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Physiological and Chemical Studies on the Bioconversion of Glycyrrhizin by *Aspergillus niger* NRRL595

Abdel-Monem H. El-Refai, Lotfy A. R. Sallam, Hassaan A. El- Menoufy and Hala Abdel Salam Amin*

Chemistry of Natural and Microbial Products Department, National Research Center, Dokki, Cairo, Egypt
E-mail: halaamin2007@yahoo.com

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ABSTRACT

Glycyrrhizin (GL), the well-known sweet saponin of licorice, has been used as a food-additive and as a medicine. Its aglycone, glycyrrhetic acid (GA) showed antiinflamatory, antiulcer and antiviral properties. GA is now produced form GL by acid hydrolysis. However, it is difficult to obtain GA in a good yield by using this method, because many by-products are also produced. Screening of different microorganisms (13 bacteria, 2 yeasts and 23 fungi) for production of GA from GL revealed that *Aspergillus niger* NRRL 595 produced the highest yield of GA. The bioconversion of GL by *A. niger* NRRL 595 for 96 h, followed by isolation and purification of the transformation products led to the separation of two conversion products, namely: GA and 3-oxo-GA. Confirmation of the identity of these products was established by determination of their Rf values, m.p., and IR, UV, MS and NMR spectra. The conditions for cultivation of this fungus with the maximum hydrolytic activity for the maximum yield of GA were investigated. Based on the results, *A. niger* NRRL 595 was cultivated with a medium composed of 1.75 % GL, 0.5 % glucose, 0.8 % corn steep liquor at pH 6.5 at 32 °C for 96 h. The cultivation of fungal cells under the latter conditions afforded GA and 3-oxo-GA in a yield of 65 % and 22 %, respectively.

Keywords: Glycyrrhizin, glycyrrhetic acid, bioconversion, A. niger NRRL 595

INTRODUCTION

Glycyrrhizin (GL) as a triterpenoid saponin derived from the root of licorice (Glycyrrhiza glabra) which is one of the most popular traditional medical herbs. GL is composed of one molecule of glycyrrhetic acid (GA) as aglycone and two molecules of glucuronic acid (Hennell et al. 2008). GA has expectorant and antitussive properties. It is widely used as a flavoring agent and is frequently employed to mask the taste of bitter drugs such as aloe and quinine. Contemporary research has identified anti-inflammatory, antiulcer, antiallergic, antiviral, antibacterial, and hepatoprotective effects demonstrated systemically by GA (Bombardelli et al. 1989; Akao 2000; Shibata 2000). Hyperkalemia is a common life-threatening problem in hemodialysis patients. Because GA inhibits the enzyme 11β-hydroxy-steroid dehydrogenase II and thereby increases cortisol availability to the colonic mineralocorticoid receptor, it has the potential to lower serum potassium concentrations (Farese et al. 2009). Recent study has shown that GA exhibits many pharmacological activities. Here a new derivative of GA, biotinylated GA, was synthesized by introducing the biotin to the C-29 carboxyl of GA, and its antitumor effects were confirmed through tests on mouse B16 melanoma cells and BEL 7402 human hepatocarcinoma cells (Shi et al. 2009). Kalaiarasi et al. (2009) reported that administration of 18β-glycyrrhetinic acid is able to reduce hyperglycemia and hyperlipidemia related to the risk of diabetes mellitus.

In principle, GA could be obtained by acid hydrolysis of the natural saponin followed by multi-stages processes for the purification of the resulting aglycone (Mahran et al. 1973). However, it is difficult to obtain GA in a good yield by this method, because many by-products are also produced. On the other hand, it has been disclosed that an enzyme named glycyrrhizinic acid hydrolase from *Aspergillus niger* selectively hydrolyzed 3-O-β-D-glucuronide linkage of GL without cleavage of the terminal glucuronobiose bond to give GA and glucuronobiose (β-D-glucuronyl-(1-2)-D-glucuronic acid) in a high yield without formation of glucuronic acid (Muro et al. 1986). The action of this enzyme was different from those of the β-glucuronidases so far reported (Akao et al. 1986; Kim et al. 1999). Hattori et al. (1985) studied transformation of GL and related compounds by bacterial strains isolated from human feces. They found that *Ruminococous* sp. PO1-3 hydrolyzed GL to GA in a yield of 34 % after incubation for 40 h. Sakano & Ohshima (1986a) studied the conversion of GA using *Streptomyces* sp. G-20 with the aim of preparing useful new products. The authors found that this culture converted GA into 22-α-hydroxy-18 β-GA (as a major product) and two minor hydrolyxlated derivatives of that later major product. In addition, the same authors Sakano & Ohshima (1986b) found that *Chainia antibiotica* IFO 12, 246 converted GA and 22α-hydroxy GA into three kinds of 3,4-seco-oleanane-type conversion products. Studies were made by El- Menoufy (1988) on 102 different microbial cultures and found that *Saccharomycoposis lipolytica* was the most active

***Corresponding author**

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organism, it catalyses the conversion of crude GL to GA in a yield of 49.6%. The capacity of 8 bacterial strains, isolated from soil, for the conversion of GL to GA was assayed. *Pseudomonas saccharophila* showed the highest β-glucuronidase activity and may be useful for industrial fermentation (Tanaka et al. 1990).

The biotransformation process was found to be markedly influenced by the fermentation conditions including medium composition (kind and concentration of nutrients, substrate/product concentration), incubation temperature, pH value, dissolved oxygen (Kuramoto et al. 1994; Kim et al. 1999; Akao 1999a). Thus El- Menoufy (1988) found that among six nutritive media differing in the nature and/or the concentration of some constituents, the one containing glycerol, peptone, yeast extract, meat extract and KH_2PO_4 was the most favorable for the formation of GA from GL with *Saccharomycopsis lipolytica*. The present study aims at the selection of the most potent microorganism able to hydrolyze GL to GA. The selected strain was cultivated under different physiological conditions to evaluate its ability to transform GL, in order to achieve the maximal conversion output.

MATERIALS AND METHODS

Microorganisms

Different microorganisms used in this work (Table 1) were donated by the Center of Cultures of Natural and Microbial Products Chemistry Department, National Research Center, Cairo, Egypt. Experimental cultures were inoculated with descendants from a single slant of pure experimental organisms on potato dextrose agar. All the slant cultures were stored in a refrigerator with regular transfer every month.

Types of media

Medium I (%, w/v): GL, 1; glucose, 1 and corn steep liquor (CSL), 0.8; pH 5.7 (Kuramoto et al. 1994).
Medium II (%, w/v): GL, 1; CSL, 0.8; yeast extract 0.4; malt extract, 2; pH 5.3.
Medium III (%, w/v): Malt extract, 2; yeast extract, 0.4; pH 5.3 (Abd-Elsalam 2000).
Medium IV (%, w/v): GL, 1; CSL, 0.8; pH 5.7.
Medium V (%, w/v): Glucose, 2; peptone, 0.5; meat extract, 0.3 ; dry yeast, 0.3; NaCl, 0.5; $CaCO_3$, 0.3; pH 7 (Sakano & Ohshima 1986b).
Medium VI (%, w/v): Glycerol, 1; peptone, 0.5; meat extract, 0.3; yeast extract, 0.3; K_2HPO_4, 0.5; pH 7.4 (Sakano & Ohshima 1986a).
Medium VII (%, w/v): Glycerol, 0.5; glucose, 0.5; peptone, 0.5; yeast extract, 0.3; meat extract, 0.3; KH_2PO_4, 0.75; pH 5.6 (El- Menoufy, 1988).
Medium VIII (%, w/v): GL, 1; glucose, 1; peptone, 0.5; yeast extract 0.3; KH_2PO_4, 0.1; $MgSO_{4\cdot7}H_2O$, 0.05, pH 5.7 (Kuramoto et al., 1994).
Media III, V, VI, and VII were supplemented with 1% GL (w/v) in the experiment studying the effect of medium composition on GL bioconversion.

Table 1: Bioconversion of GL to GA by the experimental micro-organisms as traced visually*.
TLC conditions

Microorganism	GA	Microorganism	GA
Bacteria		*M. rasimosis*	–
Bacillus sphaericus	-	*Aspergillus niger NRRL 595*	+
B. cereus	-	*A. niger NRRL*3	+
B. megaterium	-	*A. niger 1*	+
B. coagulans	-	*A. niger 2*	+
B. sp. 25	-	*A. niger 4*	+
B. sp. 38	-	*A. neveus*	–
B. subtilus	-	*A. fumigatus*	–
Mycobacterium luteus	+	*A. flavus*	–
Micrococcus sp. 360	-	*A. terreus*	+
Micrococcus sp. 6B	-	*A. ochraceous*	+
Staphylococcus aureus	-	*A. versicolor*	-
Escherichia coli	-	*A. carneus*	-
Pumillus sp. E 601	-	*Penicillium auranticum*	-
Yeasts		*P. waksmanii*	-
Candida tropicalise	-	*P. aurantigrism*	-
Saccharomyces cerevisiae	-	*P. chrysogenum*	-
Fungi		*P. islandicum*	-
Mucor roxii	-	*P. cyclopium*	+
M. pusillus	-	*Fusarium solani*	+

- Transformation medium (medium I) (%, w/v): GL, 1; glucose, 1, CSL, 0.8; pH 5.7.
- *The presence of GA was detected by TLC analysis (p. 37) and sign. (+) denotes its formation, while sign. (-) means that the microorganism fails to produce GA.

Thin layer chromatography (TLC) was performed on silica gel plates, (Fluka, silica gel 60F-254, layer thickness 0.2 mm) using the following solvent systems:
A. Chloroform – petroleum ether – acetic acid (6:6:1, v/v).
B. Acetic acid – n-butanol – 1,2-dichloroethane – water) (4:1:4:,1. v/v).

Preparation of inocula

For bacteria and yeasts, a suspension of each microbial strain (2 mL of 48h old cultures) was used to inoculate aliquots of 50 mL of liquid medium composed of (%, w/v) glucose, 1; peptone, 0.5; yeast extract, 0.3; malt extract, 0.3; agar, 2; pH 6.5 (Volesky & Philips 1995) dispensed in 250 mL Erlenmeyer flasks. The flasks were agitated on a reciprocal shaker at 200 rpm at 30°C ± 2 for 24 h. For fungi, aliquots of 50 mL of liquid growth medium III were dispensed in 250 mL Erlenmeyer flasks and sterilized.

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The sterilized medium was inoculated with 2 mL of spore suspension prepared by adding 5 mL sterile distilled water into 6 days old slant culture. The flasks were agitated on a rotary shaker (200 rpm) at 30°C for 48h.

Biotransformation process: Screening expermints

The sterilized fermentation medium I (containing 1 % GL) was inoculated with the standard volume of inoculum (2 mL each for bacteria and yeasts and 5 mL for fungi). The flasks were agitated on a reciprocal shaker (200 rpm) at 30 °C ± 2 for 48 h in case of bacteria and yeasts and for 72 h in case of fungi.

Biotransformation process using *A. niger* NRRL 595

Unless otherwise stated, medium I (containing 1 % GL) (Kuramoto *et al*. 1994) was used for GA production by *A. niger*. The medium was adjusted to pH 5.7 using NaOH or HCl solution, dispensed in 50 mL portions in 250 mL flasks and sterilized by autoclaving at 121°C for 15 min at 1.5 atmospheric pressure. These flasks were inoculated with 5 mL of the preculture suspension. Then, the flasks were fixed on a rotary shaker at 200 rpm and 30°C for 72 h.

Extraction and determination of the converted products

At the end of the transformation period, contents of each flask (cells and medium) were acidified with 1 N HCl to pH 2. The reaction mixture was extracted twice with double its volume of ethyl acetate. Then, the organic layer was washed three times with water to get rid of any residual HCl, dried over anhydrous sodium sulphate and evaporated to dryness in vacuo to give solid residue (test material) (Kim et al. 1999). The residue was dissolved in chloroform - methanol mixture (1:1) and mounted on TLC plates. The plate was first chromatographed for GA and 3-oxo-GA with solvent system A, and secondly for GL with solvent system B (Akao et al. 1991). GL, GA and 3-oxo-glycyrrhetic acid (3-oxo-GA) were detected on TLC plates under UV light or by acid charring (10 % H_2SO_4, 110°C, 10 min).These compounds were quantitatively analyzed with TLC scanner (Shimadzu CS-9000 dual wave-length flying spot, thin layer chromato-scanner, Japan) at λs =250 nm and λr = 400, by using calibration lines obtained from authentic samples (Hattori et al. 1985).

$$\text{GA yield (\%)} = \frac{\text{GA concentration}}{\text{GL concentration}} \times 1.75 \times 100\%$$

1.75 = Factor of conversion of GA to GL on molecular base.

$$\text{3-oxo-GA yield (\%)} = \frac{\text{3-oxo-GA concentration}}{\text{GL concentration}} \times 1.76 \times 100\%$$

1.76 = Factor of conversion of 3-oxo-GA to GL on molecular base.

Separation and identification of the transformation products

Preparative TLC was carried out for separation of considerable amounts of transformed products. The test material dissolved in small volume of chloroform was applied to silica gel plates. The plates were eluted using solvent system A and the resulted separated zones were scrapped off and extracted with chloroform. The solvent was filtered off and evaporated to dryness in vacuum. This leads to separation of two crude compounds I (150 mg) and II (80 mg). Compounds were further purified separately on Sephadex LH-20 column (2.5 X 60), using methanol as an elution solvent. The purified transformed products I and II were then analyzed by infrared (IR), UV, FD-mass and NMR.

Compound I: It was identified as glycyrrhetic acid from its m.p., IR, UV, MS and NMR. It was obtained as colourless crystals (110 mg); m.p. 293 – 295 °C (Reported m.p. 294-296, Hattori et al. 1983); UV λ_{max} ($CHCl_3$): 250 nm; IR (potassium bromide) cm^{-1} : 3438 (OH), 1706 (C=O),1664 (conjugated C=O) 2875, 2944, 2968 (CH aliphatic); MS : m/z 470 (M^+, 16.74 %), 303 (100 %), 262 (81 %), 248 (19 %), 216 (17 %), 175 (49 %), 135 (80 %); ^{1}H NMR (DMSO-d_6) ppm : δ 00.68, 00.74, 00.91, 01.02, 01.12, 01.33, 01.46 (each 3H, s,C-CH_3), δ 03.04 (1H, m, CH-OH), δ 05.44 (1H, C=CH), 12.2 (br, 1H, OH, disappears after exchanged by D_2O); ^{13}C-NMR: δ 016.0 – 055.3 (25 SP^3 carbons corresponding to C_1, C_2, C_4-C_{10}, C_{14}-C_{29}), δ 076.6 (CH-OH, corresponding to C_3), δ 127.3, 169.7 (2 SP^2 carbons, C=C, corresponding to C_{12}, C_{13}), δ 177.7, 199.0 (2 SP^2 carbonyl carbons, C=O, corresponding to C_{11}, C_{30}).

Compound II (3-oxo- glycyrrhetic acid): was obtained as colourless crystals (60 mg); mp. 305°C (Reported m.p. > 300, Hattori et al., 1983); UV λ_{max} ($CHCl_3$): 250 nm; IR (potassium bromide) cm^{-1}: 3312 (COOH), 1727, 1682 (2C=O), 1645 (conjugated C=O), 2875, 2924, 2968 (CH aliphatic) ; MS : m/z 468 (M^+, 47 %), 453 (25 %), 440 (27 %), 422 (17 %), 303 (94 %), 262 (93 %), 216 (27 %), 137 (19 %), 135 (100 %) ; ^{1}H-NMR (DMSO-d_6) ppm : δ 00.76, 00.94, 00.98, 01.05, 01.07, 01.13, 01.33 (each 3H, s, C-CH_3), δ 05.44 (1H, s, C=CH) ; ^{13}C-NMR : 015.8 – 060.5 (25 SP^3 carbons corresponding to C_1, C_2, C_4-C_{10}, C_{14}-C_{29}), δ 127.4, 171.1 (2 SP^2 carbons, C=C corresponding to C_{12}, C_{13}), δ 178.4, 199.0, 216.1 (3 SP^2 carbonyl carbons, C=O, corresponding to C_{30}, C_{11}, C_3, respectively).

RESULTS AND DISCUSSION

Screening experiments

The screening experiments were carried out with ultimate goal of selecting the most active GL transforming microorganism. Studies were made on 38 different microbial cultures (13 bacteria, 2 yeasts and 23 fungi) using GL as a carbon source. These cultures manifested large variations in capacity to produce GA. Thus, while

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some cultures failed to perform the desired reaction (14mould fungal, 13 bacterial and 2 yeast cultures), the other experimental organisms could perform the desired reaction with the production of different amounts of GA, depending on the type of culture employed (Table 1). On the other hand, *Mycobacterium luteus, A. niger* NRRL 595, *A. niger* NRRL 3, *A. niger* 1, *A. niger* 2, *A. niger* 4, *A. terreus, A. ochraceous*, *Penicillium cyclopium* and *Fusarium solani* successfully converted GL to GA (Table 2).Under the experimental conditions employed, the tested *A. niger* NRRL 595 was proved to be the most potent microorganism that could hydrolyze GL leading to formation of promising amounts of GA. It could transform about 18% of the added GL with the formation of about 1mg/mL GA. In this connection, Muro et al. (1986) and Sasaki et al. (1988) reported that glycyrrhizinic acid hydorlase produced by *A. niger* GRM3 selectively hydrolyzed 3-O-β-D-glucuronide linkage of GL to give the aglycon, GA and 2-O-β-D-glucuronyl-D-glucuronic acid (glucuronobiose). Recently, it was reported that GL biotransformation by *A. niger* included two stages: first, the two glucuronic acid residues at the C-3 position of GL were hydrolyzed to produce GA; and second, GA was oxidized and hydroxylated to compounds 1 and 2. These were 7β,15α-dihydroxy-3,11-dioxo-oleana-12-en-30-oic acid (1) and 15α-hydroxy-3,11-dione-oleana-12-en-30-oic acid (2) (Hongzhi et al., 2009).

Table 2: The amounts of GA produced by the promising tested microorganisms

Microorganism	GA out put	
	mg/100 mL	Conversion %
Mycobacterium. luteus	16.9	2.96
Aspergillus niger NRRL 595	101.3	17.73
A. niger NRRL 3	6.90	1.21
A. niger 1	20.76	3.63
A. niger 2	24.8	4.34
A. niger 4	53.63	9.39
A. terreus	20.42	3.57
A. ochraceous	13.38	2.34
Penicillium cyclopium	1.35	0.27
Fusarium solani	8.66	1.52

GL concentration = 1000 mg/100 ml medium

Isolation and identification of the transformation products encountered after transformation of GL by A. niger NRRL 595.

Efforts have been imposed to isolate and confirm the identity of the encountered conversion products. Therefore, the experimental fungus was cultivated for 96h in GL containing medium (namely medium I). Two conversion products, main product I (70 %) and minor product II (30 %) were isolated. The TLC analysis reveals that the main product I which has Rf 0.33 is glycyrrhetic acid (GA) and the other minor product II, whose Rf = 0.68 is 3-oxo-glycyrrhetic acid (3-oxo-GA). This preliminary identification was based on studying the TLC profile of products I and II as compared with authentic samples of the same compounds. The identification of the produced GA and 3-oxo-GA was furthermore confirmed by studying their IR, MS and NMR characteristics.

Table 3: ^{1}H NMR and ^{13}C NMR assignments of the isolated compounds

Carbon No.	GA		3-oxo-GA	
	^{1}H	^{13}C	^{1}H	^{13}C
1	-	38.6	-	40.8
2	-	27.1	-	28.3
3	3.04	76.6	-	216.1
4	-	40.4	-	41.2
5	-	54.1	-	54.0
6	-	18.3	-	21.2
7	-	32.1	-	33.9
8	-	42.9	-	43.5
9	-	61.1	-	60.5
10	-	36.7	-	36.4
11	-	199.0	-	199.0
12	5.44	127.3	5.44	127.4
13	-	169.7	-	171.1
14	-	44.9	-	47.3
15	-	25.7	-	26.0
16	-	26.1	-	26.4
17	-	31.5	-	31.8
18	-	48.0	-	48.3
19	-	40.6	-	43.3
20	-	43.1	-	44.9
21	-	30.3	-	31.5
22	-	37.5	-	37.7
23	0.68	27.8	0.76	28.7
24	0.74	16.0	0.94	15.8
25	0.91	16.2	0.98	18.3
26	1.02	17.2	1.05	18.5
27	1.12	23.0	1.07	23.2
28	1.33	28.1	1.13	28.8
29	1.46	28.4	1.33	30.8
30	12.2	177.7	-	178.4

The maximum absorption of the main conversion product (I) in the UV at 250 is attributed to the characteristic conjugated carbonyl in ring C. IR spectrum displayed an absorption band at 3438 cm^{-1} (br, OH) and two carbonyl absorption bands at 1706 and 1664 cm^{-1}. Its mass spectrum shows a molecular ion peak appeared at m/z 470 (16.47 %) which matched with the molecular formula of GA ($C_{30}H_{46}O_4$). Its ^{1}H-NMR (Table 3) shows 7 signals at δ 00.68-0146 ppm (s, $7CH_3$, corresponding to methyl groups of carbons C_{23} – C_{29}), δ 03.04 (d, 1H, CH – OH, corresponding to C_3), δ 5.44 (s, 1H, C = CH, corresponding to C_{12}). Furthermore, ^{13}C-NMR (Table 3) shows 26 SP^3 carbon atoms corresponding to C_1-C_{10} and C_{14}-C_{29} and $4SP^2$ carbons corresponding to C_{11}, C_{12}, C_{13}, C_{30}. This proves the presence of one carbon attached to hydroxyl group (C_3) that recorded at δ 76.6; two unsaturated carbon atoms (C_{12}, C_{13}) that recorded at δ 127.3 and δ 169.7, respectively; and two carbonyl carbon atoms (C_{11}, C_{30}) which recorded at δ 199 and δ 177, respectively. Therefore, the structure of I was identified as

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glycyrrhetic acid. The spectral data was found to be in good agreement with those published (Hattori *et al*. 1983; Akao *et al*. 1991; Kim *et al*., 1999).

The IR spectrum of the minor conversion product (II) displayed a broad absorption band at 3312 cm^{-1} (COOH) and three carbonyl absorption bands at 1727, 1682 and 1645 cm^{-1}. Its mass spectrum shows a molecular ion peak at m/z 468 (46.46 %) which matches with the molecular formula of 3-oxo-GA ($C_{30}H_{44}O_4$). Its ^{1}H-NMR (Table 3) shows 7 signals δ 00.76 – 01.33 ppm (s, 7CH_3, corresponding to methyl groups of carbons C_{23}-C_{29}), δ 05.44 ppm (s, 1H, C=CH, corresponding to C_{12}). In agreement, with this suggestion, the ^{13}C-NMR spectrum of II (Table 3) shows 25 SP^3 carbon atoms corresponding to C_1, C_2, C_4 - C_{10}, C_{14} – C_{29} and 5 SP^2 carbon atoms corresponding to C_3, C_{11}, C_{12}, C_{13}, C_{30}. These findings reveal the presence of 3 carbonyl carbon atoms (C_3, C_{11}, C_{30}) recorded at δ 216.1, 199.0 and 178.4, respectively and two unsaturated carbon atoms C_{12}, C_{13} recorded at δ 127 and 171.1 respectively. Therefore the structure of II was identified as3-oxo- glycyrrhetic acid. In agreement with our results, Hattori et al. (1983) reported that GL was transferred into three metabolites (GA, 3-epi-GA and 3-oxo-GA) by human intestinal flora. Also, 3-oxo-GA was obtained as a metabolite in the metabolism of GL by cultivation of rat gastrointestinal bacteria (Akao, 1997).

Physiology of the transformation process

Suitability of the cultivation medium

Results in Table 4 show that the composition of the fermentation medium greatly affected GL convertibility. Among tested media, medium I composed of (%, w/v): GL, 1; glucose, 1; CSL, 0.8; pH 5.7 (Kuramoto et al. 1994) was proved to be the best fermentation medium that favored the hydrolysis of GL to GA and 3-oxo-GA (17.73, 7.4%, respectively). This was accompanied by the highest total GL bioconversion estimate (25.19%). Good GL bioconversion efficiency (16.2%) was achieved on medium IV which is composed of (%, w/v): GL, 1; CSL, 0.8. These media (I & IV) were characterized by the presence of GL as a carbon source and CSL as a sole nitrogen source. Similar results have also been reported by El-Menoufy (1988) who stated that, CSL could successfully replace peptone and meat extract. Thus, the superiority of medium I & IV may be due to the presence of CSL which was not present in the other tested media.

Effect of glucose, CSL, GL levels

In examining effect of cultivation medium composition on the bioconversion process, different levels of either glucose, CSL or GL were used (Figures. 1, 2 and 3, respectively). The low level of glucose (0.5 %) supported maximum GL bioconversion activity (44.45 %), while increase of glucose level over the latter concentration resulted in a gradual decrease in GL bioconversion efficiency. The enhanced bioconversion efficiency in the presence of glucose might be due to increased growth of the fungus on this carbon source. However, increasing glucose concentration to 2% was accompanied by sharp decrease in the bioconversion efficiency, possibly because the cells preferred the easily oxidizable d-(+)-glucose as exclusive carbon source and repressed induction of GL hydrolyzing activity (Manosroi et al. 2003).

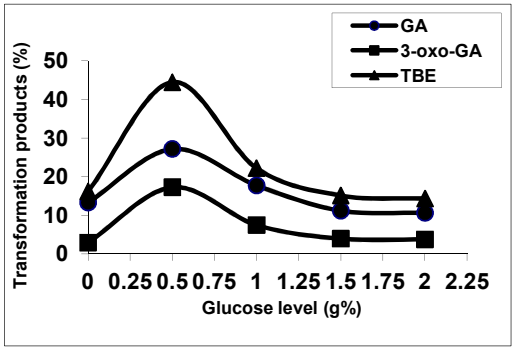

Figure 1: Effect of glucose level on GL bioconversion by *A. niger* NRRL 595

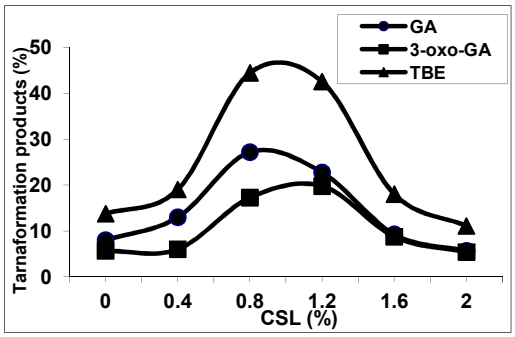

Figure 2: Effect of corn steep liquor level on GL bioconversion by *A. niger* NRRL 595

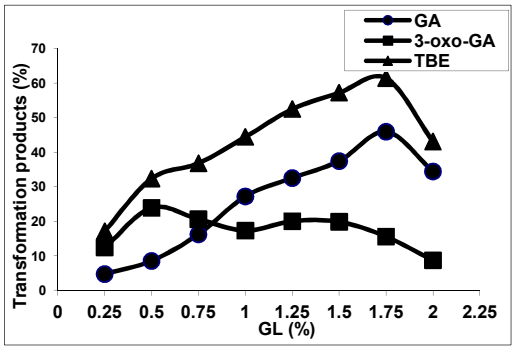

Figure 3: Effect of GL level on GL bioconversion by *A. niger* NRRL 595

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Table 4: Effect of composition of the fermentation medium on GL bioconversion by *A. niger* NRRL 595

Medium	Residual GL (%)	Transformation products		TBE (%)
		GA (%)	3-oxo-GA (%)	
I	64.9	17.73	7.46	25.19
II	93.9	0.82	0./14	0.96
III	92.8	1.86	0.33	2.19
IV	60.5	13.3	2.90	16.2
V	93.7	1.3	0.23	1.53
VI	92.9	1.75	0.3	2.05
VII	89.2	2.66	0.49	3.15
VIII	86.5	6.07	2.48	8.55

Total bioconversion efficiency T.B.E (%) = GA yield (%) + 3-oxo-GA yield (%)

Table 5: Bioconversion of GL by *A. niger* NRRL 595 as influenced by the initial pH value of the fermentation medium*

Initial (pH)	Residual GL (%)	Transformation products		TBE (%)
		GA (%)	3-oxo-GA (%)	
4	76.7	16.80	0.24	17.04
4.5	70.2	20.06	5.28	25.34
5	50.5	28.95	8.97	37.92
5.7**	31.3	45.91	15.55	61.45
6	24.1	51.25	17.48	68.73
6.5	22.3	51.45	18.25	69.70
7	24.8	49.86	18.02	67.88

- *Medium composition (%, w/v): GL, 1.75; glucose, 0.5 and CSL, 0.8
- Transformation period: 72 h
-**Control treatment: pH of the fermentation medium was adjusted to pH 5.7

The influence of different CSL concentrations on GL bioconversion indicated that as the amount of CSL increased the GA yields were gradually increased and reached maximum (27.18 %) at 0.8 % CSL level. On the other hand, the maximum 3-oxo-GA yield (19.8 %) and total GL bioconversion efficiency (47.5 %) were recorded on using 1.2 % CSL. Also, Kuramoto et al. (1994) found that CSL was effective for the hydrolysis activity. These findings may be of practical importance, since CSL is a cheap raw material. The steeping water had been reported to contain considerable amounts of sugars, nitrogenous compounds, organic acids (namely, lactic acid) amino acids and vitamins (Liggett & Koffler 1948).

The effect of GL level on the transformation process revealed that the bioconvertibility of GL increased from 17.1 to 61.45 % by increasing GL concentration from 0.25 to 1.75 %, respectively. Maximum GA output (45.91%) was recorded at 1.75 % GL level, while the highest yield of 3-oxo-GA was recorded at 1.25 % GL level. These results suggest that a fermentation medium composed of (%, w/v) glucose 0.5, CSL 0.8, and GL 1.75, affords the best bioconversion estimates of GL, together with the formation of the highest yield of GA. In most cases, increase of GA is accompanied by increase of 3-oxo-GA. This may be explained by the enzymic reversibility between GA and 3-oxo-GA, which has been demonstrated by some investigators (Hattori et al. 1983, 1985; Akao 1997, 1999b). These authors suggested that, the equilibrium may be displaced in the direction of oxidation or reduction of GA or 3-oxo-GA according to fermentation conditions.

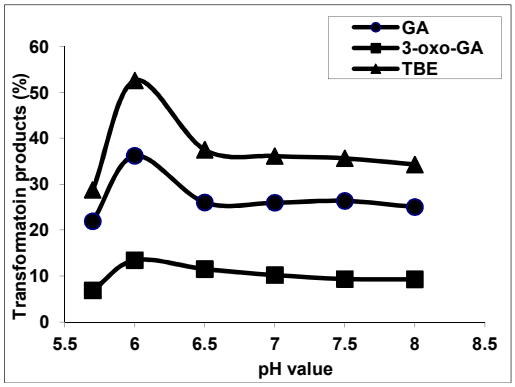

Figure 4: Bioconversion of GL by *A. niger* NRRL 595 grown on the fermentation medium adjusted with phosphate buffer at different pH values

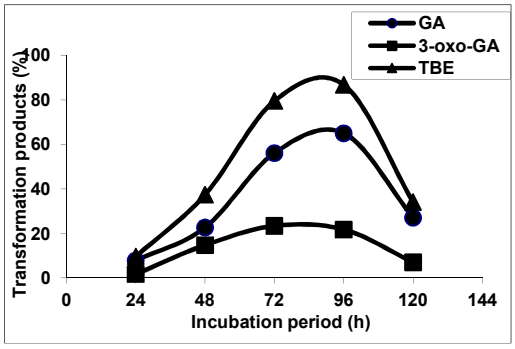

Figure 5: Effect of incubation period on GL bioconversion by *A. niger* NRRL 595

pH value relation

Control of pH value of the fermentation medium (initially or with buffer solutions) is an essential factor for the efficient enzymatic hydrolysis of GL by *A. niger* NRRL 595. The results presented in Table 5 show that high conversion activities of GL were maintained within pH range of 6-7, however the bioconversion process was markedly retarded at pH values below 5.7. These findings support the data presented by El-Menoufy (1988) and Akao (1997) who reported that the enzymatic hydrolysis of GL is

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difficult in the acidic medium. Maximum GA and 3-oxo-GA yieldsof 51.45 and 18.25 %, respectively, corresponding to total bioconversion efficiency of 69.7 %, were recorded at initial pH of 6.5. In agreement with the present results, Akao (1997) found that GL was hydrolyzed in the rat intestinal tract between pH 6 and 7. However, Akao et al. (1987), El-Menoufy (1988), Akao (1999a) and Kim et al. (1999) reported that the optimal pH value of GL β–glucuronidase activity was 5.6.

It is noteworthy to mention that, relatively good activities (52.57 %) were recorded on performing the transformation process in phosphate buffered medium (pH 6) (Figure 4). However, the capacity of the microorganism to transform GL was greatly suppressed in acetate buffered medium at pH values from 3.6 to 5.6, indicating the deleterious effect of the buffer constituents (data not shown).

Effect of incubation temperature

Concerning the effect of different incubation temperatures on GL bioconversion by *A. niger* NRRL 595, results show that relatively high bioconversion yields were obtained at temperature range of 30-35 °C (data not shown). Maximum GA output was obtained at 30 °C. The optimum temperature for production of GA differs according to the used strain. For example, the intestinal human bacteria acted optimally when incubated at 37 °C (Hattori et al. 1983). However, Muro et al. (1986) had cultivated *A. niger* GRM3 at 27 °C to achieve good conversion of GL to GA.

Transformation time course

The capacity of the experimental organism to transform GL proved to be markedly affected by transformation period (Figure 5). Consumption of GL and the total bioconversion efficiencies were increased with the increase of the incubation period. Maximal yield of GA (65%) was obtained after 96 h of incubation, with an obvious decline thereafter. However, the highest yield of 3-oxo-GA (23.43%) was recorded after 72 h. Moreover, the maximal total conversion value (86.78%) was achieved after 96 h with consumption of about 90% of the added GL. During the different phases of the transformation process, GA was proved to be the major product. Hattori et al. (1985) reported that, GL was transformed into three metabolites (GA, 3-epi-GA, 3-oxo-GA) by human intestinal flora. The reaction seems to include hydrolysis of GL to GA and glucuronic acid and reversible transformation of GA to 3-epi-GA via a metabolic intermediate (3-oxo-GA) by the intestinal flora. They also demonstrated that, GL could not be converted to the three metabolites mentioned above by the action of a single species of bacterium but could be converted by the cooperative action of bacteria responsible for different steps of the metabolic pathways (Hattori et al. 1985). A fairly large number of bacteria showed β-glucuronidase activity to hydrolyze GL to GA, while a moderate number of the bacteria had activity to reduce 3-oxo-GA to either GA or 3-epi-GA (Hattori et al. 1983; 1985; Akao 1997).

CONCLUSION

Aspergillus niger NRRL 595 was screened and selected on the basis of its ability to hydrolyze GL producing high yield of GA. Maximum total conversion value of 86.78% was obtained using production medium composed of (%, w/v): 1.75 GL, 0.5glucose, 0.8 corn steep liquor; pH 6.5; with the consumption of about 90% of the added GL. The medium was inoculated with 15% (v/v) inoculum and incubated at 30 °C for 96 h. Under these optimal conditions, GA yield became about 3 times higher than 3-oxo-GA yield (65 % and 22 %, respectively) and the cells bioconversion efficiency increased from 25.19 to 86.78%.

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