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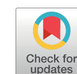
Mould starter selection for extended solid-state fermentation of quinoa



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ABSTRACT

Solid-state fermentation of plant materials with food grade moulds is considered a method of obtaining bioactive food products and additives. However, the use of quinoa seeds as a substrate in such a process has not been thoroughly studied to date. In this paper, extended fermentation with *Rhizopus oligosporus*, *Aspergillus oryzae* and *Neurospora intermedia* (tempe, koji and oncom moulds, respectively) was performed in order to enhance the antioxidant potential of coloured quinoa (as measured by the total soluble phenols, $ABTS^{\cdot+}$ and $^{\cdot}OH$ scavenging activities, and the reducing power).

The most efficient of the examined strains was *Rhizopus*, which caused an average improvement in the aforementioned qualities of 220% (black seeds) and 129% (red seeds), as compared to pre-cooked seeds. Four-day fermentation with *Rhizopus* resulted in a drastically enhanced amount of vanillin in the free phenolic fraction (130-fold in black quinoa seeds). However, the overall level of free and bound phenolics (as measured by the HPLC-DAD-ESI-TOF-MS method) in the material decreased. Both total peptides and individual free amino acid levels were significantly increased, which corresponded with the high antioxidant potential of the material. The fermented quinoa was also significantly enriched with total dietary fibre with dominant insoluble fraction.

1. Introduction

Solid-state fermentation of plant materials is usually performed in order to obtain valuable compounds produced by microorganisms, namely enzymes and various secondary metabolites. This kind of biotreatment is also used in food production to enrich substrates in bioactive compounds, such as vitamins and phenolics of potential antioxidant activity, as well as to enhance their nutritional value, e.g. by a partial digestion of macromolecules. One of the most important food grade fungal species in solid-state food fermentations is *Aspergillus oryzae* – koji mould, essential for the production of soy sauce and soy paste. *Rhizopus oligosporus* and *Neurospora intermedia* are used in Indonesia to obtain tempe and oncom, respectively (Owens & Astuti, 2015). The moulds mentioned above belong to the GRAS (generally recognized as safe) species. They are not capable of toxic secondary metabolite synthesis (Blumenthal, 2004; Jennessen et al., 2005; Partida-Martinez et al., 2007; Perkins & Davis, 2000), which in case of *A. oryzae* may be considered exceptional among *Aspergillus* spp. (Mikušová, Sulyok, Santini, & Šrobárová, 2014).

Soybeans are the most popular plant substrates in the above processes, although other plant materials may also be used. Quinoa seeds are a source of gluten-free protein of nutritional value comparable to that of milk protein, good quality starch, dietary fibre, polyunsaturated fatty acids, vitamins and minerals (Repo-Carrasco-Valencia & Serna, 2011). They have been successfully used as a substrate in the preparation of tempe- and oncom-like food products (Starzyńska-Janiszewska, Bączkowicz, Sabat, Stodolak, & Witkowicz, 2017a; Starzyńska-Janiszewska, Stodolak, Duliński, Mickowska, & Sabat, 2017b). In this kind of biotreatment, the usual period of mould incubation with the substrate is rather short, up to 30 (tempe) and 72 (oncom) hours. Extended incubation leads to undesirable changes, namely ammonification of the product (tempe) and further mycelium degradation. This does not, however, occur in high-starch substrates. Previously, we found that the prolongation of tempe-type incubation from 30 to 40 h significantly improved the antioxidant potential of quinoa seeds (Starzyńska-Janiszewska, Duliński, Stodolak, Mickowska, & Wikiera, 2016). Our present study is focused on the extended procedure (up to a few days), as this method proved effective in the case of cereals resulting in products enriched with bioactive and nutritional compounds (Yigzaw, Gorton, Solomon, & Akalu, 2004; Zhang et al.,

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2012). As far as we know, such studies have not yet been conducted on pseudocereal seeds, including quinoa. The substrate choice can further be supported by observations of Davey, Peñaloza, Kell, and Hedger (1991), who found that *R. oligosporus* NRRL 2710 growing on quinoa, in contrast to legume seeds, showed a stable cellular viability for a long incubation time (a slight decrease was noted no sooner than after 60 h).

The fermented quinoa seeds obtained may be, in our opinion, taken into consideration as a bioactive and fibre-enriched gluten-free component of composite flours. Such flours containing pseudocereals are regarded as a valuable ingredient of baked goods and pasta products (Zannini, Pontonio, Waters, & Arendt, 2012). Different kinds of bioprocessing (e.g. submerged fermentation with yeasts and lactic acid bacteria) have already been recognized as an effective tool to improve the bioactive and nutritional quality of gluten-free materials (Zannini et al., 2012).

Therefore, the aim of the present study was to verify the effectiveness of *Aspergillus oryzae*, *Rhizopus oligosporus* and *Neurospora intermedia* during the extended (up to 5–6 days) solid-state fermentation of coloured quinoa seeds in terms of enhancing their antioxidant potential. Next, products of the best antioxidant activity obtained from black and red seeds after the chosen treatment combination (mould kind and incubation time) were selected and further characterized (with regard to the phenolic profile, free amino acids and peptides, dietary fibre). On the basis of the presented results, an assumption concerning the possible nature of the antioxidant potential of fermented quinoa was proposed.

2. Material and methods

2.1. Raw materials, and microorganism strains

Black and red quinoa seeds (Bolivia, debittered by polishing and washing; packaged by Bio Planet S.A.) were purchased from a health food store in Kraków, Poland. The physical characteristic of the material was described previously (Starzyńska-Janiszewska et al., 2017a).

Rhizopus oligosporus ATCC 64063 strain recommended for cereal fermentation (Berg, Olsson, Swanberg, Schnurer, & Eriksson, 2007), *Aspergillus oryzae* DSM 1861 (koji strain) and *Neurospora intermedia* DSM 1965 (oncom strain) were grown on potato dextrose agar (PDA) slants for 12 days at 24 °C.

2.2. Precooking of seeds before fermentation

Red and black quinoa seeds were cooked for 15 min in tap water (1:3 m/v) acidified with lactic acid to pH 4.5–5 (in order to provide proper conditions for fungal growth). Then, water was discarded and the seeds were dried on a surface with a sterile cloth.

2.3. Fermentation of quinoa seeds with Rhizopus oligosporus and Aspergillus oryzae

Cooled (< 30 °C) pre-cooked seeds of black and red quinoa were aseptically inoculated with *R. oligosporus* or *A. oryzae* spore suspension (10^4 spores per g of seeds). Each inoculum was obtained by flooding the surface of the agar slant cultures with a sterile saline solution (8 g/L, supplemented with peptone (0.01 g/L) and Tween 80 (0.1 mL/L)), followed by filtration (nylon net filters ø 11 μm). The spore density was measured using a spore counting method in a Thoma chamber. The inoculated material was tightly packed in sterile petri dishes (ø 10 cm, 5 replications for each fermentation period) and incubated at 31 °C (*R. oligosporus*) or 25 °C (*A. oryzae*) for 2, 4 and 6 days. The fungal growth was stopped by steaming the material for 10 min.

2.4. Fermentation with Neurospora intermedia

Cooled (< 30 °C) precooked seeds of black and red quinoa were aseptically inoculated with fragments of *N. intermedia* aerial mycelium, mixed (5 min) and tightly packed in sterile petri dishes (ø 10 cm, 5 replications for each fermentation period). The material was incubated (30 °C, > 60% air humidity) for 24 h (allowing the mycelium to overgrow the seeds), and next the petri dishes were opened. The fermentation was continued for the next 24 h (at that point, the aerial mycelium with orange spores tightly covered the surface of the seeds), after which the material was turned over and the process was conducted for further 24 h (a 3-day incubation period in total) and 72 h (a 5-day period in total). The fungal growth was stopped by steaming the material for 10 min.

The precooked and fermented material was lyophilized and stored at 3 °C until analysed. The analysis was performed on black and red quinoa seeds: raw, precooked, and fermented with *R. oligosporus*, *A. oryzae* – for 2, 4 and 6 days, and *N. intermedia* – for 3 and 5 days.

2.5. Analytical methods

The samples for the analysis of the antioxidant parameters and total phenolic compounds were prepared by 2 h-long shaking of a 0.5 g sample with 15 mL buffer, centrifugation and filtration of the supernatant. The $ABTS^{\cdot+}$ scavenging activity (Starzyńska-Janiszewska, Stodolak, & Mickowska, 2014) was expressed as μmol Trolox equivalents/g dwb. The hydroxyl radical ($^{\cdot}OH$) neutralization was measured according to Marambe, Shand, and Wanasundara (2008) and expressed as EC_{50} (efficient concentration) which amounts to mg of the sample used for the extraction that is required for the inhibition of 50% free radicals in the reaction conditions. A lower value of EC_{50} indicates higher radical scavenging activity. Reducing power (Ardestani & Yazdanparast, 2007) was expressed as $RP_{0.5}$, defined as the amount of a lyophilized sample (mg) used for the extraction that produces 0.5 absorbance units at 700 nm. A lower value of $RP_{0.5}$ indicates higher reducing power. Total soluble phenols were measured by way of the reaction with Folin-Ciocalteu reagent and expressed in mg gallic acid/g dwb, as described in Starzyńska-Janiszewska et al. (2014).

Dry matter was determined with a moisture analyzer (WPS 110S, Radwag, Radom, Poland). The extraction and determination of free and bound phenolic compounds (mg/100 g dwb) by means of liquid chromatography with diode array detection and electrospray ionization time-of-flight mass spectrometry method (HPLC-DAD-ESI-TOF-MS) was performed according to Gómez-Caravaca, Segura-Carretero, Fernández-Gutiérrez, and Caboni (2011).

The amino acid (g/100 g dwb) profile was measured by the ion-exchange chromatography as described in Starzyńska-Janiszewska et al. (2017b).

Free amino acids (g/100 g dwb) were extracted with 0.1 mol/L HCl. After protein precipitation with solid sulfosalicylic acid, supernatants were lyophilized, dissolved in sodium citrate buffer pH 2.2 and filtered through a 0.45 μm syringe filter. Next, a chromatographic amino acid analysis was performed as described in Starzyńska-Janiszewska et al. (2017b).

SDS-PAGE Electrophoresis (supplement): samples were prepared by way of extraction of 25 mg sample in 250 μl of buffer (125 mmol/L Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 5% beta-mercaptoethanol, coloured by Coomasie Brilliant Blue G-250). Extraction was performed by vigorous shaking for 1 h at room temperature, followed by centrifugation and boiling for 5 min at 100 °C. Aliquots of 1.5 μl (approx. 15 μg of proteins/lane) were analysed by Tris-Tricine SDS-PAGE under reducing conditions according to the Schägger method (Schägger & von Jagow, 1987).

Total peptides (g/100 g dwb) were measured in phosphate buffer extracts (0.1 mol/L, pH 7.4) according to the o-phthaldialdehyde method as described in Zhu, Cheng, Wang, Fan, and Li (2008), with a standard curve prepared from L-glutathione (reduced form).

Total soluble and insoluble dietary fibre (g/100 g dwb) was estimated according to the enzymatic-gravimetric method AOAC 991.43

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