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Simultaneous enrichment of cereals with polyunsaturated fatty acids and pigments by fungal solid state fermentations

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ABSTRACT

Four *Mucor* strains were tested for their ability to grow on four cereal substrates and enriched them with gamma-linolenic acid (GLA) and β-carotene. *M. circinelloides* CCF-2617 as the best producer accumulated of both GLA and β-carotene in high amounts during utilization of rye bran/spent malt grains (3:1). The first growth phase was characterized by rapid GLA biosynthesis, while distinct β-carotene formation was found in the stationary fungal growth. Therefore various cultivation conditions were tested in order to optimize the yield of either GLA or β-carotene. The fungus grown on cereal substrate supplemented with glucose produced maximal 8.5 mg β-carotene and 12.1 g GLA in 1 kg fermented substrate, respectively. On the other hand, the highest amount of GLA in the fermented substrate (24.2 g/kg) was achieved when 30% of sunflower oil was employed to the substrate. Interestingly, β-carotene biosynthesis was completely inhibited when either whey or linseed oil were added to the substrate.

1. Introduction

Humanity has become dependent upon cereal grains for the majority of its food supply. However, there are lots of evidences suggesting that cereal grains are less than optimal foods for humans. They lack a number of nutrients, such as polyunsaturated fatty acids (PUFAs) and pigments which are essential for human well-being due to their healthy, dietary and functional properties (Cordain, 1999; Pennington, 2002).

Nevertheless, cereals could be considered as challenging sources of PUFAs/pigments providing that they will be naturally modified with the aim to enrich them with these essential compounds. One possible approach may lay on application of gene engineering techniques in order to prepare new cereal varieties with desired fatty acid and/or pigment profile. These methods are, however, limited not only for difficulties with genes transformation to various cereals but also because transgenic cereals containing PUFA or carotenoids may not be approved for application in the food industry in many countries. Therefore, another promising and effective process is based on solid state fermentations (SSF) where suitable microorganisms utilize cereals and convert them to new cereal-derived bioproducts with high content of valuable metabolites (Singhania et al., 2009; Barrios-González, 2012). In a fact, Slovak team has pioneered SSF process where microorganisms belonging to Zygomycetes easily and efficiently used cereals and accumulated lipid with dietetic valuable PUFAs (Slugeň et al., 1994). Zygomycetous fungi have been often described for their capacity to effectively produce various types of PUFAs (Čertík and Shimizu, 1999). Moreover, these fungi also partially hydrolyze biopolymers and decrease anti-nutrient compounds in cereal substrates (Čertík et al., 2010). The biotechnological processes for preparation of various cereal-based products enriched with gamma-linolenic acid (GLA), dihomo-gamma-linolenic acid, arachidonic acid and eicosapentaenoic acid by lower oleaginous fungi (*Thamnidium* sp., *Cunninghamella* sp., *Mucor* sp., *Mortierella* sp.) during SSF have been reported (FSB 2012) and these products have been successfully tested as food or feed additives (Čertík and Adamechova, 2009).

However, simultaneous production of both PUFAs and carotenoid pigments has not been reported yet. Although a range of microorganisms including mainly yeasts (Yurkov et al., 2008; El-Banna et al., 2012), fungi (Iturriaga et al., 2000; Dufosse, 2006; Kuzina and Cerdá-Olmedo, 2007; Nanou et al., 2012) and algae (Del Campo et al., 2007) are able to synthesize carotenoid pigments (primarily β-carotene and lycopene) in sufficient quantities, only some of them can be applied for cereal utilization by SSF process. Mucorales fungi are potential microbial candidates for enrichment of cereals with both PUFAs and carotenoids because of active biosynthetic apparatus for overproduction of these compounds as well as for their excellent growth on cereal substrates (Čertík et al., 2010). Nevertheless, in order to ensure high yields of

Abbreviations: GLA, gamma-linolenic acid; PUFA, polyunsaturated fatty acid; SMG, spent malt grains; SSF, solid state fermentations.

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PUFAs and pigments in prefermented cereals, optimization of SSF process is required for successful development of this biotechnological method for production of novel functional cereals enriched with these metabolites. This work is therefore focused on selection of useful fungal strain and optimization of substrate for maximal content of GLA and β-carotene in prefermented cereal-based mass.

2. Material and methods

2.1. Microorganisms

Fungal strains *Mucor petrinsularis* CCF-2409, *Mucor dimorphosporus* f. *dimorphosporus* CCF-2583, *Mucor circinelloides* f. *lusitanicus* CCF-2617, *Mucor hiemalis* f. *hiemalis* CCF-2698 were obtained from the Culture Collection of Fungi (CCF), Department of Botany, Charles University, Prague, Czech Republic. The cultures were maintained on modified Czapek-Dox agar slants with yeast extract (2.5 g/L) at 4 °C. The spore suspension for inoculation was prepared by washing the mycelium with sterilized aqueous solution of 0.1% (w/v) Tween 80 to reach the final concentration of 1–2.10^6 spores per mL. All chemicals and solvents used in the present study were of analytical grade unless otherwise specified.

2.2. Substrates and solid state fermentation conditions

Various types of cereal substrates (wheat bran – WB, rye bran – RB, oat flakes – OF, barley groats – BG) and spent malt grain (SMG) were employed for SSF experiments. SMG served as an inert support and was kindly provided by Heineken company. Autoclavable microporous polypropylene bags (160 mm × 270 mm) were filled with 10 g of dry substrates [WB, WB/SMG (3:1), RB, RB/SMG (3:1), OF, OF/SMG (3:1), BG, BG/SMG (3:1)], moistened by the addition of 10 mL of distilled water, soaked for 2 h at laboratory temperature and sterilized in autoclave (120 kPa, 120 °C, 20 min). SMG was washed twice before the addition to substrates in order to remove undesirable compounds. Cereal substrates were inoculated with 2 mL of the spore suspension (prepared as mentioned above). Each bag was closed with sterile cotton plug and inoculated substrates were arranged to obtain substrate layer of about 1 cm thickness in the bags. Cultivation was carried out statically at 25 °C for up to 180 h. In order to ensure homogenous growth of the fungi, prefermented material was gently mixed once a day during the cultivation.

To test effect of addition of glucose, whey and oils (sunflower oil, olive oil, linseed oil) to the substrate, microporous polypropylene bags (160 mm × 270 mm) were filled with 10 g of dry RB/SMG (3:1), moistened by the addition of 10 mL of distilled water, soaked for 2 h at laboratory temperature and sterilized in autoclave (120 kPa, 120 °C, 20 min). Sterilized water solution of glucose or whey (calculated as lactose, whey consisted of 80% lactose) was added to the substrate to reach the final concentration of 10, 20, 30, 40 and 50% in the substrate. Similarly, sterilized oils were added to the substrate to reach the final concentration of 5, 10, 20 and 30% in the substrate. Substrates were inoculated with 2 mL of the spore suspension (prepared as mentioned above). Each bag was closed with sterile cotton plug and inoculated substrates were arranged to obtain substrate layer of about 1 cm thickness in the bags. Cultivation was carried out statically at 25 °C for 140 h. In order to ensure homogenous growth of the fungi, prefermented material was gently mixed once a day during the cultivation.

To assess reproducibility, triplicate SSF experiments for each substrate were prepared and analyzed individually (values are presented as the means of triplicates, the reproducibility varied in the range of 92–96%).

2.3. Lipid and carotenoid pigment isolation

Prefermented cereal materials were gently dried at 65 °C for 10 h and weighed. Lipids containing carotenoid pigments were extracted from dry homogenized prefermented cereals 2-times by 100 mL chloroform/methanol (2:1, v/v) for 3 hours at laboratory temperature with occasional stirring (Čertík et al., 1996). After extraction the mixture was filtered to remove mycelium and the extracts were collected. Then 0.9% KCl (1.2-fold of total extract volume) was added, the mixture was stirred vigorously for 1 min and centrifuged to effect phase separation. The chloroform–lipid and pigment containing layer was filtered through anhydrous Na_2SO_4 and evaporated under vacuum. Total lipids with pigments were determined gravimetrically (triplicate standards of dry yeast cells were used to assess reproducibility) and used for further analysis.

2.4. Fatty acid analysis

Fatty acids from total lipids were converted to their methylesters by methanolic solution of sodium methoxide and methanolic HCl (Christoperson and Glass, 1969) and analyzed by gas chromatography (GC-6890N, Agilent Technologies) using a capillary column DB-23 (60 m × 0.25 mm, film thickness 0.25 μm, Agilent Technologies) and a FID detector (constant flow, hydrogen 40 mL/min, air 450 mL/min, 250 °C) under a temperature gradient (150 °C held for 3 min; 150–175 °C at program rate 7.0 °C/min; 175 °C held for 5 min; 175–195 °C at program rate 5.0 °C/min; 195–225 °C at program rate 4.5 °C/min; 225 °C held for 0.5 min; 225–215 °C at program rate 10 °C/min; 215 °C held for 7 min; 215–240 °C at program rate 10 °C/min; 240 °C held for 7 min) with hydrogen as carrier gas (flow 2.5 mL/min, velocity 57 cm/s, pressure 220 kPa) and a split ratio of 1/20 (Inlets: heater 230 °C; hydrogen flow 51 mL/min for 2 min, then hydrogen flow 20 mL/min; pressure 220 kPa) (Sukrutha et al., 2012). The fatty acid methylester peaks were identified by authentic standards of C_4–C_{24} fatty acid methylesters mixture (Supelco, USA) and evaluated by ChemStation B 01 03 (Agilent Technologies). To identify the unknown peaks in lipid structures, GC-MS analysis of fatty acid methyl esters was also performed. The samples were introduced through gas chromatograph (GC-6890N, Agilent Technologies) and separated as mentioned above. The effluent was detected by total ionization monitoring with a mass selective detector (MS-5975, Agilent Technologies). The electron impact mass spectra were set at a voltage of 70 eV. All peaks were evaluated by MSD ChemStation E.00.00.202 (Agilent Technologies) with combination of NIST Mass Spectral Search Program version 2.0 d (Agilent Technologies).

2.5. Carotenoid analysis

Carotenoid pigments were analyzed by high-performance liquid chromatography (HPLC). Analysis was carried out with an HP 1100 chromatograph (Agilent) equipped with a UV-VIS detector. Pigments extracts in chloroform (10 μL) were injected onto LiChrospher® 100 RP-18 (5 μm) column (Merck). The solvent system (the flow rate was 1 mL/min) consisted of solvent A, acetonitrile/water/formic acid 86:10:4 (v/v/v), and B, ethyl acetate/formic acid 96:4 (v/v), with a gradient of 100% A at 0 min, 100% B at 20 min, and 100% A at 30 min. The carotenoid pigments were identified by authentic standards (Sigma, Germany) and evaluated by ChemStation B 01 03 (Agilent Technologies).