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Enhanced extraction genistein from pigeon pea [*Cajanus cajan* (L.) Millsp.] roots with the biotransformation of immobilized edible *Aspergillus oryzae and Monacus anka* and antioxidant activity evaluation

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ABSTRACT

A new method of enhanced extraction genistein from pigeon pea [*Cajanus cajan* (L.) Millsp.] roots with the biotransformation of immobilized edible *Aspergillus oryzae* and *Monacus anka*, was investigated. It showed that immobilized *Aspergillus oryzae* and *Monacus anka* on sodium alginate effectively supported the highest genistein extraction yield by screening microorganism tests. After biotransformation process with immobilized *Aspergillus oryzae* and *Monacus anka* under 30 °C, pH 6.0, 2 days, liquid-solid ratio 12:1 (mL/g), the extraction yield of genistein reached 1.877 mg/g, which was 2.65-fold to that of normal extraction yield. Moreover, IC_{50} values of the extracts measured by DPPH-radical scavenging test and β-Carotene-linoleic acid bleaching test were 0.737 mg/mL and 0.173 mg/mL (control sample 1.117 mg/mL and 0.216 mg/mL), respectively. SOD (Super Oxygen Dehydrogenises) activity of the extracts treated with immobilized microorganism which was stronger than that of the untreated pigon pea roots (1.44 U/mg) at the concentration of protein (0.9375 μg/mL) was 1.83 U/mg. The developed method could be an alternative method for the enhanced extraction of genistein from plants and could be potentially applied in the food industry

1. Introduction

Pigeon pea [*Cajanus cajan* (L.) Millsp.], a famous and multipurpose grain legume crop, is widely distributed in subtropical areas and tropical areas. Nowadays, pigeon pea is cultivated for forage and edible beans. It is also an important source of protein [1,2]. Its roots can be used as healthy additive for cooking soup in China. The phytochemical study indicates that pigeon pea roots are rich in flavonoid constituents such as genistein and genistin. Genistein (4, 5, 7-trihydroxyisoflavone), a most potential and naturally occurring isoflavonoid, is commonly used as a dietary supplement [3]. It displays a range of potential health beneficial effects, and it has stimulatory effect on tyrosine kinase inhibition and possesses antioxidant [4], anti-inflammatory, low toxicity properties [5]. The common source of genistein is soybean, but its availability is limited because of the low genistein content. Genistin (genistein-7-O-β-d-glucopyranoside) is a glycoside of genistein, which shows weaker potential for absorption in intestine than genistein [6]. Furthermore, genistin has weaker antioxidant potency in the LDL oxidation assay [7] and DPPH radical-scavenging assay than genistein. Therefore, extraction of active genistein is much more interesting than genistin.

The extraction of genistein from pigeon pea roots can be carried out by a variety of ways [8]. However, their extraction yields of genistein were still very low because of its lower content in plant. Their methods are inapplicable in industrial scale-up production due to the high cost for equipments and limited material throughput. Nowadays, enzymes were used to hydrolyze genistin to genistein [9], however, the application of enzymes in industrial scale is challenging due to the high costs and the discontinuity activity. Actually, the microorganisms can produce enzymes more economically and continuously. Further more, the immobilized microorganisms facilitate separation of cells from product, allow reuse,and enhance enzymes stability [10].

Application of microorganisms for the biotransformation of target compound has been taken into practice because of microorganisms enzyme's high specificity and environmental

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compatibility [11–15]. Such biotransformation possesses high stereo and region selectivity, ease of handling, low cost and environmental friendliness [16]. It was also reported that *Monascus naka* produced various enzymes, including β-Glucosidase, α-amylase and glucoamylaseas. It was reported that *Aspergillus oryzae* can produce β-Galactosidase [17–19]. β-Glucosidase can cleave β-1,4 linkages and is able to hydrolyze cellooligosaccharides and cellobiose into glucose. Some reports testified β-Glucosidase can transform genistin to genistein [20].

Immobilization of cells to solid carriers is a widely used strategy to improve stability of biocatalysts such as storage and operational stabilities. Moreover, this strategy can increase selectivity towards other substrates compared with the corresponding free cells. Additionally, cell immobilization is highly thermal stable due to the molecular rigidity introduced by binding it to a rigid support and creation of a protected microenvironment for the biocatalyst [21]. Compared with the immobilization of enzymes, the immobilization of living cells could produce enzymes continuously.

In the present study, enhanced extraction genistein from pigeon pea roots using *Aspergillus oryzae* and *Monacus anka* co-immobilized with sodium alginate was investigated for the first time. Considering the food safety of microorganisms, potential edible strains were screened from eight kinds of microorganisms and were evaluated by the biotransformation ability from genistin to genistein. Then the fermentation parameters including pH, temperature, time and liquid-solid ratio were optimized in a continuous recirculation bioreactor to obtain higher productivity of genistein. Antioxidant activities of the extracts of pigeon pea roots incubated with immobilized cells were determined by DPPH-radical scavenging test, β-Carotene-linoleic acid bleaching test and SOD activity test.

2. Materials and methods

2.1. Materials and microorganisms

The strains of *Yeasts*, *Aspergillus niger*, *Aspergillus oryzae*, *Rhizopus arrhizus*, *Saccharomyces cerevisiae*, *White-rot fungus*, *Lactic acid bacteria* and *Monacus anka* (*Yeast* DQY-1, *Yeast* JB, *Yeast* CICC 1912, *Aspergillus. niger* 3.3883, *Aspergillus niger* M85, *Aspergillus niger* 3.3148, *Aspergillus oryzae* 3.951, *Aspergillus oryzae* Y29, *Aspergillus oryzae* 3.302, *Rhizopus arrhizus* 3.130, *Saccharomyces cerevisiae* BX24, *Monacus anka* 3.554, *Monacus anka* 3.782, *White-rot fungus* 5.776 and *White-rot fungus* F-9 were purchased from the Institute of microbiology, Heilongjiang, China.

Genistein (4′,5,7-trihydroxyisoflavone) and genistin (4′,5,7-trihydroxyisoflavone-7-glucoside) were purchased from Fluka (Buchs, Switzerland). 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) and β-Carotene-linoleic acid were supplied by Sigma–Aldrich (Taufkirchen, Germany). SOD assay kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Ethanol and other organic reagent obtained from Tianjin Chemical Reagents Co. (Tianjin, China) were of analytical grade. Methanol was of HPLC grade (J & K Chemical Ltd., China). Formic acid of HPLC grade was purchased from Dima Technology INC. (USA). Distilled deionized water was supplied using a Milli-Q water purification system (Millipore, MA, USA).

2.2. Preparation of raw material

Dried pigeon pea roots were collected during the autumn season from Hainan province, China, and authenticated by Prof. Shao-Quan Nie. The whole dried pigeon pea roots flour was obtained with knifeblade mill (Marconi, Piracicaba, SP, Brazil).

2.3. Co-immobilization of cells and Screening of the potential strain

For *Yeasts*, *Aspergillus niger*, *Aspergillus oryzae* and *White-rot fungus* strains, *Monascus anka*, *Rhizopus arrhizus*, *Saccharomyces cerevisiae and Lactic acid bacteria* were used in this study. The pure culture of the filamentous fungus, each strain was initially reactivated with successive inoculations for test tubes with PDA (Potato Dextrose Agar), incubated at 30 °C for 4 days, transferred to 250 mL conical flasks containing 100 mL PDA medium and incubated at 30 °C for more 6 days for sporulation of the microorganisms on the surface of the culture medium. After this period, 50 mL of Tween 40 solution (15 mg/L) (Atlas Chemical Industries, Inc.) were added to each flask plus a set of glass beads, stirred for 1 min and then transferred to an empty, sterilised flask, after homogenisation, the inoculum was adjusted to 1.75×10^7 spores/mL, which were stored at 4 °C for subsequent inoculation.

For immobilization, 10 mL of spore suspension (1.75×10^7 cells/mL) were mixed thoroughly with 10 mL of 6% sterilized sodium alginate solution (final concentration). After proper mixing, the suspension was dropped by a 5 mL disposable plastic syringe into 100 mL of 2% $CaCl_2$ solution with continuous stirring. The Ca-alginate gel beads were formed and kept to harden in the same $CaCl_2$ solution for 2 h at 4 °C. Then, the beads were washed using sterile distilled water for several times to remove the excess of $CaCl_2$ solution and free cells. The mean diameter of beads obtained was about 0.5 mm. Then, all the beads were grown in 100 mL of the 20% potato extract, 2% carbon sources and 1% nitrogen sources mediums with one gram pigeon pea roots by shaking at 30 °C and 130 rpm for 1–5 days.

2.4. Sample solution preparation

The pigeon pea roots were extracted with 100 mL of ethanol-water (80: 20, v/v) solution by ultrasound-assisted extraction method in 30 min for three times. The extracts were gathered and concentrated to dryness by removing the ethanol solvent in a rotary evaporator (RE-52AA, Shanghai Huxi Instrument Co., China) at 50 °C to dryness and dissolved in 10 mL of methanol to obtain the sample solution for determined by HPLC analysis.

2.5. Determination of enzyme activity

Enzyme units were calculated using PNPG as a substrate, under standard assay conditions in the described method [22]. Enzyme activity of the immobilized cells was calculated by the absorbance measured at 400 nm. The activity was defined as mmol of p-nitrophenol produced per minute under the assay conditions (U). The enzyme activity was calculated as per gram with the immobilized cell (U/g) and the activity of co-strains was 1.04 U/g. The assays were done in triplicate on each sample and the average values were used.

2.6. Fermentation process parameters

The effects of various fermentation process parameters such as incubation time, incubation temperature, initial pH, etc. were investigated. Control experiments were similarly carried out by inoculating 10 mL viable cells or without cells. All the experiments were carried out with three independent repetitions.

2.7. Determination of genistein and genistin

The analysis was carried out using an Agilent 1200 reversed-phase HPLC system. A HIQ Sil C18 W reversed-phase column (250 mm × 4.6 mm i.d., 5 μm) was used with an oven temperature