http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 16 (24): 2046-2049, 2013
ISSN 1028-8880 / DOI: 10.3923/pjbs.2013.2046.2049

Alginate Immobilization of *Escherichia coli* MTCC 1652 Whole Cells for Bioconversion of Glycyrrhizinic acid and into 18-β Glycyrrhetinic Acid

M. Ahmad and B.P. Panda
Microbial and Pharmaceutical Biotechnology Laboratory,
Centre for Advanced Research in Pharmaceutical Science, Faculty of Pharmacy,
Jamia Hamdard, New Delhi-110062, India

Abstract: Microbial biotransformation of Glycyrrhizinic acid (GL) into 18-β Glycyrrhetinic Acid (GA) was achieved using *Escherichia coli* MTCC 1652 whole cell. The *E. coli* whole cell was immobilized by entrapment method within calcium alginate beads using cell suspension of equal volume with sodium alginate 8%. The pH of solution, reaction volume and % of GL were optimized during the immobilization procedure and optimum pH 6.5, reaction volume of 4 mL and at 3% GL concentration for 12 h of incubation time showed highest concentration of GA (72.649 μg mL^{-1}) with 76% bioconversion of GL to GA. Under optimized condition the immobilized cell produces 58.663 μg per mL of GA in licorice root extract containing 95.118 μg of GL per mL of the extract with 61% conversion at 12 h.

Key words: *E. coli*, immobilization, calcium alginate, glycyrrhizinic acid, 18-β glycyrrhetinic acid

INTRODUCTION

Licorice extract and its principle component, Glycyrrhizinic acid (GL) have extensively used in food as a sweetener and a flavoring ingredient and finds wide application in both traditional and herbal medicine (Amin *et al*., 2011). GL is composed of one molecule of 18-β Glycyrrhetinic Acid (GA) as aglycone and two molecules of glucuronic acid (Hennell *et al*., 2008). The pharmacological activity is due to the presence of triterpene aglycone 18-β Glycyrrhetinic Acid (GA) and in lesser measures, to its glycoside Glycyrrhizin (GL) (Gisbon, 1978; Samuelsson, 1993). Biotransformation of GL into GA can be implemented through the function of β-glucuronidase which is produced by *Escherichia coli* MTCC 1652 and can hydrolyze glucoside bond. β-glucuronidase from *E. coli* MTCC 1652 have been produced and analyzed and the specificity and productivity of conversion of GL to GA was achieved successfully and has a wide range of pH, high substrate specificity and high efficiency for GL conversion.

Alginate is a naturally occurring binary linear heteropolymer that contains 1, 4-linked β-D-mannuronic acid and α-L-glucuronic acid residue. Because of its good biocompatibility and processing capacity, it is one of the best entrant matrixes for cell entrapment (Zhang *et al*., 2010). Entrapment is one of the simplest methods available for enzyme or cell immobilization under mild conditions and results in minimal denaturation of the immobilized biocatalyst compared with other immobilization methods (Roy *et al*., 2004).

To the best of our knowledge, no studies have yet reported on the use of immobilized whole cells for the bioconversion of GL to GA. In the present research, *E. coli* MTCC 1652 whole cells were immobilized with alginate. Very stable immobilized cells were achieved that allowed the continuous production of GA.

MATERIALS AND METHODS

Plant materials, microorganism and chemicals: The roots of *G. glabra* were obtained from local market of New Delhi, India. The crude drug was identified and a specimen voucher No./NISCAIR/RHMD/consult/2012-2013/2085-92 is issued by NISCAIR, CSIR, New Delhi, India. Bacterial strain *E. coli* MTCC 1652 were collected from MTCC, IMTECH, Chandigarh, India. The bacterial strain were grown and maintained in nutrient agar slants at 4°C. Glycyrrhetinic acid (GA) and glycyrrhizin 98% pure (GL) obtained from Sigma Aldrich, Bangalore, India. All the chemicals, reagents and microbiological medium obtained from Hi-media, Bombay, India.

Corresponding Author: B.P. Panda, Microbial and Pharmaceutical Biotechnology Laboratory,
Center for Advanced Research in Pharmaceutical Science, Department of Pharmaceutical Biotechnology,
Faculty of Pharmacy, Jamia Hamdard, New Delhi, 110062, India Tel: +919990335013

Cell suspension production: *E. coli* MTCC 1652 cell suspension was prepared in nutrient broth. To the 50 mL of nutrient broth consisting of 5 g peptone, 5 g NaCl, 1.5 g beef extract, 1.5 g yeast extract and 1000 mL distilled water, pH was adjusted to 7.2, bacterial inoculums was added and incubate at 37°C, for 24 h in orbital shaker at 150 RPM. The cells were harvested from the culture broth by centrifugation at 5000 RPM for 10 min and the pellets were stored at 4°C for use.

Immobilization of *E. coli* MTCC 1652 whole cell in calcium alginate: Sodium alginate powder in the range of 4-10% w/v was added to 100 mM Tri-HCl buffer, pH 7. The sodium alginate powder was added slowly to the buffer solution in order to prevent flocculation. Stirring was performed by magnetic stirrer for 45 min to obtain a homogenous solution of sodium alginate. The cell suspension was then slowly added (volume ratio of 1:1) to the sodium alginate solution. The resulting mixture was added drop wise using a syringe to $CaCl_2$ solution of 0.1-0.5 M. Each drop led to the formation of a calcium alginate bead inside which the *E. coli* cells were entrapped. The beads were stabilized by stirring for 1 h and were stored at 4°C (Barin *et al*., 2007).

Determination of entrapment efficiency in beads: To determine the total number of immobilized cells within the beads, three beads were selected at random from a sample. The selected beads were ruptured to make homogenous solution. The homogenous solution was diluted 10 times. The cells within the diluted solutions were allowed to grow in petri dishes containing nutrient agar and incubated at 37°C for 24 h. The cell density was obtained by colony counts on petri dishes (Greenberg *et al*., 1995).

Effect of pH, volume and time on bioconversion rate: Effect of pH, volume and time of the synthetic medium (0.05% NH_4Cl, 0.005% of $(NH_4)_2{\bullet}SO_4$, 0.4% dextrose, 0.01% NaCl, 0.01% $MgCl_2{\bullet}6H_2O$, 0.6% Na_2HPO_4 and 0.3% KH_2PO_4), pH of the medium was adjusted to 7.2 (Spizzizen *et al*., 1951) on bioconversion were determined by incubating the alginate entrapped cells. Different concentrations (1, 2 and 3%) w/v of pure GL in different reaction volume (2, 4 and 6 mL) of synthetic medium was used for bioconversion. The optimum pH for bioconversion was determined by incubating the GL at different pH of 5.5, 6.5 and 7.5 at 37°C for 4, 8 and 12 h, respectively with 10 no of beads.

Enzyme assay: β-glucuronidase activity was measured in terms of Hydrolytic Unit (HU) by incubating enzyme with 3 mM GL solution for 10 min at 35°C. The enzymatic reaction was blocked by adding 200 mM glycine buffer solution pH 10.4. The amount of GA formed after hydrolysis was monitored by High Performance Liquid Chromatography (HPLC). One HU is defined as μg of GA released per 10 min from 1 μg μL^{-1} of GL solution.

Analysis of 18-β glycyrrhetinic acid: Samples consisting of unconverted GL and GA were analyzed by high performance liquid chromatography (Analytical technologies, Boroda, India). The chromatography was carried out by column Lichrospher 100 RP C18 (temperature 25°C), the mobile phase consist of methanol: water (85:15v/v) at flow rate of 1 mL min^{-1} with run time 10 min. Detection of GL and GA was carried out by UV detector at 254 nm (Wang *et al*., 2010).

RESULTS

Optimization of the immobilization conditions: For better entrapment of microbial cells, optimization of different parameters improves the rigidity and permeability of the beads (Anisha and Prema, 2008). The effects of alginate concentration and Ca^{2+} concentration were investigated in the current study. The concentration of sodium alginate and molarity of calcium chloride was optimized at different range. When sodium alginate powder in range of 1-10% was added into 0.1 to 0.5 M $CaCl_2$ solution, the beads formed were stable only at 8% with 0.4 M $CaCl_2$ solution. The optimal conditions for maximal immobilization efficiency were determined to be 0.4 M Ca^{2+} and 8% alginate concentration, the entrapment efficiency was 900 CFU/beads and size of the beads were in the range of 3-5 mm.

Bioconversion of GL to GA: The effect of percentage of standard GL i.e., 1, 2 and 3% under different volume and pH was observed in the present study. The standard chromatogram of GA, GL and extracted GA from the broth is presented in Fig. 1a-c.

At 1% GL concentration at pH 5.5, with 10 beads the conversion rate and GA% was undetectable under all the reaction volume i.e., with 2, 4 and 6 mL after 4, 8 and 12 h incubation. However, the HU of enzyme was observed with 4 and 8 mL reaction volume with maximum 40.779 HU and there is decrease in GL concentration in the reaction mixture. At pH 6.5 with 10 beads only bioconversion was observed after 12 h incubation in 4 and 6 mL reaction volume with GA concentration of 48.503 and 42.774 μg per mL with 35.048 and 31.570 HU, respectively (Fig. 2). At pH 7.5, the conversion rate and %

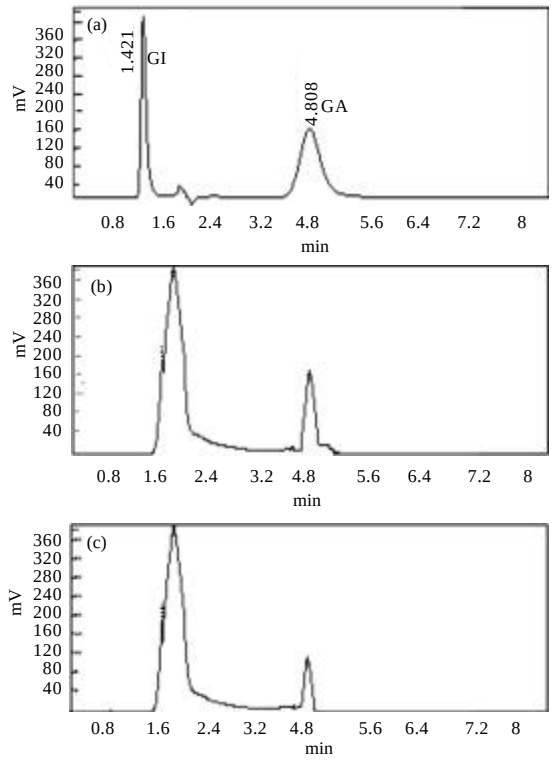

Fig. 1(a-c): (a) HPLC Chromatograms of pure GL and GA (b) Degradation of 3% GL into GA at pH 6.5 and 12 h and (c) Degradation of 3% plant extract of *G. glabra* root powder into GA at pH 6.5 and 12 h

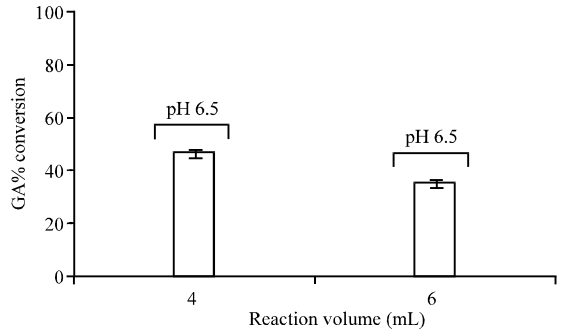

Fig. 2: Bioconversion of 1% of GL to GA at 12 h with pH of 6.5

GA was undetectable under all the reaction volume i.e., with 2, 4 and 6 mL after 4, 8 and 12 h incubation with very less enzyme activity.

At 2% GL concentration at pH 5.5, the concentration of GA was undetectable under all the conditions with very less enzyme activity. However, there is sharp decrease in the GL concentration to 13.455 μg mL^{-1} after 12 h

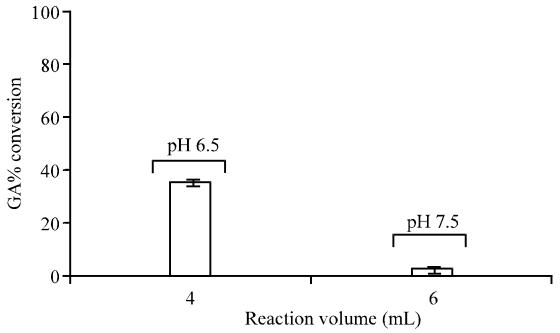

Fig. 3: Bioconversion of 2% of GL to GA at 12 h with pH of 6.5 and pH 7.5

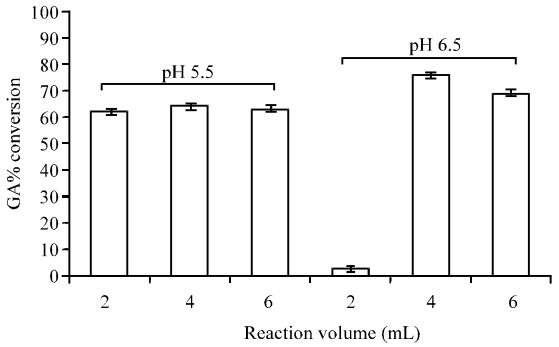

Fig. 4: Bioconversion of 3% of GL to GA at 12 h with pH of 5.5 and pH 6.5 with reaction volume of 2, 4 and 6 mL

incubation. At pH 6.5, 28.522 μg of GA per mL was observed after 12 h of incubation with enzyme activity of 29.981 HU in 6 mL reaction volume. At pH 7.5, very less amount of GA was observed in 4 mL reaction volume and GL was completely exhausted from the reaction mixture. However at 6 mL reaction volume 33.875 μg of GA was observed per mL of the reaction volume after 4 h of incubation with enzyme activity of 20.192 HU, thereafter i.e., at 8 and at 12 h GA was undetected in the reaction mixture (Fig. 3).

At 3% GL concentration at pH 5.5, considerable amount of GL is converted in to GA after 12 h incubation with 59. 387 μg, 61.426 μg and 60.818 μg per mL with enzyme activity of 40.003, 39.021 and 24.010 HU in 2, 4 and 8 mL reaction volume, respectively. At 6.5 pH, maximum amount of GL is converted to GA with a concentration of 72.694 μg mL^{-1} and enzyme activity of 46.039 HU in 4 mL reaction volume at 12 h incubation (Fig. 4).

Under optimized condition the immobilized cell produces 58.663 μg per mL of GA, in plant extract containing 95.118 μg of GL mL^{-1} of the extract with 61.67% conversion rate at 12 h.

DISCUSSION

Bioconversion of GL to GA is efficiently carried out by entrapped *E. coli* cells in alginate beads. The enzyme responsible for bioconversion is β-glucuronidase. The conversion rate is highly depend on the immobilization method, materials used for cell entrapment, bead size, no of cell entrapped in each bead, reaction volume, pH of the medium and substrate concentration (Zhang *et al*., 2010).

In the present research, alginate method was selected for immobilization of *E. coli* cells since alginate is a widely used method of choice for whole cell immobilization (Barin *et al*., 2007). The immobilization procedure is optimized for better entrapment of *E. coli* cell and bead stability. The bioconversion reaction was optimized for substrate concentration form low to high under diverse pH condition with diverse reaction mixture volume with different time of incubation. At very less concentration of GL the conversion is undetectable under acidic condition however the enzyme was activity was observed. Since the β-glucuronidase activity was optimum at pH 6 (Amin *et al*., 2011), as there is lower enzyme activity at acidic condition less amount of GL is converted in to GA. A very similar conversion rate was observed under slight alkaline pH i.e., at 7.5. However under low acidic conditions i.e. at pH of 6.5, maximum bioconversion was observed with all the concentration of GL irrespective of reaction mixture. This shows that bioconversion of GL to GA is optimum at lower acidic condition. At pH 5.5 and at 7.5 the GL concentration is decreases as the time of incubation increases, this may be due to conversion of GL to other unknown molecules.

CONCLUSION

Enzyme activity and continuous conversion of GL into GA in immobilized whole cell system depends on cell concentration, % of GL, pH, reaction volume and incubation time. Alginate immobilized *E. coli* cell showed higher enzyme activity with 46.039 HU and 72.649 μg per mL of GA production at 12 hr of incubation time. Under actual condition, 61.67% conversion was achieved with crude water extract of *G. glabra* root containing 95.118 μg of GL per mL.

ACKNOWLEDGMENT

We acknowledge Dr. H. B Singh, taxonomist and chief scientist, NISCAIR, CSIR, New Delhi, India for authenticating our herbal crude drugs.

REFERENCES

Amin, H.A.S., H.A. El-Menoufy, A.A. El-Mehalawy and E.S. Mostafa, 2011. Biosynthesis of glycyrrhetinic acid 3-O-mono-β-d-glucuronide by free and immobilized *Aspergillus terreus* β-d-glucuronidase. J. Mol. Catal B-Enzym., 69: 54-59.

Anisha, G.S. and P. Prema, 2008. Cell immobilization technique for the enhanced production of α-galactosidase by *Streptomyces griseoloalbus*. Bioresour. Technol., 99: 3325-3330.

Barin, M., M. Otadi, F. Khorasheh and A. Kheirolomoom, 2007. Effect of cell concentration on acylation of penicillin G enzymatic reaction in immobilized cells. Sci. Iran., 16: 69-73..

Gisbon, M.R., 1978. *Glycyrrhiza* in old and new perspectives. Lloydia., 41: 348-353.

Greenberg, N., B. Tarakovsky, G. Yrime, S. Ulitzur and M. Sheintuch, 1995. Observations and modeling of growth of immobilized microcolonies of luminous *E. coli*. Chem. Eng. Sci., 51: 743-756.

Hennell, J.R., S. Lee, C.S. Khoo, M.J. Gray and A. Bensoussan, 2008. The determination of glycyrrhizic acid in *Glycyrrhiza uralensis* Fisch. ExDC. (Zhi Gan Cao) root and the dried aqueous extract by LC-DAD. J. Pharm. Biomed. Anal., 47: 494-500.

Roy, I., S. Sharma and M.N. Gupta, 2004. Smart biocatalysts: design and applications. Adv. Biochem. Eng. Biotechnol., 86: 159-189.

Samuelsson, G., 1993. Drugs of Natural Origin. A text book of Pharmacognosy. Swedish. Pharmaceutical Press, Stockholm, pp. 127-135.

Spizzizen, J., J.C. Kenney and B. Hampil, 1951. Biochemical studies on the phenomenon of virus reproduction: III. The inhibition of coliphage T2r+ multiplication by sodium by sodium salicylate and sodium genistate. J. Bacteriol., 62: 323-329.

Wang, J., Q. Sun, P. Gao, J.F. Wang and C. Xu *et al*., 2010. Bioconversion of glycyrrizinic acid in liquorice into 18-β-glycyrrhetinic acid by *Aspergillus parasiticus* Speare BGB. Appl. Biochem. Micro., 46: 421-466.

Zhang, W.Y., P. Prabhu and J.K. Lee, 2010. Alginate immobilization of recombinant *Escherichia coli* whole cells harboring L-arabinose isomerase for L-ribulose production. Bioprocess. Biosyst. Eng., 33: 741-748.