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RESEARCH ARTICLE

IMPROVED INULINASE ACTIVITY BY *PENICILLIUM PURPUROGENUM* GROWN IN MICROWAVE PRETREATED COFFEE SPENT BY L_{16} ORTHOGONAL DESIGN OF EXPERIMENT

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Abstract

Inulin belongs to non-digestible carbohydrate, storage polysaccharide, widely distributed in grasses and other plants. Recently, inulin has gained significant economic importance and received great interest because they can represent as cost effective and abundant substrate for the production of microbial inulinases with commercial viability. In this aspect, we have investigated the microwave assisted pretreatment process of coffee spent for effective inulin extraction and thereby, better inulinase activity. In the present study, fungal species showing high inulinase activity have been isolated from rhizosphere region of soil samples collected from Non such estate, Coonoor and Yercaud, India. Two different species viz., *Penicillium purpurogenum* (0.953 U/ml) and *Aspergillus tamarii* (0.432 U/ml) showed maximum inulinase activity. Under normal inulin extraction conditions the inulinase activity by *Penicillium purpurogenum* has been observed as 28.08 U/ml (29.46 fold increase) whereas, maximum inulinase activity was recorded as 42.57 U/ml (44.67 fold increase) by the pretreatment (inulin extraction) of coffee spent using microwave assisted extraction (MAE). The optimum conditions of MAE of inulin with improved inulinase activity were found to be 540 watts, 1: 25 material ratio and 30 seconds. Based on the results MAE may be considered as significant and cost effective method for inulin extraction.

Keywords: coffee spent, inulin, inulinase, microwave assisted extraction (MAE), *Penicillium purpurogenum*

Introduction

Inulin is a widespread naturally occurring polyfructan present in plants that consist of linear chains of β(2,1) linked fructose residues attached to a terminal sucrose molecule (Vandamme and Derycke, 1983; Marchetti, 1993). It is distributed as a major reserve carbohydrate in the roots or tubers of plants such as *Helianthus tuberosus*, *Jerusalem artichoke*, dahlia , chicory, banana, garlic etc. (Nagem *et al*., 2004; Singh and Gill, 2006; Bonciu *et al*., 2010; Chi *et al*., 2011). Inulins can be differentiated according to the degree of polymerization with various functional properties (Molina *et al*., 2005) and it depends upon various parameters such as plant source, climate, growth conditions and storage time (Chi *et al*., 2011).

Inulinases (2,1-β-D-frutan fructanohydrolase, EC 3.2.1.7) generally catalyze the hydrolysis of the glycosidic linkages present in the inulin to produce inulo-oligosaccharides, fructose and glucose (Neagu and Bahrim, 2011). Fructose formation from inulin by inulinases offers greater advantage that it involves only a single enzymatic step yielding up to 95-98% fructose. In this aspect, microbial inulinases can be classified according to their mode of action on inulin hydrolysis. Exo-inulinases (β -D-fructopyranoside fructohydrolase, EC 3.2.1.8) release fructose from the fructosyl terminal of inulin whereas, endo-inulinases (β-D-fructan:fructan hydrolase, EC 3.2.1.7) acts on the internal glycosidic linkages of the inulin and

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normally lack invertase activity (Ertan *et al*., 2003; Chi *et al*., 2011). Previous literature survey has reavealed that several fungal and bacterial species like *K. marxianus* var. *bulgaricus*, *A. fumigatus*, *A. niger*, *Staphylococcus. sp* RRL1, *P. pastoris, P. purprogenum, Pseudomonas sp., B. polymyxa* and *C. pseudoropicalis* produce different forms of inulinases (Prabhjot *et al*., 2006). Attempts have been made on utilization of several cheap and cost effective substrates like chicory, dahlia, onion, garlic, banana, wheat, rye and barley for effective production of inulinases. They can be employed in various industrial applications like production of ultra-high fructose syrup, bioethanol, inulo-oligosaccharides, single-cell protein, citric acid, butanediol and lactic acid (Chi *et al*., 2011; Pandey *et al*., 1999).

In the light of the aforementioned reports, it was found that there is no scientific documentation on the formulation of optimal process for inulin extraction from cheap substrates using experimental designs. In this connection, our laboratory has focused on the extraction optimization of inulin (pre-treatment) from the coffee spent (substrate) using orthogonal design of experiment for improved inulinase activity.

Materials and methods

Isolation of microorganisms

The soil samples from coffee plantation were collected from Non such estate, Coonoor and Yercaud, Tamil Nadu, India. According to Warcup (1950), the procedure was followed. The rhizosphere soil samples (1 g) were suspended in 100ml of sterilized distilled water (1:100 dilution) and subsequently 1ml of this suspension was added into 9 ml of sterilized distilled water and serially diluted upto 10^{-6}. Petri dishes containing potato dextrose medium plus streptomycin were inoculated into the diluted soil suspensions and were kept at room temperature. The growth of the colonies were monitored up to 72 h. Fragments of the individual colonies were transferred separately to the same medium containing 10 mg of streptomycin and the growth was observed for 72 h. Finally, the strains were maintained at temperature of 4°C.

Screening Procedure

Primary screening

All fungal colonies were point inoculated on agar plates and incubated at temperature of 30°C for 5 days. Colonies that displayed rapid growth, i.e. attained large colony diameter per unit growth time, were selected for further experiments and transferred to fresh plates**.**

Secondary screening

Selected fungi and other isolates were inoculated into separate 250 ml shake flasks that contained 50 ml of Potato Dextrose Broth (potato infusion form 20 g, dextrose 2 g in 100 mL of distilled water, pH range: 5.1 ± 0.2) and incubated at temperature of 30°C at 200 rpm for 5 days. The samples were withdrawn periodically and analyzed for activity of inulinases. The lactophenol cotton blue (LPCB) wet mount preparation is mainly used for the identification of fungal species with high inulinase activity (Parija *et al*., 2003).

Extraction of inulin

The inulin from the coffee spent wash was extracted as per the protocol described by Sadasivam and Manickam (1996).

Optimization of inulin extraction from coffee spent (pretreatment) by microwave assisted L_{16} orthogonal design of experiment

The main factors selected for the extraction process were power (180 to 720 watts), materials ratio (weight of the coffee spent (g) : volume of the extracting agent (ml), 1:25 to 1:100) and extraction time (10 to 40 seconds). The optimum extraction conditions were determined by using L_{16} (4^3) orthogonal design of experiment (Table 1) (Arunkumar *et al*., 2011). A single factor analysis of variance (One way ANOVA) was adopted to investigate the effect of each factor on the extraction of inulin and expressed in terms of inulinase activity. To about 100 ml of the pretreated inulin extract, inoculated 7 days old stock culture and incubated at temperature of 37°C for 24 hr on a rotary shaker (REMI Orbital Shaking Incubator, Model CIS - 24 BL, India) at

200 rpm. The culture broth was centrifuged at 8000 rpm for 20 min at temperature of 4°C and the supernatant was used to record inulinase activity.

Table 1. *Orthogonal design parameters in microwave assisted extraction*

Level	Power (watt)	Sol:Liq (g/ml)	Time (sec)
1	180	1:25	10
2	360	1:50	20
3	540	1:75	30
4	720	1:100	40

Inulinase assay

Inulinase assay was carried out according to slightly modified method proposed by Skowronek and Fiedurek (2004). To about 2 ml of 0.2 % inulin (pH 3.5), added 0.05 ml enzyme extract and incubated at temperature of 37°C for 20 minutes. The same reaction mixture without enzyme extract was used as the control. The amount of the reducing sugars in the reaction mixture was assayed using the DNS method (Miller, 1959). One unit of inulinase activity (U) was defined as the amount of enzyme, which forms 1 µmol fructose per min under special conditions.

Statistical analysis

The inulinase activity was expressed as mean ± SD. One way ANOVA analysis was used to express the level of significance and all the analyses were carried out using MS Excel 2007 and GraphPad Prism Trial version 5.0.

Results and Discussion

Serial dilution of soil samples in PDA agar plates has revealed the growth of various fungal species and when cultured those organisms in the coffee spent (unprocessed) alone as nutrient medium, only few fungal isolates were identified as inulinase producers. Out of that isolate 1 (Coonoor soil sample) has proved to possess maximum inulinase activity (0.732 U/ml) than isolate 2 (0.432 U/ml). Similarly, fungal colonies screened in Yercaud soil samples showed that isolate 1 possess maximum inulinase activity (0.953 U/ml) than isolate 2 (0.238 U/ml). The isolate 1 and 2 from both the soil samples (Coonoor and Yercaud) were identified as *Penicillium purpurogenum* and *Aspergillus tamarii*, respectively based upon the macroscopic (colour, texture, appearance) and microscopic characteristics (microstructures) by a local mycologist (Dr. K. Kannan, Dept. of Biotechnology, Bannari Amman Institute of Technology, Sathyamangalam, Tamil Nadu, INDIA). The color of *Penicillium purpurogenum* was visualized as blue or green and its texture revealed the presence of conidiophores that branch out into racemules at the apex with the chains of colored, unicellular spores, or conidia (Figure 1). Similarly, *Aspergillus tamarii* was visualized as black color with well defined hyphae and conidiospores (Figure 2). Hence, *Penicillium purpurogenum* isolated from the Yercaud soil sample was considered as efficient inulinase producer and subsequently selected for further experimental analysis.

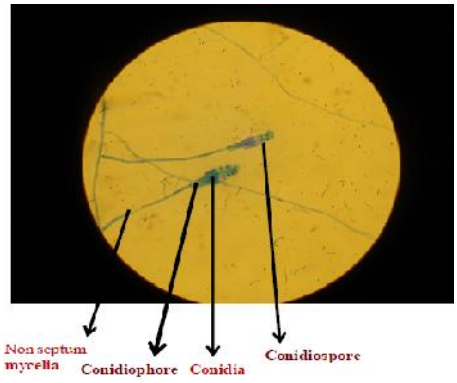

Figure 1. *Microscopic apperance of Pencillium purpurogenum*

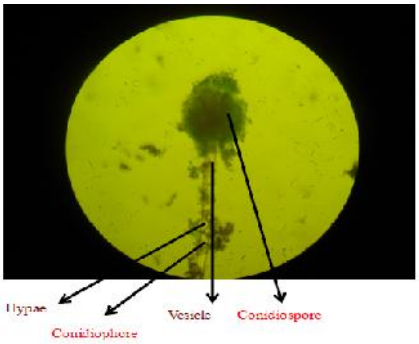

Figure 2. *Microscopic apperance of Aspergillus tamarii*

The amount of inulin extracted under normal conditions from the coffee spent was found to be 40 µg/ml and the activity of inulinase produced by *Penicillium purpurogenum* cultured in the inulin extract was observed as 28.08 U/ml (29.46 fold increase).

Table 2. *Experimental result and range analysis in microwave assisted extraction*

Experiments	Power (watt)	Sol:Liq (g/ml)	Time (sec)	Inulinase activity (U/ml)
1	1	1	1	12.42
2	1	2	2	16.33
3	1	3	3	20.07
4	1	4	4	19.12
5	2	1	2	31.86
6	2	2	1	18.40
7	2	3	4	10.35
8	2	4	3	4.99
9	**3**	**1**	**3**	**42.57**
10	3	2	4	24.21
11	3	3	1	11.79
12	3	4	2	11.47
13	4	1	4	31.00
14	4	2	3	20.08
15	4	3	2	11.43
16	4	4	1	4.99
K1	67.95	117.85	47.61	
K2	64.61	79.03	71.10	
K3	90.04	53.64	87.71	
K4	67.51	40.59	84.69	
k1	16.98	29.46	11.90	
k2	16.40	19.75	17.77	
k3	22.51	13.41	21.92	
k4	16.87	10.41	21.17	
R	6.11	19.32	10.02	

Process optimization techniques can act as powerful tools in acheiving the expected response and may be approached heuristically (Bolboaca and Jantschi, 2007). The concept of design of experiments was introduced by Fisher as early in 1920s and popularized by Taguchi by applying the techniques in many sectors like medical, agriculture, environment, chemical sciences etc., (Taguchi, 1986). Instead of using full factorial analysis for process optimization, Taguchi method has suggested that the orthogonal array of approach may provide less cost and time. Such an approach was adopted in the extraction optimization of inulin from coffee spent in order to improve the inulinase activity.

Microwave assisted extraction optimization of inulin (4^3, L_{16} orthogonal design) from coffee spent was found to be 540 W, 1:25 material ratio and 30 seconds with a maximum inulinase activity of 42.57 U/ml. One way ANOVA results proved that material ratio was significantly different at 5% ($p < 0.05$) and an equivalent effect has been observed for the microwave power, even though it was not found to be significantly different at 5% level. The entire results have been depicted in Table 2 and 3.

Table 3. *One way ANOVA of microwave assisted extraction*

Levels	Sum of squares	Degrees of freedom	Mean square	F-value
Power (watt)	100.19	3	33.39	0.20
Sol:Liq (g/ml)	1858.52	3	619.38	3.71
Time (sec)	250.35	3	83.43	0.50

Effect of microwave power on inulinase activity

Several techiques like Soxhlet, solvent assisted, temperature assisted, ultrasonication, microwave and supercritical fluids have been proposed for the extraction of natural compounds. Among these techniques microwave assisted extraction (MAE) was considered as a new method for the extraction of phytochemicals and also processing various raw materials. MAE was also considered to be the superior method than other conventional methods (Gallo *et al.*, 2010). Previously several researchers have successfully extracted biopolymers using MAE (Chen *et al.*, 2005; Leonelli and Mason, 2010). Microwave heating leads to the expansion and degradation of cell wall and also alteration in the membrane fluidity with the liberation of cytosolic contents into the solvent (Eskilsson and Bjorklund, 2000; Latha, 2007). Normally, rapid rupture of cell wall and membrane takes place at higher temperature when kept at higher power, as a result the desired analytes may be leached out into

the solvent whereas, at low power levels the cell wall rupture might take place gradually. An increase in the inulinase activity (42.57 U/ml) has been observed between 180 to 540 watts was due to an increase in the yield of inulin content (Figure 3). Whereas, a decrease in the inulinase activity was observed at 720 watts proved that an increase in the power (increase thermal energy) leads to not only the disruption of cell wall and membrane, but also a reduction in the inulin content because of the hydrolysis of β-glycosidic linkages of inulin that yield fructo oligosaccharides.

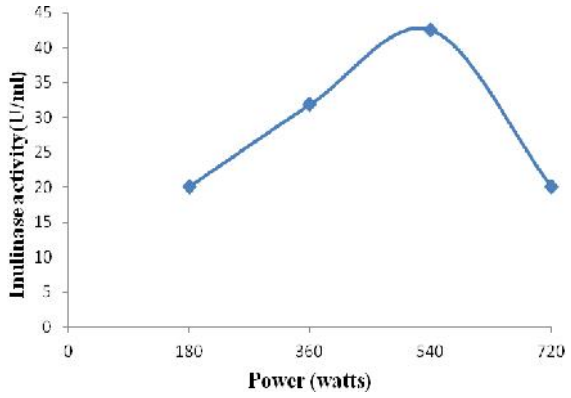

Figure 3. *Effect of microwave power on the extraction of inulin*

Effect of material ratio (Sol:Liq) on inulinase activity

The inulinase activity reached maxima (42.57 U/ml) at an initial level i.e., 1:25 materials ratio because of an increased inulin extraction. Further increase in the material ratio leads to a gradual decrease in the inulinase activity was due to the fact that when the material ratio reached a steady state level, the inulin content has become well saturated and thereby, inhibit the diffusion of itself from the cell that leads to a subsequent reduction in the activity (Xu *et al.*, 2005; Sathishkumar *et al.*, 2008). In conventional extraction techniques, higher solvent volume may give lower yield and thereby, this kind of experimental design based approach may reduce the consumption of solvents and also increase the yield. In general, the material ratio play a vital role in affecting the concentration gradient between solid and liquid phase and improve the yield of the desired analyte (Wang *et al.*, 2009).

Effect of irradiation time on inulinase activity

The inulinase activity was found to be maximum (42.57 U/ml) at an irradiated time of 30 seconds because of increased inulin content. Investigation of irradiation time is extremely important because the efficiency of extraction may be varied. Generally, by increasing the extraction time, the quantity of the components extracted may be increased, although there is a risk of degradation. Irradiation time is also influenced by the dielectric properties of the solvent. At an initial stage of extraction (10 seconds to 30 seconds), microwave irradiation may induce and increase the kinetic energy of inulin present in the cell that may influence its diffusion rate. Furthermore increase in time (40 seconds) may lead to hyperthermal and hypervaporisation reflux in the system that cause a decrease in the inulin content and thereby, record poor inulinase activity (Wang *et al.*, 2009). A similar report regarding the role of irradiation time has been documented by Arunkumar *et al.* (2011) for the extraction of phenolic acids.

Conclusions

From different isolates, *Penicillium purpurogenum* grown in the raw coffee spent was found to possess maximum inulinase activity (0.953 U/ml).

The optimal conditions of MAE of inulin (pretreatment) was found to be 540 watts, 30 seconds and 1:25 material ratio with an improved inulinase activity (42.57 U/ml, 44.67 fold increase).

Normally, MAE presents advantages like less time consumption, high extraction efficiency and environmentally benign.

In conclusion, coffee spent may be pretreated with MAE procedure and can be used as inoculum for the growth of *Penicillium purpurogenum* that effectively improve the inulinase production. In future, this kind of experimental approach can be adopted for cost effective processing of any agro waste for better enzyme production.

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