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Using wheat bran and soybean meal as solid state fermentation substances for the production of *Xylaria nigripes* with bioactivities

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ABSTRACT

Different combinations of wheat bran (WB) and soy meal (SM) were used as solid state fermentation substrates for the growth of functional *Xylaria nigripes* (*XN*, *Wu Ling Shen*, in Chinese). Hot water as well as ethanol (70%) were used to extract active compounds with antioxidant, anti-inflammatory and neuroprotective properties. In general, ethanol was a more effective solvent than hot water to extract active compounds. When WB was used as the sole fermentation substrate, the ethanol extract of *XN*-fermented substances obtained the highest antioxidant activities, especially DPPH radical scavenging and reducing activities; anti-inflammation, as measured by the inhibition of COX-2 activity, was used as an active index. Fermentation substrates containing equal amounts of WB and SM increased the protective effects against H_2O_2-induced damage in neuronal cells (PC12 cells) for 70% ethanol-extracted *XN*. Both *XN* and residues of fermentation substrates may have contributed to the biological activities of *XN*-fermented substances.

1. Introduction

Oxidative stress and inflammation play important roles in the pathogenesis of many chronic diseases, such as atherosclerosis, cancer, diabetics, rheumatoid arthritis, post-ischemic perfusion injury, myocardial infarction, cardiovascular diseases, chronic inflammation, stroke, septic shock and aging [1,2]. In addition, neurodegenerative disorders are induced by inflammation and oxidant stress as well as an imbalance between the generation of free radicals and antioxidant defenses *in vivo*, leading to many health problems, including Alzheimer's disease, encephalitis, epilepsy, hydrocephalus, stroke, Parkinson's disease, multiple sclerosis, Huntington's disease and prion diseases [3]. Currently, researchers have shown a great deal of interest in searching for natural bioactive compounds that have antioxidant and anti-inflammatory activities [1].

In Chinese folk medicine, *Xylaria nigripes* (*XN*) is used as a diuretic and nerve tonic and is also used to treatment insomnia and trauma. Ko et al. [4] reported that hot water and the 95% ethanol extract of *XN* showed anti-inflammatory and immunomodulatory behaviors by inhibiting iNOS and COX-2 expression through blocking phosphorylation of NF-κB. The antioxidant ability of *XN* has also been shown to provide protection against carbon tetrachloride-induced liver injury in mice [5]. *XN* contains high contents of polyphenols, flavonoids and exopolysaccharides and thus exhibits potent antioxidant, anti-inflammatory and antitumor activities [4,6,7]. *XN* has a tranquilizing effect on the central nervous system; a study conducted by Zhao et al. [8] further demonstrated that *XN* could induce rapid eye movement sleep deprivation and could reduce spatial memory impairment.

XN is usually found several feet underground in *Odentotermes* termite nests [9,10]. In ancient times, *XN* was obtained by digging up termite nests, which is a very laborious, time consuming and economically un-feasible process. In recent decades, submerged fermentation has been applied to grow *XN* mycelium [4,5,7,8]. Compared to submerged fermentation, solid state fermentation (SSF) is a feasible process that provides controlled release of nutrients [11] and is very useful in long-term fermentation for slow growing fungi [9]. Additionally, SSF provides a natural habitat for microorganisms with effective growth and better product characteristics, leading to reduced fermentation costs (due to the advantages of using a smaller fermenter-size), reduced downstream processing, reduced stirring and lower sterilization costs [12].

To our knowledge, this is the first report describing solid-state fermentation of *XN*. In this study, *XN*-SSF was carried out by using two different agricultural waste materials, wheat bran (WB) and soybean meal (SM). *XN* fermented substances were used to extract bioactive compounds and their bioactivity was studied, including their DPPH radical scavenging activity, inhibition of lipid peroxidation, reducing power, inhibitory effects on

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activity of cyclooxygenase-2 (COX-2), production of nitric oxide (NO), and protection against H_2O_2-induced damage, using pheochromocytoma (PC12) cells as a neuronal cell model.

2. Materials and methods

2.1. Chemicals

All chemicals and solvents used were of analytical grade. Dulbecco's Modified Eagle Medium (DMEM) was purchased from Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from Biological industries (Beit-Haemek, Israel). Soy lecithin was obtained from Wako Pure Chemical Industries (Osaka, Japan). Gallic acid, rutin hydrate, α-Diphenyl-β-picrylhydrazyl (DPPH), lipopolysaccharide (LPS), N-(1-Naphthyl) ethylenediamine dihydrochloride, sulfanilamide, thiazolyl blue tetrazolium bromide (MTT), L-N^G-nitroarginine methyl ester (L-Name), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma Chemicals (St. Louis, MO, USA). *Xylaria nigripes* (Klotzsch) M.C. Cooke (BCRC No. 34,219), RAW264.7, mouse macrophage cells and PC12, rat adrenal pheochromocytoma cells were purchased from the Bioresource Collection and Research Centre (BCRC; FIRDI, Hsinchu, Taiwan). Wheat bran (WB) and soy meal (SM) were kindly provided by Top food industry corp. (Taichung, Taiwan) and Central union oil corp. (Taichung, Taiwan), respectively.

2.2. Preparation of stock culture of Xylaria nigripes (XN)

The *X. nigripes* (*XN*) culture provided by BCRC on malt extract agar (MEA) plates was sub-cultured by cutting out 5 mm^2 of the agar plate culture with a sterilized cutter and transferring the cut section to a fresh 20 g/l malt extract agar (MEA) plate. The plates were incubated at 25 °C for 12–14 days. The stock cultures were prepared by submerging the sub-cultured *XN* (in an agar cube of 5 mm^2) in a tight-capped glass tube filled with sterilized MilliQ water and storing it at room temperature. The viability of the stock cultures was checked every month by culturing in a MEA plates.

2.3. Inoculum preparation and solid state fermentation (SSF)

Stock cultures were inoculated into malt extract broth (20 g/l malt extract, 10 g/l glucose) and incubated at 25 °C for 7 days on a rotary shaker with shaking at 60 rpm. Five fermentation substances were used, including: (1) wheat bran (WB), (2) wheat bran mixed with soybean meal (SM) at a ratio of 4:1 (WB_4SM_1), (3) wheat bran mixed with soybean meal at a ratio of 1:1 (WB_1SM_1), (4) wheat bran mixed with soybean meal at a ratio of 1:4 (WB_1SM_4), and (5) soybean meal (SM). The fermentation substance (50 g) was inoculated with 10% *XN* in a 1-l Erlenmeyer flask and incubated at 25 °C with controlled humidity (60%) for 30 days.

2.4. Extraction of fermented materials

The fermented samples were harvested and stored at −20 °C until used for extraction. Fermentation substances without inoculating *XN* were used as controls (unfermented samples). All samples were extracted with 90 °C hot water or 70% ethanol with constant stirring for 1 h according to Ko et al. [6]. After extraction, the samples were filtered through muslin cloth and centrifuged at 10,000 × g for 10 min. The supernatant was dried by lyophilization (Model FDU540, Eyela Co., Japan), and the lyophilized powder was stored at −20 °C. The yield of hot water extracted and ethanol extracted samples ranged from 12 to 20% and 8 to 11%, respectively.

2.5. Measurements of antioxidant activity

2.5.1. DPPH scavenging activity

The DPPH radical scavenging activity was estimated according to the method of Yamaguchi et al. [13]. An aliquot of the *XN*-fermented, un-fermented extract (0.1 ml, 0.39–12.5 mg/ml) or Trolox (0.1 ml, 50–500 μM) was mixed with 0.4 ml of 100 mM Tris-HCl buffer (pH 7.4) and then added to 0.5 ml of a DPPH solution (500 μM in ethanol). After shaking vigorously for 20 s, the mixture was left in the dark at room temperature for 20 min. The absorbance of the resulting solution was measured by a spectrometer at 517 nm. The ability to scavenge DPPH radicals was calculated by the following equation.

$$\text{DPPH scavenging activity (\%)} = \left[1 - \frac{\text{absrobance of sample at 517 nm}}{\text{absorbance of control at 517 nm}} \times 100\right]$$

2.5.2. Inhibitory activity of lipid peroxidation

The antioxidant effect of *XN* samples on lipid peroxidation was measured by the method described by Chen et al. [14] with slight modifications. Liposomes were prepared from soybean lecithin by using a chloroform–methanol system and further dried with nitrogen gas. An aliquot of the *XN*-fermented and un-fermented extracts (15 μl, 0.15–12.5 mg/ml) and 20 mM sodium phosphate buffer (165 μl, pH 7.2) were mixed with 300 μl of liposome. Liposome peroxidation was induced by $FeCl_3$-ascorbate. The formation of malondialdehyde–thiobarbituric acid (MDA–TBA) was used as an index of lipid peroxidation by measuring the absorbance at 535 nm with a spectrometer. The inhibitory activity against liposome peroxidation was calculated by the following equation:

$$\text{Inhibition effect (\%)} = \left[1 - \frac{\text{absrobance of sample at 535 nm}}{\text{absorbance of control at 535 nm}} \times 100\right]$$

2.5.3. Reducing power

The reducing power of the tested samples was determined according to the method of Jayanthi and Lalitha [15] with some modifications. The reaction mixture, containing an equal volume (0.25 ml) of the *XN*-fermented and un-fermented extract (0.39–12.5 mg/ml) or ascorbic acid (0.005–0.100 mg/ml), 0.2 M phosphate buffer (pH 6.6) and 1% potassium ferricyanide was incubated at 50 °C for 20 min. After cooling, the mixture was mixed with 0.25 ml of 10% trichloroacetic acid and then centrifuged at 4500 × g for 10 min. The suspension (0.5 ml) was mixed with distilled water (0.5 ml) and a freshly prepared 0.1% ferric chloride solution (0.1 ml). The absorbance was measured at 700 nm with a spectrometer. The control was prepared in a similar manner excluding samples. A higher absorbance of the reaction mixture indicated a higher reducing power.

2.6. Anti-inflammation activities

2.6.1. Inhibitory effect on cyclooxygenase −2 (COX-2) activity

All samples were tested for their ability to inhibit COX-2 activity by using a COX-(human recombinant)-inhibitor screening kit (Catalog No. 560,131, Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions. Cyclooxygenase catalysis is the first step in the biosynthesis of arachidonic acid (AA) to PGH2 (the first inducer of inflammation pathway at a site of injury). PGH2 is subsequently converted to a variety of eicosanoids that include PGE2, PGD2, PGF2α, PGI2, and thromboxane (TX) A2 [16]. In this assay, PGF2α is produced from the reduction of PGH2 with stannous chloride and is measured by enzyme immunoassay. Stock solutions of samples at a concentration of 5 mg/ml were dissolved in