Recent Research in Science and Technology 2011, 3(4): 101-104
ISSN: 2076-5061
www.recent-science.com

MICROBIOLOGY

IN VITRO STUDIES ON STIMULATION OF GYMNEMIC ACID PRODUCTION USING FUNGAL ELICITOR IN SUSPENSION AND BIOREACTOR BASED CELL CULTURES OF *GYMNEMA SYLVESTRE* R.BR.

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Abstract

Gymnemic acids have become a valuable drug in diabetes treatment due to their potent antidiabetic activity. These compounds are extracted commercially from large quantities of *Gymnema sylvestre.* Since the intact plant contains low concentrations of active compound, plant cell cultures have employed as an alternative to produce large amounts of these secondary metabolites. Moreover using a bioelicitor the secondary metabolite production can be increased. The objective of this study was to develop a rapid system for the enhanced production of gymnemic acid. *Aspergillus niger* cell extract was used as an elicitor to stimulate the production of secondary metabolite. Comparatively 9 fold increase of gymnemic acid yield was obtained in elicited cultures.

Keywords: Bioelicitation, Gymnema sylvestre, gymnemic acid, cell culture

Introduction

The recent development of elicitation has opened a new avenue for the production of secondary compounds. Secondary metabolite synthesis and accumulation in cell cultures can be triggered by the application of elicitors to the culture medium. Elicitors are microbe derived molecules which can enhance secondary metabolite production in cultured cells (Dicosmo and Misawa, 1985). The general strategy for obtaining tissue culture lines for the production of a particular compound is establishing the culture strain from the explant obtained from the high production site, such as the leaf explant for gymnemic acid production. The leaf of *Gymnema sylvestre* contains higher concentration of gymnemic acid than other organs. Previous investigation carried out by Komalavalli and Rao (2000) and Ashokkumar (2002) demonstrated the *in vitro* propagation of *Gymnema sylvestre* and Subathra Devi *et al* (2006) and Gopi *et al* reported the *Gymnema sylvestre* cell culture We have developed a novel cell culture system for *in vitro* growth and production of *Gymnema sylvestre*, suggesting a possible technology for large scale production of gymnemic acid.

Methodology

Preparation of fungal elicitor (Staniszewska *et al.*, 2003)

Aspergillus niger(Fig.1) was grown in 250 ml flasks containing SD broth. The flasks were incubated at room temperature under static conditions. At stationary phase, after 21 days, the flasks were autoclaved and the fungal mat separated from the culture medium / filtrate. The culture filtrate was filtered through Whatman No.1 filter paper and made up to a known volume, autoclaved and stored at 4°C and designed as culture media filtrate. The fungal mat was washed several times with distilled H_2O and an aqueous extract was prepared by homogenizing in a mortar and pestle using acid washed neutralized sand. This extract was filtered through muslin cloth or centrifuged and the clear supernatant was taken. The supernatant is made up to a known volume, autoclaved and stored at 4°C and designated as mat extract. Suspension of *A. niger* cell extract, OD_{600} = 1.2 (15 ml/l) was added to MS medium. Elicitor was added to MS medium directly before planting cell suspension culture to a new flask.

Fig.1. *Aspergillus niger*

Aspergillus niger colonies on SDA medium

Microscopic view of ***Aspergillus niger***

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Fig.2. Stirred tank Bioreactor

Fermentor configuration

The fermentor (Fig.2) used for the suspension culture was a stirred tank batch reactor (5 l) with the working volume of 2 l. It contains single four bladed rushton turbines. All the components of the vessel were made up of stainless steel. The culture broth was aerated by passing sterile filtered air from the bottom of the vessel and just below the impeller. The whole set up was autoclaved with medium before running.

Mass production of gymnemic acids using Bioreactor (Wookseo *et al.*, 2003)

Cell suspension culture of *Gymnema sylvestre* produced in shake flask culture were transferred to 5-litre batch type bioreactor (Fig.2) containing 2 l MS medium with (BA 0.5 mg/l and IAA 1.5 mg/l). Initial inoculum was adjusted to 10 g for the bioreactor culture. After 25 days of culture, the fresh weight of cells was measured. In the next batch, 30 ml of *Aspergillus niger* cell extract was added into the medium. Gymnemic acid production rate of elicited and non-elicited cultures were analysed.

Results and Discussion

Aspergillus niger cell extract was added to medium on which *G. sylvestre* cells were inoculated. The growth rates of the elicited and the non-elicited cultures were not significantly different. The kinetics of dry weight accumulation and gymnemic acid yield for the suspension culture over a 28 day growth period was determined (Fig.3 and Fig.4). The amount of initial inoculum inoculated was 200 mg/l. The dry weight accumulation of elicited culture was maximum on day 21 (4012 ± 114.2 mg/l) and in case of non-elicited culture it was 3985 ± 114.3 mg/l. The maximum gymnemic acid yield of elicited suspension culture was 974.79 ± 1.28 mg/l on day 21 after the inoculation.

Fig.3. Growth index of cell suspension culture of *Gymnema sylvestre* on MS medium with and without elicitors

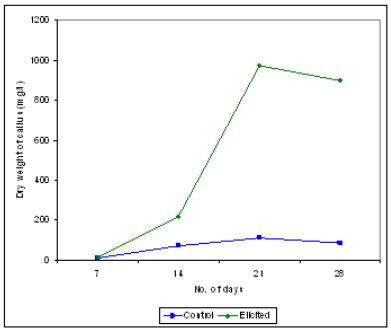


Fig.4 Content of gymnemic acid in cell suspension culture of *Gymnema sylvestre* before and after treatment with biotic elicitor

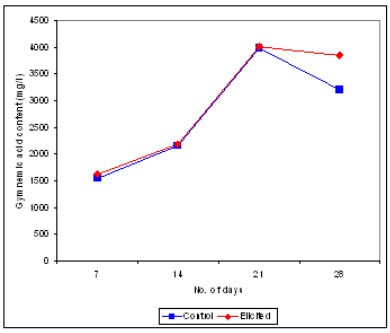


Fig.5.Suspension cultures of *Gymnema sylvestre*

Callus that was established that can be propagated in a suspension culturing system (Fig.5) and produced gymnemic acids under a defined culture condition. During the process, the profound influence of external phytohormones for the production of gymnemic acids from *Gymnema sylvestre* suspension culture was demonstrated. The proper combination of IAA and BA influence the rate of gymnemic acid production and a faster growth rate with the gymnemic acid content as high as that of the plant leaf. Results showed that acidic culture medium and moderate shaking speeds favoured the gymnemic acid production and the cells grown in the dark condition

showed significant gymnemic acid yield(subathra devi *et al*.,2006). This information is essential in designing a scale up system for commercial production of gymnemic acids. It is also interesting to note that elicited cell suspension cultures express maximum gymnemic acid. Dunlop and Curtis (1991) reported that a fungal elicitation synergistically increased production of secondary metabolites. In the current study, when *Aspergillus niger* cell extract was used as a elicitor, the productivity increase was considerably greater than that obtained with non-elicited cultures. Although the mechanism by which elicitors increase the productivity of secondary plant metabolites has not been elucidated their stimulating activity is quite significant if an appropriate elicitor is chosen to stimulate synthesis of a particular product.

This current study reported the establishment of mass production of gymnemic acids of *Gymnema sylvestre* through the shake flask and bioreactor culture. The synthesis of chemical compounds is often linked with morphological and biochemical differentiation process. Chemical gradients in a differentiated tissue or callus aggregates may facilitate the synthesis of particular secondary metabolites (Dorenburg and Knorr, 1995). Precursor feeding has been an effective method to increase the production of secondary metabolites in several culture systems (Wu *et al*., 2003). The effects of precursors feeding on the product yield often indicate the distance between the precursor and product in the biosynthesis pathway. To meet the commercial demand, this plant needs further investigation. Future studies would involve the addition of precursors other biotic and abiotic elicitors and other chemical factors to increase the gymnemic acid yield. Adaptation of this method for commercial production also can compensate the natural resource shortage of *Gymnema sylvestre*.

Mass production of gymnemic acids

10 gms of cell suspension produced in shake flask culture was transferred to 5-liter batch fermentor containing 2 litre MS medium with (BA – 1 mgl^{-1} and IAA – 0.5 mgl^{-1}). Fresh weight was increased 65 times (6.5 g) after 28 days of culture and the gymnemic acid production achieved was 900 mg/l. While 8 times (80 g) increase of freshweight was gained in elicitor added MS medium, when compared to shake flask culture, 8-9 fold increase in gymnemic acid production was observed(Fig.6 and 7).

Fig.6. Production rate of Gymnemic acids before elicitor treatment

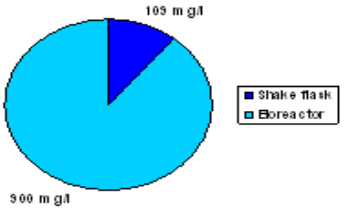


Fig.7. Production rate of Gymnemic acids after elicitation

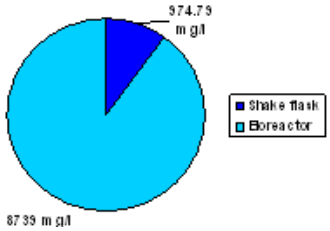
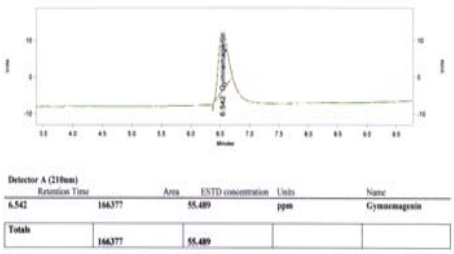


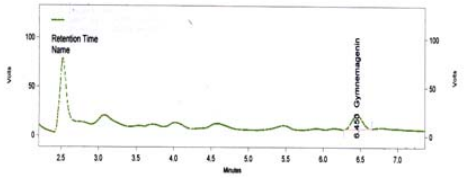
Fig.8. HPLC Profile for Gymnemagenin standard



Detector A (210nm)

Retention Time	Area	ESTD concentration	Units	Name
6.542	166377	55.489	ppm	Gymnemagenin
Totals	166377	55.489		

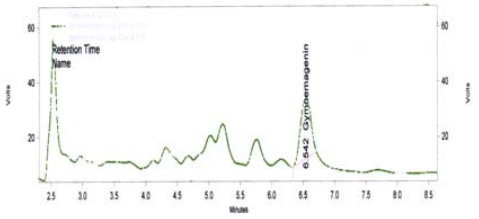
Fig.9. HPLC Profile for Gymnemagenin test sample 1



Detector A (210nm)

Retention Time	Area	ESTD concentration	Units	Name
6.450	144258	48.112	ppm	Gymnemagenin
Totals	144258	48.112		

Fig.10. HPLC Profile for Gymnemagenin test sample 2



Detector A (210nm)

Retention Time	Area	ESTD concentration	Units	Name
6.542	247912	82.681	ppm	Gymnemagenin
Totals	247912	82.681		

HPLC analysis

The samples extracted from dried callus and medium were subjected to HPLC analysis (Yokota *et al*., 1994). The total content of gymnemic acids was determined by HPLC analysis as gymnemagenin, which was the main sapogenin obtained on the hydrolysis of the mixture of gymnemic acids present in

the extract. The HPLC profile for gymnemagenin was elucidated. The retention time of gymnemagenin was 6.5 (Fig.8, 9 and 10). The gymnemagenin content of each sample was measured from the corresponding peak and the quantity calibrated with that of the external standards. The content of gymnemagenin in cell suspension culture of *Gymnema sylvestre* before and after treatment with biotic elicitor was determined (Table 1). When compared to non-elicited cultures very high yield was achieved in elicited cultures.

Table 1. Content of gymnemagenin in cell suspension culture of *Gymnema sylvestre* before and after treatment with biotic elicitors

Metabolite	Cell suspension	
Contents (mg/100 ml)	Control	Elicited by *A. niger* extract
Gymnemagenin	4.8112	8.2681

Callus that was established could be propagated in a suspension culturing system and it produced gymnemic acids under a defined culture condition. During the process profound influence of external phytohormones on the production of gymnemic acids from *Gymnema sylvestre* suspension culture was demonstrated. The proper combination of IAA and BA influenced the rate of gymnemic acid production.

In vitro propagation of medicinal plants with enriched bioactive principle and cell culture methodologies for selective metabolite production is found to be highly useful for commercial production of medicinally important compounds. The increased use of plant cell culture systems in recent years is perhaps due to an improved understanding of the secondary metabolite pathway in economically important plants (Vanisree *et al.*, 2004).

Because of the complex and incompletely understood nature of plant cells in *in vitro* cultures, case-by-case studies have been made within last few years (Vanisree *et al.*, 2004). These new technologies will serve to extend and enhance the continued usefulness of the potent anti-diabetic plant *Gymnema sylvestre*. Advances in *Gymnema sylvestre* cell culture could provide new means for the cost-effective and commercial production. Hopefully it can be concluded that a continuation and intensification efforts in this field will lead to controllable and successful biotechnological, production of specific, valuable and as yet unknown plant chemicals.

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