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Research Article

Microbial Screening for Biotransformation of Glycyrrhizin into 18α-Glycyrrhetinic Acid and 18β-Glycyrrhetinic Acid and their HPLC Analysis

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Abstract

Glycyrrhizin is the main compound of *Glycyrrhiza glabra* which can be biotransformed into 18α-glycyrrhetinic acid and 18β-glycyrrhetinic acid for diverse therapeutic properties. In the present study different microbial strains were screened for highest β-glucuronidase enzyme activity for biotransformation of glycyrrhizin into 18α-glycyrrhetinic acid and 18β-glycyrrhetinic acid. The maximum enzyme activity (62.126 U/ml) was obtained from sonicated *Escherichia coli* MTCC 1652 among all the microbial strains. The highest concentration of 18α-glycyrrhetinic acid and 18β-glycyrrhetinic acid from sonicated *E. coli* was found to be 705.985 µg/ml and 133.036 µg/ml respectively. However, among all the fungal strains, sonicated *Penicillium citrinum* has produced maximum (37.012 U/ml) β-glucuronidase activity with concentration of 120.601 µg/ml 18α-GA and 9.513 µg/ml 18β-GA. *Penicillium chrysogenum* have produced maximum amount of 217.799 µg/ml and 46.9856 µg/ml of 18α-GA and 18β-GA respectively with lower (21.329 U/ml) β-glucuronidase activity. The present study could be applied as potential source of simultaneous production of plant phytomolecules which has tremendous health benefit.

Keywords: Glycyrrhizin; β-glucuronidase; 18α-glycyrrhetinic Acid; 18β-glycyrrhetinic Acid; *Escherichia coli*; *Penicillium citrinum*

Introduction

Glycyrrhiza glabra (Fabaceae), commonly known as licorice, is widely used in Indian system of medicine for the treatment of various disease. Roots and stems of *Glycyrrhiza glabra* has main active constituent glycyrrhizin (GL), a triterpenoids saponin. GL is the major bioactive compound of licorice and has been recommended for prevention of hepatic lipotoxicity, chronic hepatitis C, and lipopolysaccharide- (LPS-) induced inflammation [1-3]. GL exhibits bioactive properties through the functions of its biologically active metabolite, 18β-glycyrrhetinic acid (GA), which is produced via enzymatic hydrolysis [1]. GA is mainly used for its multiple functions such as anti-tumour, anti-virus, antibacterial and as antioxidant in processed food. GA can exist as trans and cis isomers, the transform 18α-glycyrrhetinic acid and the cis form 18β-glycyrrhetinic acid [4-6]. Both of isomers showed biological activity but 18β-glycyrrhetinic acid has been more extensively studied because of its abundance in root extract. Hence it was widely believed that 18β-glycyrrhetinic acid has more biological activity, than 18α-glycyrrhetinic acid [7,8].

In the past decade set, several studies have demonstrated that microbial transformation is a versatile tool to enlarge the structural diversity of triterpenoids [9]. Biocatalysis and biotransformation have many advantages, such as high substrate specificity and mild reaction conditions [4]. A great challenge for the realization of a desired biotransformation is finding the appropriate microorganism. Thus, classical screening of a series of microbial strains is still the most widely used technique. Very recently, a comprehensive review on microbial transformation of triterpenoids was published [10]. Isolation of *Aspergillus parasiticus* producing β-glucuronidase was screened and enzyme activity was determined by using phenolphthalein-β-D-glucuronide (PPG) [11]. Enzymatic conversion of glycyrrhizin of *G. glabr*a into GA using commercial preparation from *Aspergillus niger* was also reported [12]. Due to structural similarity of GL and GA to steroids, they have mineralocorticoid like effects and inhibit metabolic enzymes for adrenocorticosteroids [13]. GL is found naturally as β-isomer (18β-GL, 18β-GA) which can be isomerized to their α-isomers (18α-GL, 18α-GA) under alkaline condition [14]. Stereochemistry play important role for different biological and physicochemical properties of 18α-GA and 18β-GA

[15]. Screening of different microbial strains is required to investigate the presence of β-glucuronidase enzyme for bioconversion of GL into 18α-GA and 18β-GA.

The present study focused on screening of different bacterial and fungal strains for higher activity of β-glucuronidase enzyme and for conversion of GL into 18α-glycyrrhetinic acid and 18β-glycyrrhetinic acid in fermented *G. glabra* root extract. Biotransformed compounds were analysed by high performance liquid chromatography (HPLC) method.

Materials and Methods

Microorganism

Seven fungal strains and two bacterial strains were taken for screening. Fungal strains *Penicillum chrysogenum* MPBL1 and *Rhizopus oryzae* MPBL2 were isolated from the soil and identified in Indian Agriculture Research Institute, Pusa, New Delhi. *Monascus purpureus* 369 and *Monascus purpureus* 410 fungal strains were obtained from Institute of Microbial Technology (IMTECH), Chandigarh, India. *Rhizopus oligosporous* NCIM1215 and *Penicillum citrinum* NCIM 768 were received from National Collection of Industrial Microorganism (NCIM), Pune, India. *Monascus pilosus* CBS290.34 was obtained from American Type Culture Collection (ATCC). All the fungal strains were grown and maintained in Potato Dextrose Agar (PDA) slants and subcultured every 30 days. The bacterial strain *Escherichia coli* MTCC 1652 and *Bacillus subtilis* MTCC 2616 were taken from the Institute of Microbial Technology (IMTECH), Chandigarh, India which were grown and maintained in Nutrient Agar (NA) and subcultured every 30 days.

Preparation of seed culture

Spore suspension of *Penicillum chrysogenum* MPBL1, *Rhizopus oryzae* MPBL2, *Monascus purpureus* 369, *Monascus purpureus* 410, *Rhizopus oligosporous* NCIM 1215, *Penicillum citrinum* NCIM 768 was prepared from actively growing slants in sterile water and diluted to a concentration 5.7x10. Spore counting was carried out by using hemocytometer. Spore suspension (15%) was inoculated to conical flasks containing the potato dextrose broth (PDB). These culture was inoculated at 27˚C for 48 hrs in a shaker incubator at 110 rpm. Bacterial seed culture of *Escherichia coli* MTCC 1652 and *Bacillus subtilis* MTCC 2616 were prepared by culturing bacterial inoculums in nutrient broth (NB) for 48 hrs at 37 ˚C at 150 rpm in rotary shaker.

Preparation of *Glycyrrhiza glabra* root extract

G. glabra roots were cleaned, dried and milled to prepare coarse powder. Twenty-five grams of coarse powder were extracted with soxhlet apparatus by using 100% water for 72 hr. The extract was concentrated under reduced pressure in rotary evaporator at 40˚C.

Biotransformation of glycyrrhizin to 18α-glycyrrhetinic acid and 18β-glycyrrhetinic acid

Submerged biotransformation of GL was carried out in 250 ml Erlenmeyer conical flask. Czapedox medium of pH 5.5 was prepared and sterilized at 121˚C and 1.06 Kg/cm2 for 20 min. The fungal seed culture (5 ml) was inoculated to Czapedox medium and biotransformation of all fungal strains was carried out at 27˚C and 110 rpm for 96 hrs. Synthetic media was prepared and pH was adjusted to 7.2. Bacterial seed culture (5 ml) was inoculated to 50 ml of synthetic media and bacterial biotransformation was carried out at 37˚C and 150 rpm for 24 hrs [16].

Screening of bacterial and fungal strains

The fungal and bacterial strains were screened on the basis of β-glucuronidase enzyme activity and their bioconversion capability. These microbial strains were assayed in two ways: non-sonication and sonication. Fermented broth was sonicated with the help of probe sonicator to disrupt the cells so that entire enzyme can be leaked out and a part of fermented broth was kept as non-sonicated. The enzyme activity was performed by HPLC method [17].

β-glucuronidase enzyme assay

Hydrolytic Unit (HU) was used for enzyme activity by incubating the enzyme with 3 Mm GL solution at 35˚C for 10 min. The enzyme reaction was stopped by adding 200 mM glycine buffer solution of pH 10.4. The amount of 18α-GA and 18β-GA was analysed by HPLC. Here one HU is defined as weight of 18α-GA and 18β-GA in microgram released from 1 µg/µl of pure GL solution in 10 min [18].

Extraction of GL, 18α-GA and 18β-GA

The fermented broth was subjected to sonication for 10 min. The disrupted cell was then centrifuged at 5000 rpm for 10 min. Supernatant having 18α-GA, 18β-GA and unconverted GL was filtered and analysed by HPLC [17].

Chromatographic condition and analysis of GL, 18α-GA and 18β-GA

The analysis of GL was performed by high performance liquid chromatography (HPLC) method. The chromatography was carried out by RP C18 column. The mobile phase consists of methanol: water (85:15 v/v) at a flow rate of 1 ml/min with run time of 10 min and detection was carried out at 254 nm [11,18]. Simultaneous analysis of 18α-GA acid (20 µl) and 18β-GA (20 µl) was performed by HPLC method. The mobile phase was used acetonitrile: tetrahydrofuran: water (10: 80: 10 v/v) at flow rate of 1 ml/min with run time of 10 min. The UV detection was carried out at 254 nm [19].

Results and Discussion

Bioconversion of glycyrrhizin to 18α-GA and 18β-GA

Bioconversion from enzymatic approach is better than traditional chemical method as the sugar molecules can be removed from the glycoside in mild reaction conditions and the formation of by-products can be avoided. Bioconversions of GL into GA have already been carried out by using enzymes derived from animal livers and human intestinal bacteria [11]. β-glucuronidases enzymes are members of the glycosidase family 2 of enzymes that hydrolyse the glycosidic bond between two or more carbohydrate or between a carbohydrate and noncarbohydrate moiety [20]. In

the present study enzymatic conversion of glycyrrhizinic acid (GL), from root extract of Glycyrrhiza glabra L., into 18α-GA and 18β-GA, was achieved by using β-glucuronidase from different microbial strains.

E. coli, B. subtilis, P. chrysogenum, R. oryzae, P. citrinum, M. purpureus 369, *M. purpureus* 410, *R. oligosporous* and *M. pilosus* were screened on the basis of their β-glucuronidase enzyme activity (Table 1, Figure 2) and their bioconversion capability (Table 2, Figure 2). All the standard chromatograms are shown in figure 1. Enzyme activity of sonicated bacterial strain was found to be higher than non sonicated bacterial strain. The maximum enzyme activity (62.126 U/ml) was obtained from sonicated *E. coli* MTCC 1652 as compared to sonicated *B. subtilis* (19.536 U/ml). However, among all the fungal strains, sonicated *P. citrinum* produced maximum β-glucuronidase activity (37.012 U/ml). All the sonicated fungal strains have produced higher quantity of β-glucuronidase enzyme activity as compare to non-sonicated fungal strains except *R. oligosporous* and *M. purpureus.* Higher enzyme activity of sonicated bacterial and fungal strains might be due to presence of β-glucuronidase as an intracellular enzyme.

Microbial Strains	β-glucuronidase (Unit/ml)	
	Non-Sonicated (NS)	Sonicated (S)
E. coli MTCC 1652	23.419	62.126
B. subtilis 2616	17.235	19.536
P. chrysogenum MPBL 1	19.166	21.329
P. citrinum NCIM 768	32.219	37.012
R. oryzae MPBL 2	23.085	33.129
R. oligosporous NCIM 1215	33.356	27.174
M. purpureus MTCC 369	33.009	27.272
M. purpureus 410	10.108	17.437
M. pilosus ATCC CBS 290.34	15.531	17.721

Table 1: Concentration of β-glucuronidase enzyme under submerged biotransformation process.

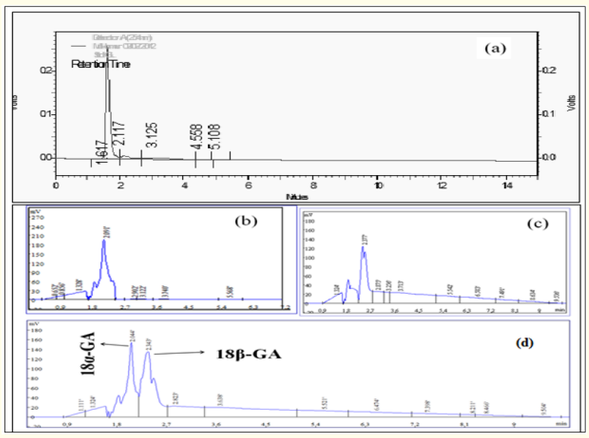

Figure 1: HPLC chromatogram of standard glycyrrhizin (a), 18α-glycyrrhetinic acid (b), 18β-glycyrrhetinic acid (c) and standard mixture of 18α-GA and 18β-GA (d).

Microbial Strains	GL (µg/ml)	Sonicated (S) (µg/ml)		Non-sonicated (NS)(µg/ml)	
		18α-GA	18β-GA	18α-GA	18β-GA
E. coli MTCC 1652	70.499	705.985	133.036	266.133	37.698
B. subtilis MTCC 2616	43.900	202.00	14.132	195.860	14.048
P. chrysogenum MPBL 1	45.366	202.375	46.985	217.799	14.744
P. citrinum NCIM 768	52.103	120.601	9.513	166.131	14.194
R. oryzae MPBL 2	48.311	176.476	13.355	162.388	8.320
R. oligosporous NCIM 1215	39.929	108.805	10.289	179.047	13.146
M. purpureus MTCC 369	30.628	109.915	8.656	147.830	7.631
M. purpureus MTCC 410	91.965	198.150	11.471	114.872	13.682
M. pilosus ATCC CBS 290.34	56.877	101.378	9.956	176.497	5.570

Table 2: Concentration of glycyrrhizin, 18α-glycyrrhetinic acid and 18β-glycyrrhetinic acid under submerged biotransformation process by different microbial strains.

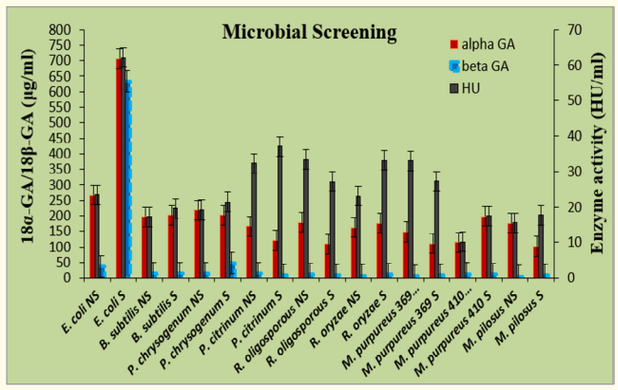

Figure 2: Screening of different microbial strains.

Quantitative determination of 18α-GA acid and 18β-GA was carried out by using a HPLC with RP C18 column. and an UV detector operating at 254 nm (Figure 1). The highest concentration of 18α-GA and 18β-GA from sonicated *E. coli* was found to be 705.985 µg/ml and 133.036 µg/ml respectively. This may be due to higher enzyme activity of *E. coli.* Among all the fungal strains, *P. chrysogenum* have produced maximum of 217.799 µg/ml (non sonicated) and 46.9856 µg/ml (sonicated) of 18α-GA and 18β-GA respectively. Sonicated *R. oligosporous* and *M. purpureus* have produced almost equal quantity of 18α-GA. Non-sonicated *B. subtilis, P. chrysogenum* and *P. citrinum* have also produced almost equal concentration of 18β-GA. Non-sonicated *M. purpureus* and *M. pilosus* have produced 7.631 µg/ml and 5.570 µg/ml of 18β-GA respectively (Table 2, Figure 2).

Earlier it has been reported that sonication cause cell lysis and release of intracellular enzyme in soil [21]. Short time sonication is responsible for activation of extracellular enzymes while long time sonication would lyse the microbial cell and release the intracellular enzymes [22]. Our results also suggest that sonication might be a promising factor for differentiating between extracellular and intracellular enzyme activity.

Conclusion

Biotransformation of natural compounds is an important tool to produce various new phyomolecules which may be used in different diseases. In the present study nine microbial strains (bacterial and fungal) were screened for biotransformation of GL to 18α-GA and 18β-GA in *Glycyrrhiza glabra* root extract. Result showed that sonicated *E. coli* produced maximum bioconversion as compared to all microbial strains. Sonicated *E. coli* was found to be the best source of β-glucuronidase enzyme for biotransformation among all the microbial strains. It was found that bacterial strains produced higher amount of β-glucuronidase enzyme than fungal strains during submerged biotransformation process. As the microbial β-glucuronidase enzyme is an intracellular enzyme, it is very important to understand its role in hydrolysis of glycyrrhizin molecule during biotransformation process. Moreover, biotransformation processes depend upon various factors including microorganism, media components, pH and temperature [23,24]. Therefore, exact mechanism of microbial biotransformation needs to be verified.

Bibliography

1. Wu X., *et al*. "Prevention of free fatty acid-induced hepatic lipotoxicity by 18beta-glycyrrhetinic acid through lysosomal and mitochondrial pathways". *Lipotoxicity* 47 (2008): 1905-1915.

2. Wang C Y., *et al.* "Glycyrrhizic acid and 18β-glycyrrhetinic acid modulate lipopolysaccharide-induced inflammatory response by suppression of NF-κB through PI3K p110δ and p110γ inhibitions". *Journal of Agriculture and Food Chemistry* 59 (2011): 7726-7733.

3. Rossum V T., *et al.* "Glycyrrhizin as a potential treatment for chronic hepatitis C". *Alimentary Pharmacology and Therapeutics* 12 (1998): 199-205.

4. Feng S J., *et al.* "Screening strains for directed biosynthesis of β-D-mono-glucuronide-glycyrrhizin and kinetics of enzyme production". *Journal of Molecular Catalysis B: Enzymatic* 43 (2006): 63-67.

5. Zeng C X., *et al.* "A comparison of the distribution of two glycyrrhizic acid epimers in rat tissues". *European Journal of Drug Metabolism and Pharmacokinetics* 31 (2006): 253-258.

6. Ha Y M., *et al.* "Chiral separation of glycyrrhetinic acid by high-performance liquid chromatography". *Journal of Pharmaceutical and Biomedical Analysis* 9 (1991): 805-809.

7. Rossi T., *et al.* "Selectivity of action of glycyrrhizin derivatives on the growth of MCF-7 and HEP-2 cells". *Anticancer Research* 23 (2003): 3813-3818.

8. Amagaya S., *et al.* "Comparative studies of the stereoisomers of glycyrrhetinic acid on anti-inflammatory activities". *Journal of Pharmacobiodynamics* 7 (1984): 923-928.

9. Parra A., *et al.* "Microbial transformation of triterpenoids". *Mini Reviews in Organic Chemistry* 6 (2009): 307-320.

10. Wang J., *et al.* "Bioconversion of glycyrrhizinic acid in liquorice into 18-β-glycyrrhetinic acid by *Aspergillus parasiticus* speare BGB". *Applied Biochemistry and Microbiology* 46 (2010): 421-466.

11. Morana A., *et al.* "Enzymatic production of 18-β-glycyrrhetinic acid *G. glabra* L*". Biotechnology Letters* 24 (2002): 1907-1911.

12. Ahmad M and BP Panda. "Alginate Immobilization of *Escherichia coli* MTCC 1652 whole cells for bioconversion of glycyrrhizinic acid and into 18-β glycyrrhetinic acid". *Pakistan Journal of Biological Sciences* 16 (2013): 2046-2049.

13. Chamoli A., *et al.* "Simultaneous determination of 18α-glycyrrhetinic acid and 18β-glycyrrhetinic acid in *Glycyrrhiza glabra* root by reversed phase high performance liquid chromatography". *Drug Development and Therapeutics* 7 (2016): 59-62.

14. Spizzizen J., *et al.* "Biochemical studies on the phenomenon of virus reproduction: III. The inhibition of coliphage T2r+ multiplication by sodium salicylate and sodium gentisate". *Journal of Bacteriology* 62 (1951): 323-329.

15. Wang Z Y., *et al*. "Inhibition of mutagenicity in *Salmonella* typhimurium and skin tumor initiating and tumor promoting activities in SENCAR mice by glycyrrhetinic acid: comparison of 18-alpha and 18-beta stereoisomers". *Carcinogenesis* 12 (1991): 187-192.

16. Kaisha MK. U.K Patent. 2071665: 1983.

17. Ahmad M., *et al.* "Screening and bioconversion of glycyrrhizin of *Glycyrrhiza glabra* root extract to 18-β glycyrrhetinic acid by different microbial strain". *Drug Development Therapeutics* 7 (2016): 69-72.

18. Nocca G., *et al*. "Quantitative determination of 18-*β*-glycyrrhetinic Acid in HepG2 cell line by High Performance Liquid Chromatography method". *International Journal of Analytical Chemistry* 2018 (2018): 1-5.

19. Kondo M., *et al*. "Physiochemical properties and applications of α and β glycyrrhizins, natural surface active agents in licorice root extract". *Journal of the Society of Cosmetic Chemist* 37 (1986): 177-189.

20. Amin HAS., *et al.* "Biosynthesis of glycyrrhetinic acid 3-O-mono- β -d-glucuronide by free and immobilized *Aspergillus terreus* β -d-glucuronidase". *Journal of Molecular Catalysis B: Enzymatic* 69 (2011): 54-59.

21. Cesare F., *et al*. "Use of sonication for measuring acid phosphatase activity in soil". *Soil Biology and Biochemistry* 32 (2000): 825-832.

22. Qin S., *et al*. "Differentiating intracellular from intracellular alkaline phosphatase activity in soil by sonication". *Plose One* 8 (2013): e58691.

23. El-Refai A H., *et al.* "Physiological and chemical studies on the bioconversion of glycyrrhizin by *Aspergillus niger* NRRL 595". *Malaysian Journal of Microbiology* 6 (2012): 75-82.

24. Manosroi J., *et al.* "Factors affecting the biotransformation of chlormadinone acetate to delmadinone acetate". *Journal of Molecular Catalysis B: Enzymatic* 23 (2003): 37-42.

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