Optimization in Bioconversion of Quercetin Glucosides Using *Aspergillus acueletus* LS04-3

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ABSTRACT

Microbial transformation is known to obtain more active or less toxic compounds and achieve selective compounds conversion to more active or less toxic derivatives. This study aims to explore several factors that affect the optimization of bioconversion of quercetin glucosides using *Aspergillus acueletus* LS04-3. The fungus was cultured under several conditions by varying the number of days of fermentation, the concentration of substrate, carbon, and nitrogen source. The transformation product was analyzed using HPLC and LCMS-MS. The results revealed that quercetin production reached the highest amount on the third day of incubation and the optimum concentration was at 50 ppm of quercetin glucoside based on HPLC analysis. In addition, from various carbon sources, glucose yielded the highest biotransformation product, while nitrogen accelerated the reaction. In this research, media of *A. acueletus* LS04-3 containing carbon and nitrogen could increase quercetin production. This research finding provided several factors for optimizing quercetin biotransformation by *A. acueletus* LS04-3.

1. INTRODUCTION

Microbial transformation is known to be useful for obtaining more active or less toxic compounds and achieving selective compounds conversion to more useful derivatives, which are difficult to be produced synthetically [1]. Microbial factory poses advantages including rapid growth, ease of cultivation, convenient genetic manipulations, and high-level production of natural product biotransformation.

Moreover, microbial production not only increases the product selectivity, but it also reduces the usage of toxic chemicals while conserving energy consumption [2]. Microbial transformation usually makes use of enzyme catalysed reactions such as oxidation reduction, hydrolysis, degradation and formation of regio- and sterio- specific bonds [3,4].

Microbial transformation of quercetin and rutin has long been investigated and reviewed, and occurrence of microbial glycosylation, oxidation, sulfation, methylation, hydroxylation and aromatic ring degradation has been reported [5]. However, only a few studies have done on the metabolic modification of quercetin-3-O-rhamnoside (Quercitrin) [6].

Quercetin is the most common flavonoid in nature and mainly present in its glycosylated forms such as quercitrin (3-rhamnosyl-quercetin) or rutoside (3-rhamnosy-glucosyl quercetin)

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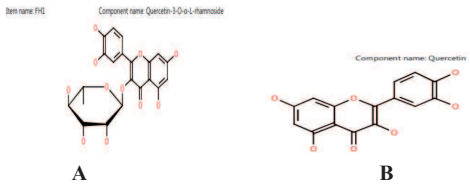

Fig. 1 chemical structure of flavonoid glicoside quercitrin (a) and quercetin (b)

(Fig. 1) [7]. Biotransformation of flavonoid by many microorganisms including species of Cunninghamella, Penicillium, and Aspergillus strains has been observed and they can perform almost all of the reactions with excellent yields. *Aspergillus niger* is one of the most widely used microorganisms in the flavonoid biotransformation [3,4]. However, biotransformation of organic compounds by *A. acueletus* is rarely reported.

A. aculeatus is a species that is often isolated from soil and root, it has black spores and morphological criteria such as colour, shape, size, and ornamentation of conidia have been used to classify strains. It belongs to Section *Nigri* and is closely related to *A. niger.* It has been used to produce various important industrial enzymes (cellulases, hemicellulases, proteases) that are utilised commercially in the food and feed industry [8].

There have been any studies reporting the microbial transformation of flavonoid glycoside by *A. acueletus*. This study is to explore the ability of *A. acueletus* LS04-3 in the biotransformation of quercitrin and the effects of different carbon and nitrogen sources and other factors in enhancing the biotransformation efficiency.

2. EXPERIMENTAL SECTION

2.1 Materials

2.1.1 Microorganism and Chemical

Filamentous fungi *A. acueletus* LS04-3 were obtained from the Indonesian Culture Collection of the Research Centre for Biology, Indonesian Institute of Sciences. The voucher specimen was deposited at -20ºC, while the working stocks were prepared on a Potato Dextrose agar (PDA) petri dish and stored at 4ºC prior to use. Quercetin and quercitrin standard were purchased from Sigma-Aldrich. HPLC-grade methanol, LC-grade acetonitrile, LC-grade methanol, dextrose, sucrose, and starch, from E-Merck. PDA, Peptone, yeast ware obtained from Himedia. Quercitrin was extracted from decoction of *Dentropthoe petandra* leaves purified, and then identified by TLC, LCMS-MS, and 1D-NMR as described in the previous study [9].

2.2 Methods

2.2.1 Optimization studies

The effect of incubation time, initial substrate concentration, and influence of carbon and nitrogen sources on biotransformation of quercitrin were studied using the fungi, in which maximum biotransformation was observed. The culture broth was analysed at the end of day 1, 2, 3, 4, 5, and 6 of incubation to study the effect of incubation time. Then the effect of initial substrate concentration was observed by adding 10, 20, 50, 100, and 200 mg of quercitrin, which have been dissolved in 0.5 mL methanol. Each solution was administered to different suspension culture. Furthermore, the influence of carbon source was studied by replacing the dextrose of potato broth (200 gr potatoes were cut into pieces, boiled for 1.5 hours, filtered and made up to 1L solution) of the medium with different carbon sources containing, glucose, sucrose, and amylum 2% each. The influence of nitrogen sources was evaluated by adding 0.5% of peptone, yeast, and $NaNO_3$ into PDB medium.

2.2.2 Culture biotransformation

The composition of growth medium for *A. acueletus* LS04-3 was varied depending on the purpose of the study. Two discs (5mm) of fungal *A. acueletus* LS04-3 were inoculated into ten Erlenmeyer flasks (300 mL) which contain 100 ml of potato dextrose broth (PDB). Then incubated in a rotary shaker at 200 rpm and 28º C for two days. Substrate quercitrin (50 mg/flask solubilized in methanol pa) was added as substrate, and incubation continued for five days more. The substrate addition day was counted as D-0. Samples were examined daily for five days (D1, D2, D3, D4, and D5). Culture controls

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consisted of a fermentation blank in which the microorganism was grown under identical conditions and no substrate was added.

2.2.3 Extraction and sample preparation of quercitrin and metabolites

Each culture was studied in duplicate. Sampling was carried out every 24 h. Broth of culture was filtered to separate between filtrate (F) and mycelia (M). The filtrate and mycelia were extracted three times with ethyl acetate and concentrated under reduced pressure. The crude extracts of the F and M were prepared for analysing the biotransformation product using HPLC and LCMS-MS in accordance to analytical procedures as previously reported [10].

3. RESULT AND DISCUSSION

3.1 Influence of substrate concentration

The optimal conditions to produce quercetin using the *A. acueletus* LS04-3 strain were determined by varying the substrate concentration and biotransformation time. Substrate concentration can affect the rate of transformation that occurs. Therefore it is necessary to determine the optimum substrate concentration that can be hydrolyzed by the fungus *A. ascueletus* LS04-3 with variations in the addition of substrate concentrations from 10, 20, 50, 100, and 200 mg/100 mL media.

Based on the HPLC analysis results, the conversion rate of quercitrin was calculated from the ratio of mmol of the substrate compound added to mmol of quercetin formed, as shown in Figure 2.

Conversion rate of quercetin was increased with increasing quercitrin concentration from 10 to 200 mg/100mL. However, the concentration at 50 mg/100 mL is a limit of saturation (of substrate bioconversion). Furthermore, the concentration of quercetin higher in the filtrate extract than biomass extract indicated that the reaction occurred intracellularly.

3.2 Effect of incubation time

The conversion rate of quercetin was evaluated by HPLC compared yield product after incubation for 1, 2, 3, 4, 5, 6 and 7 d, respectively (Figure 3).

The biotransformation of quercitrin into quercetin occurs since the first day of incubation after adding substrate (D-1) and reaches the maximum product after 72nd h incubation period, then decreases until end incubation time. Meanwhile, the concentration of quercitrin decreased from day 2 to the end of the incubation period. Therefore, it's probably due to physical adsorption on the biomass.

The change of biotransformation product in the media during the fermentation was determined through LC-MS/MS based on their retention time, exact mass, and compound name, the results of which are summarized in Figur 4 and Table 1.

The quercitrin concentration will decrease due to continuous transformation until the flavonoid compounds turn into simple phenolic compounds such as trihydroxy benzoic acid and protocatechuic acid [11], as shown in Figure 4.

Degradation to simple polyphenols leads to the same results with was done by Xu [11] with the microorganism *G. deliquescens* mentioned the presence of the quercetinase enzyme in molds of the *Aspergillus* genus, which causes the quercetin

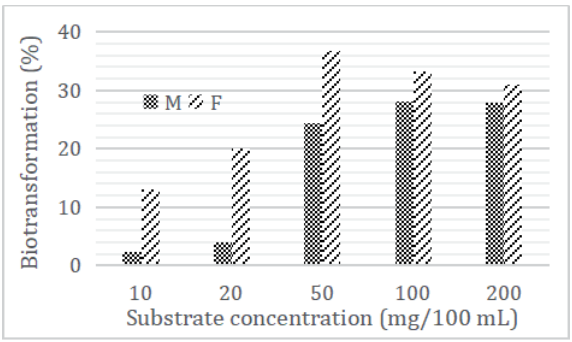

Fig. 2. The Effects of different substrate concentration on the biotransformation of quercetin

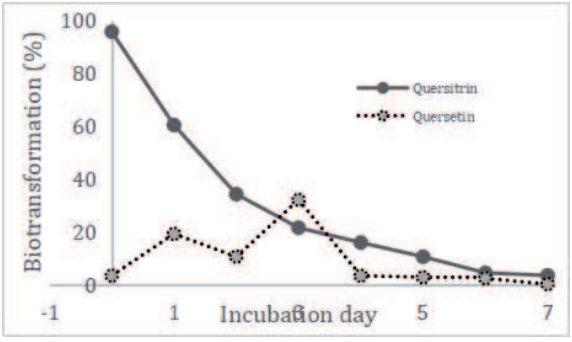

Fig. 3 The Effects of incubation time on the biotransformation of quercetin

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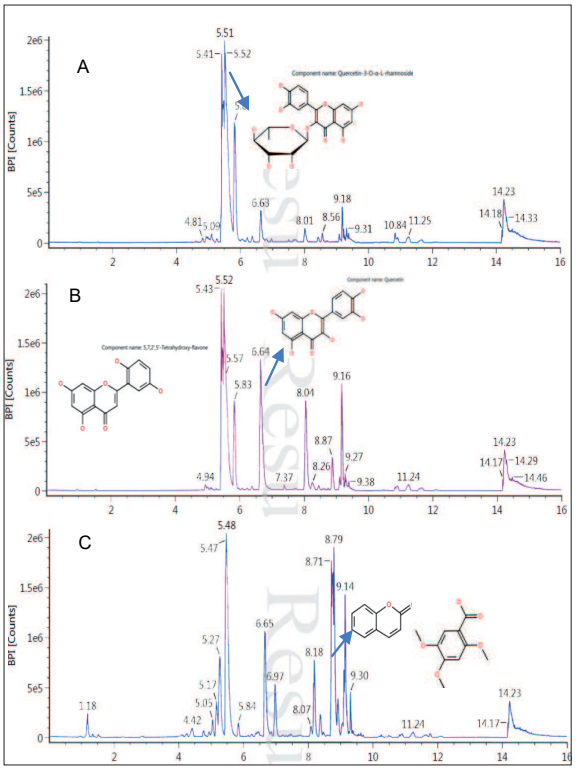

Fig 4. LCMS-MS chromatogram of biotransformation compound during incubation time. A: D-1; B: D-3; and C: D-7

formed to undergo oxidative cleavage and then degrades to simple polyphenols.

3.3 Influence of carbon and nitrogen sources

The effect of selected carbon sources on quercitrin conversion by *A. acueletus* LS04-3 in broth medium by replacing the glucose with sucrose and amylum was studied and results are given in Figure 5.

The change in carbon sources has shown differences in height and area of the peak, and glucose as carbon sources reached the heighest peak indicated the influence on the quantity of metabolite formed or on biotransformation of quercitrin by *A. acueletus* LS04-3.

Carbon is the major structural and functional component in microbial cells and plays an important role in the nutrition of fungi. From this study, glucose and sucrose were the two most carbon sources to produce quercetin. Several studies also reported that disaccharides and monosaccharides

Table 1. Identification of metabolite compounds during biotransformation of quercitrin

Day	Observed RT (min)	Observed m/z	Component name
1	5,42	449,1072	Quercetin-3-O-α-L-rhamnoside
3	5,42	449,1072	Quercetin-3-O-α-L-rhamnoside
	6,54	302,0496	Quercetin
	7,21	287,0545	5,7,2',5'-Tetrahydroxy-flavone
	9,37	288,2527	Candidate Mass 288,2527
7	4,48	227,1383	2-(p-Anisyl)-5-methyl-1-hexen
	5,06	213,0750	2,4,5-Trimethoxybenzoic acid
	6,80	147,0431	2H-1-Benzopyran-2-one
	9,52	377,0831	4-O-Caffeoylquinic acid-1
	9,52	321,2030	Ciryneol A
	6,36	227,0904	dehydromorroniaglycone
	9,32	353,1745	Dihydroguairetic acid
	7,04	409,1274	Eldutin
	8,51	249,1842	methyl arteannuate

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are more preferred by fungi as carbon sources transformation rather than polysaccharides [3, 12, 13].

Aspergillus niger has also been widely studied and is known for utilizing sugar as the sole carbon source and energy for cell growth and metabolism [14]. Hamad *et al.,* [15] described that for *A. niger*, fructose is mostly preferred after sucrose as the carbon source. Glucose and maltose are proved to be good carbon sources too as they have higher affinity than amylum, which is a polysaccharide with poor carbon source for the growth.

Nitrogen is used for functional and structural development by the fungi and play a profound influence on the biotransformation by fungi. Thus, reported studies are conflicting claims regard on comparative superiority of a particular form or source of nitrogen over the other. Differences in substrates and microorganisms used may be the main cause of the need for different nitrogen sources of each biotransformation reaction.

Influences of nitrogen source on the transformation of of quercitrin by *A. acueletus* LS04-3 was investigated by added peptone, yeast, and sodium nitrate to the medium, respectively. The result was showed in figure 6.

The HPLC analysis results showed that the addition of nitrogen sources causes the biotransformation process to be faster where quercetin can be found in the extract in the first 24th hours of the incubation period. However, the efficiency increasing still low (Fig. 6). From this study, peptone followed by yeast was the most favoured nitrogen source for the transformation of quercetin-3-O-rhamnoside to quercetin.

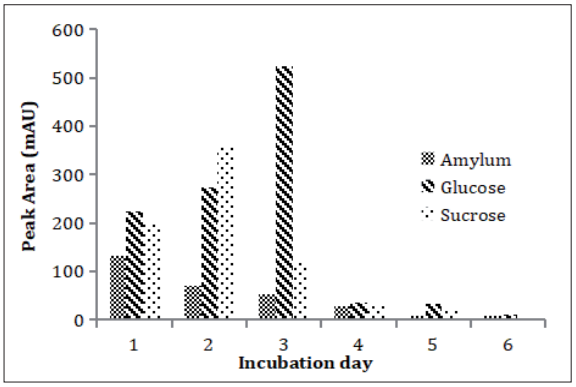

Fig. 5. The Effects of different carbon sources on the biotransformation of quercetin in filtrate extract

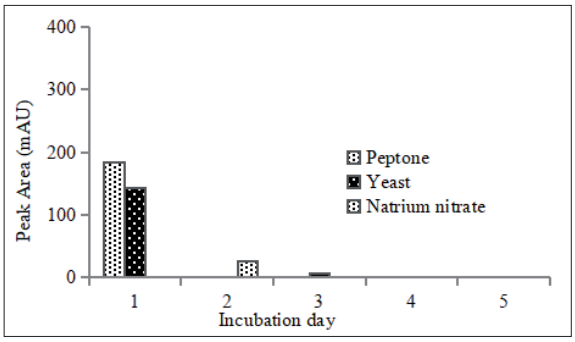

Fig. 6. The Effects of different nitrogen source on the biotransformation of quercetin

4. CONCLUSION

In the present study, it can be concluded that there is an influence of the media components, incubation periods, and substrate concentration on the biotransformation of quercetin by *A. ascueletus* LS04-3. From the present results, it was found that glucose is the best suitable carbon source compared to other carbon sources at a 2% concentration and peptone extract at 0.5% is the best suitable nitrogen source. Maximum metabolite i.e. 40% was obtained at 72nd hr of incubation period at PDB medium. It can be revealed that *A. ascueletus* can be used as an in vitro model for the study of the metabolism of flavonoid glycosides.

5. ACKNOWLEDGMENT

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