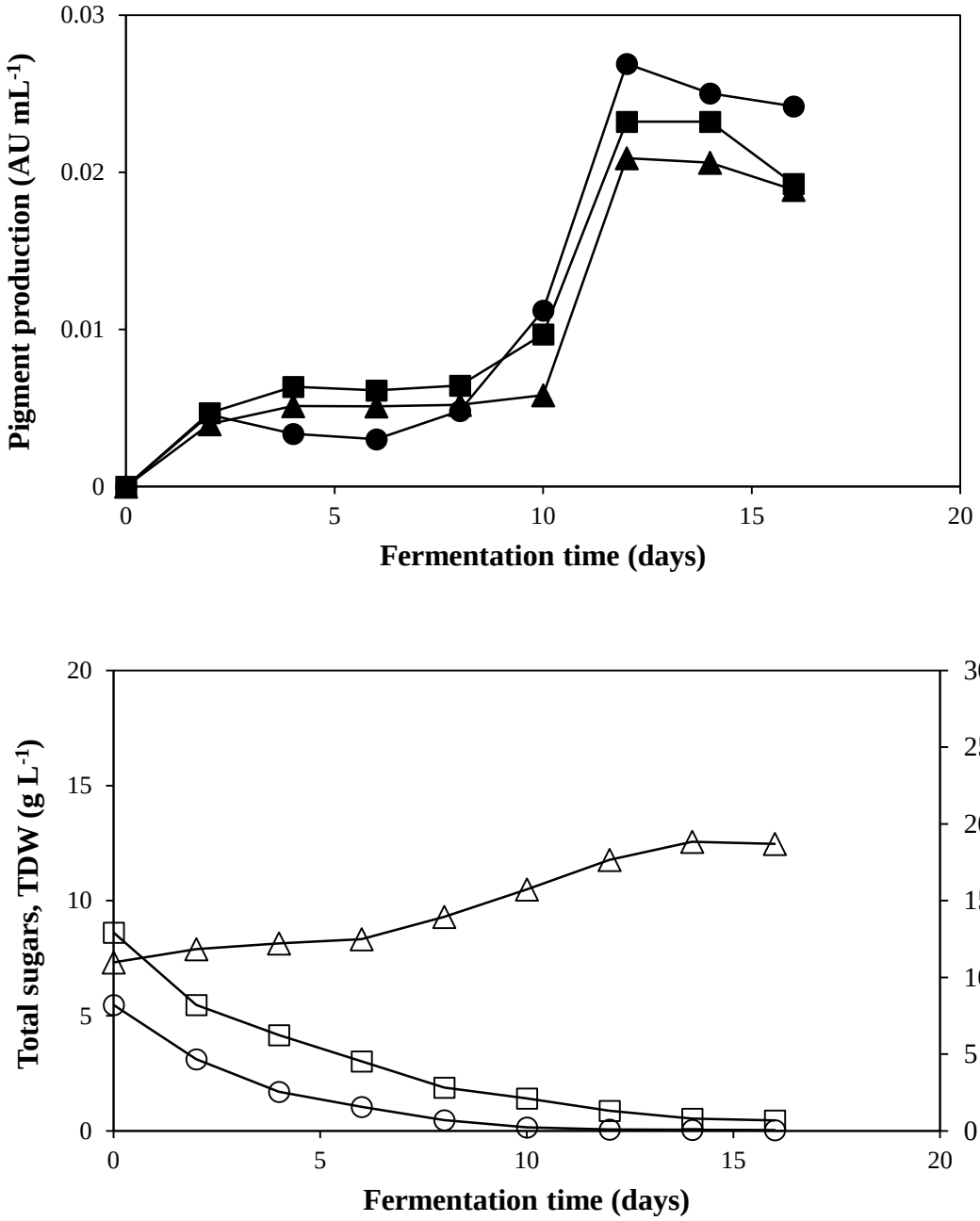
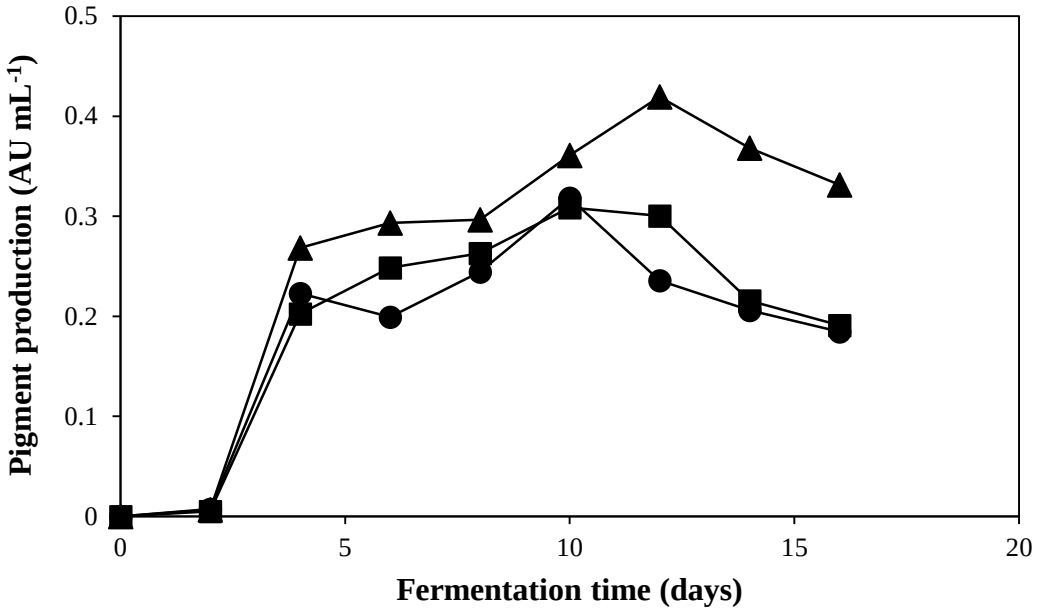

Pigment production (AU mL-1)
0.03
0.02
0.01
0
0
5
10
15
20
Fermentation time (days)
Total sugars, TDW (g L-1)
FAN (mg L-1)
20
15
10
5
0
30
25
20
15
10
5
0
0
5
10
15
20
Fermentation time (days)

Figure 4


Pigment production (AU mL-1)
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
Fermentation time (days)

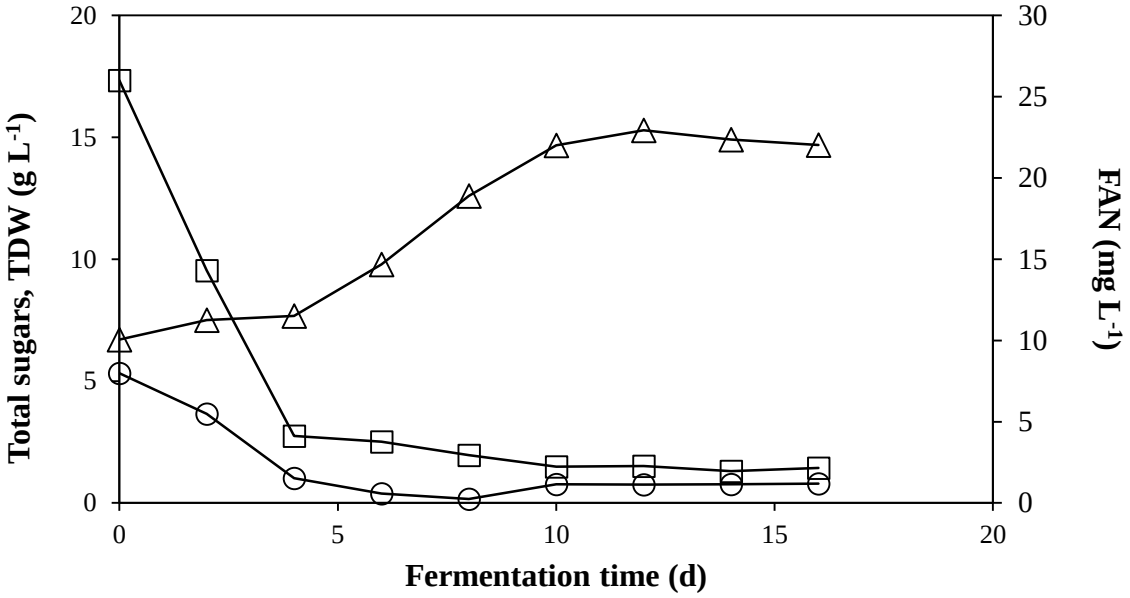

Total sugars, TDW (g L-1)
FAN (mg L-1)
20
15
10
5
0
30
25
20
15
10
5
0
0
5
10
15
20
Fermentation time (d)

Figure 5

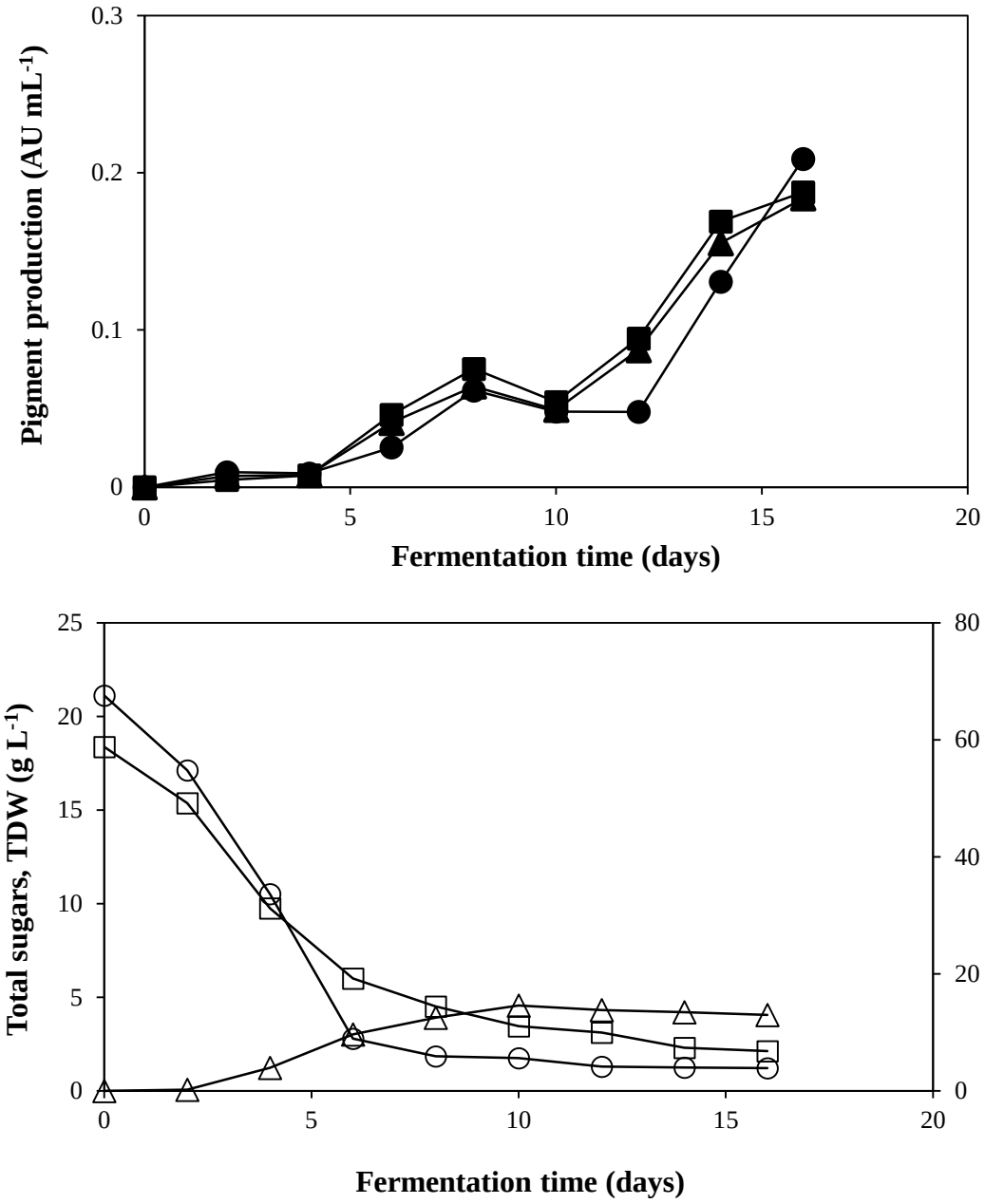
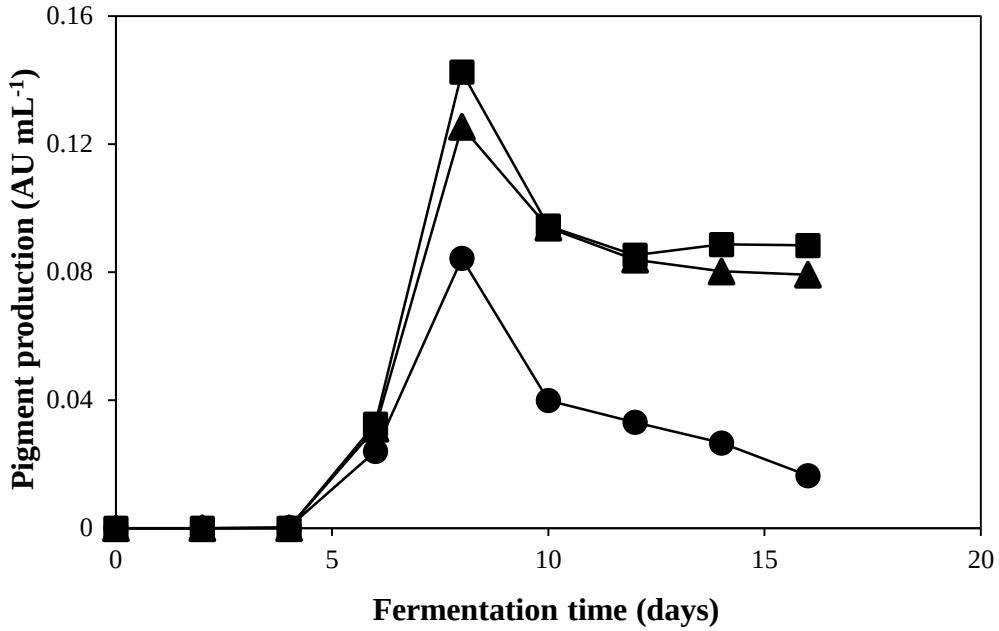

Pigment production (AU mL-1)
0.3
0.2
0.1
0
0
5
10
15
20
Fermentation time (days)
Total sugars, TDW (g L-1)
FAN (mg L-1)
25
20
15
10
5
0
80
60
40
20
0
0
5
10
15
20
Fermentation time (days)

Figure 6


0.16
0.12
0.08
0.04
0
Pigment production (AU mL-1)
0
5
10
15
20
Fermentation time (days)

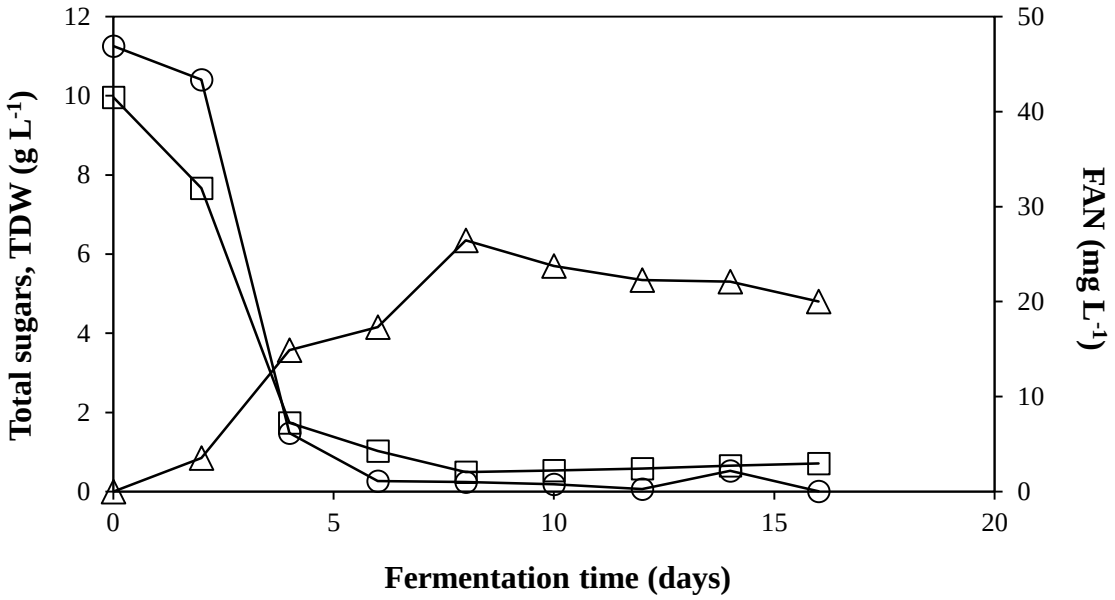

12
10
8
6
4
2
0
Total sugars, TDW (g L-1)
50
40
30
20
10
0
FAN (mg L-1)
0
5
10
15
20
Fermentation time (days)

Figure 7