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# Optimization of Process Parameters for High Yield Production of Exo – Inulinase from *Trichoderma asperellum* RSBR08 by Using Solid State Fermentation

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**Abstract**

Inulinase is an industrially applicable enzyme which is used widely in the production of ultra high fructose syrups and fructo-oligosaccharides. Production of inulinase enzyme in a cost effective manner is the major challenge faced. In the present research study, *Trichoderma asperellum* RSBR08 which has the capability to produce high exo-inulinase enzyme is produced by using the solid state fermentation. Process parameters for production of exo-inulinase by using solid state fermentation were optimized. Results showed high exo-inulinase production in garlic as substrate (199.2±6.3 U/gds), 45% moisture percentage (228.4±3.4), 26°C temperature (219.1±5.8) and 5.0 pH (213.6±4.5). Effect of different metals *viz.* $Mg^{2+}$,$Zn^{2+}$, $K^{+}$,$Ca^{2+}$, $Na^{+}$,$Mn^{2+}$ and $Hg^{2+}$ were studied on the production of exo-inulinase enzyme in which $Ca^{2+}$ induced the enzyme production (196.3±5.6) whereas $Hg^{2+}$ has inhibited the inulinase production (37.0±3.3). Based on the above results, the solid substrate fermentation parameters optimized can be used industrially.

**Key words**: *Exo-inulinase, Metal ions, Solid state fermentation, Trichoderma asperellum*

## Introduction

Inulin is a linear polymer of fructose with b, 2-1 linkage terminated with glucose unit by a sucrose type linkage at the reducing end (1). Inulin is available abundantly in roots and tubers of garlic, jerusalem artichoke, chicory, dandelion, onion, leek and dahlia (2). Inulin is an inexpensive and readily available substrate for the production of fructose syrups and fructo-oligosaccharides. Fructose is much preferred now-a-days because of its sweetening property and insignificant insulogenic effects (3).

Fructose units can be liberated from inulin by the regular acid hydrolysis process which will be carried out at temperature 80 - 90°C and 1 - 2 pH. But the acid hydrolysis process results in the degradation of fructose and formation of di-fructose anhydrides which results in the formation of colored end product. Due to these drawbacks of acid hydrolysis process, inulinase enzymes gained much importance by which 90 - 95% of fructose can be recovered in a single step without formation of any bi products (4, 5).

Initially, inulinases were isolated from plant sources but due to its less productivity, microbial inulinases were much focused (6). Bacteria, yeast and fungal strains which produces inulinases are *Aspergillus spp., Pencillium spp., Xanthomonas spp.*, *Fusarium oxysporum, Rhizopus spp., Streptomyces spp., Acetobacter spp., Artrobacter sp, Bacillus spp, Schizosacchromyces alluvius, Candida spp., Trichoderma spp., Pseudomonas spp.*, *Kluyveromyces spp.*,*Cryptococcus spp.*, *Pichia spp.*, *Sporotrichum spp.*(7, 8, 9).

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Inulinases are broadly categorized into 2 types based on the catalytic activity on inulin *i.e.* exo-inulinase and endo-inulinase. Exo-inulinase (â-2-1-D-fructan fructohydrolase) hydrolyses and liberates the individual fructose units from non-reducing end where as endoinulinase (â-2-1-D-fructan fructanohydrolase) hydrolyses randomly at the internal linkages of inulin (10).

Inulinases can be produced by both submerged and solid state fermentation. But solid state fermentation has gained much importance because of its high yield, operation ease, high product recovery and cost effectiveness (3, 11). In solid state fermentation, selection of substrate plays a major role which is core for enzymatic process. An appropriate substrate selected needs to be cost effective and readily available. In the current study, various plant materials rich in inulin and cost effective were selected for the production of inulinase (12).

In the current research work, substrate and process optimization studies were carried out for high yield production of exo-inulinase from *Trichoderma asperellum* RSBR08 using solid state fermentation.

## Materials and Methods

***Chemicals and reagents:*** All the chemicals and reagents were purchased from Fisher scientific, Mumbai, India.

***Sourcing of inulinase producing fungal strain:*** Exo-inulinase producing *Trichoderma asperellum* RSBR08 was sourced from culture collection of R&D center, SOM Phytopharma (India) Limited, Hyderabad, India. The fungal culture was subcultured on potato dextrose agar slants and stored at 4°C.

***Substrate materials collection and preparation:*** Chicory, jerusalem artichoke, garlic, onion were procured from vegetable market in Kukatpally, Telangana, India (17.4948°N, 78.3996°E). Wheat bran, coconut oil cakes were purchased from the local market of Suraram, Telangana, India (17.5412°N, 78.4338°E). Sugarcane bagasse was collected from sugarcane crushing shops in Miyapur, Telangana, India (17.5169°N, 78.3428°E). Substrates collected were washed under running tap water and chopped into small pieces. Chopped pieces were dried in hot air oven at 80°C for 24 h. The pretreated substrates were used for solid state fermentation.

***Inoculum preparation :*** Exo-inulinase producing *Trichoderma asperellum* RSBR08 was inoculated in liquid medium with the composition g/L: inulin – 10 g/L, yeast extract – 10 g/L, $NaNO_3$ – 10 g/L, $KH\ PO_4$ – 5 g/L, $MgSO_4.7H_2O$ – 1 g/L, pH – 5.0. Inoculated flask was kept on orbital shaker at 120 rpm for 96 h (12).

***Solid state fermentation:*** Solid state fermentation was carried out in HDPE autoclavable bags. 150 g of each substrate was taken in each bag and 40% moisture was maintained with the minimal media composition (g/L - yeast extract – 1 g/L, $NaNO_3$ – 1 g/L, $KH\ PO_4$ – 0.5 g/L, $MgSO_4.\ 7H_2O$ – 0.1 g/L). Substrate bags were autoclaved at 121°C and 15 psi for 30 min. $1.0x10^8$ fungal spores were added in each bag, mixed thoroughly and incubated at 28°C for 96 h (7). Substrate showing high inulinase activity was used for further optimization studies.

***Analysis of inulinase activity :*** Inulinase assay was performed for the amount of fructose units liberated from the inulin. Reaction mixture with 0.5 ml enzyme extract, 0.5 ml of 1% (w/v) inulin in 0.2 M sodium acetate buffer with pH – 5.0 and incubated at 50°C for 15 min. Amount of reducing sugars liberated were measured by using Somogyi copper reagent and absorbance was taken at 520 nm. Inulinase activity can be calculated as the amount of enzyme liberated 1 µmol of fructose per minute under the assay conditions and expressed as units of activity per gram solid substrate (U/gds) (3).

***Optimization of parameters in solid state fermentation:*** Substrate showing high inulinase activity was used further for solid state fermentation optimization studies.

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***Moisture percentage:*** Moisture percentage in the solid state bags was maintained by minimal nutrient solution. Different moisture percentages *i.e.* 25, 30, 35, 40, 45, 50, 55 and 60 were maintained in different solid state bags. Bags were inoculated with *T. asperellum* RSBR08 culture and incubated at 28°C for 96 h. Inulinase assay was carried out by using the standard protocol.

***Temperature:*** Different temperatures ranging from 20°C - 30°C (20, 21, 22, 23, 24, 25, 26, 27, 28, 29 and 30°C) were maintained. Substrate bags were inoculated with the *T.asperellum* culture and incubated at different temperatures for 96 h. Samples were drawn after the incubation period and inulinase assay was carried.

***pH:*** In solid state fermentation, pH of the substrate bags was maintained by changing the pH of the minimal nutrient solution. pH ranging from 3-8 was maintained in different bags and inoculated with the fungal culture inoculum. Inoculated bags were incubated at 28°C for 96 h and assay was carried out.

***Effect of metal ions on inulinase production:*** Effect of metals on inulinase activity was measured by using $MgSO_4$, $ZnCl_2$, KCl, $CaCl_2$, NaCl, $HgCl_2$ and $MnSO_4$ with a concentration of 0.1% w/v. Each substrate bag is inoculated with different metal and autoclaved. Sterilized substrate bags were inoculated with fungal culture and incubated at optimum conditions (13).

**Results and Discussion**

Increase in demand for inulinases and applicability in the production of fructo-oligosaccharides, inulio-oligosaccharides, and high fructose syrups *etc* created inquisitiveness to search for new inulinase sources. In this scenario, microbes have grabbed much attention with their ability to produce high inulinase yields and ease of production (14). In the current research study, attempts were made to optimize the process parameters for exo-inulinase production by using *Trichoderma asperellum* RSBR08 by solid state fermentation technology.

Potent exo-inulinase producing *Trichoderma asperellum* RSBR08 was sourced from culture collection of R&D center SOM Phytopharma (India) Limited, Hyderabad. For substrate optimization studies several substrates *viz.* Chicory, jerusalem artichoke, garlic, onion, wheat bran, coconut oil cake and sugarcane bagasse were collected and pretreated. 40% of moisture content was maintained with the minimal medium, bags were autoclaved and inoculated with the *T.asperellum* culture. After 96 h, the substrate samples were collected and inulinase activity was checked. Among all the substrates Garlic showed high exo-inulinase activity (199.2±6.3 U/gds) followed by Jerusalem artichoke (169.9±5.2 U/gds) and Chickory (157.6±5.7 U/gds). Fig. 1 Similar research reports showed 155.8 U/gds in garlic peel when substrate optimization studies were carried out by using *Aspergillus niger* (7). Similarly, in addition to garlic other nutrients like $NH_4NO_3$, $MnSO_4 \cdot 7H_2O$, Soya bean cake, and $K_2HPO_4$ showed high yield of inulinase with 76 U/gds (15). Garlic which was showing high exo-inulinase yield is used as substrate for further optimization studies.

In an attempt to optimize moisture content different percentages *i.e.* 25%, 30%, 35%, 40%, 45%, 50%, 55% and 60%. Among all moisture percentages, substrate sample from 45% showed high exo-inulinase activity (228.4±3.4) followed by 40% (198.5±5.6) and 50% (188.9.4±5.8). Fig. 2

Temperature optimization studies were carried out for the high yield production of exo-inulinase by maintaining the temperatures from 20-30°C in separate substrate bags. Substrate samples were collected at 96 h which showed high inulinase activity at 26°C (219.1±5.8). Fig. 3

Similarly, pH optimization was carried out by adjusting the pH of minimal nutrient solution. pH range maintained was 3,4,5,6,7 and 8 and the substrate samples were collected at 96 h. Among all the pH, high inulinase activity was recorded at 5.0 with an exo-inulinase activity of 213.6±4.5. Previous research reports showed 148.2 U/gds

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when garlic peels were used as substrate by using *Aspergillus niger* (7) Fig. 4.

Effect of different metal ions on the production of exo-inulinase was tested by using $MgSO_4$, $ZnCl_2$, KCl, $CaCl_2$, NaCl, $HgCl_2$ and $MnSO_4$. In the current research study, $Ca^{2+}$ induced the exo-inulinase production with an enzyme activity of 196.3±5.6 followed by $Mn^{2+}$ (174.4±6.2). Whereas $Hg^{2+}$ inhibited the inulinase production with a minimum enzyme activity of 37.0±3.3.Fig 05 Research reports indicate the inducing of inulinase activity by $Ca^{2+}$ ions by using *Aspergillus fumigatus* (16). In *Bacillus* sp. B51f, $Ca^{2+}$ showed increase in the yield of inulinase enzyme (17).

## Conclusion

From the current study, it can be concluded that *Trichoderma asprellum* RSBR08 can be produced by solid state fermentation by taking Garlic as the substrate which showed exo-inulinase activity of 199.2±6.3 U/gds. From the results of process optimization studies it can be concluded that at 45% moisture content, pH - 5.0 and temperature - 26°C, high yield of inulinase enzyme can be produced. When effect of metal ions was tested on inulinase enzyme production, $Ca^{2+}$ induced the enzyme production whereas $Hg^{2+}$ inhibited the inulinase production. By using the above process parameters, high inulinase production can be observed and this can be effectively used in industrial applications.

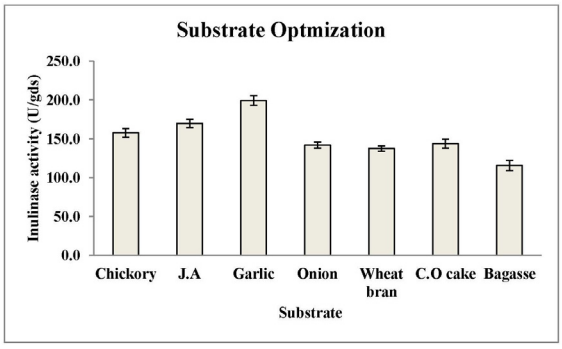


**Fig. 1:** Substrate optimization studies

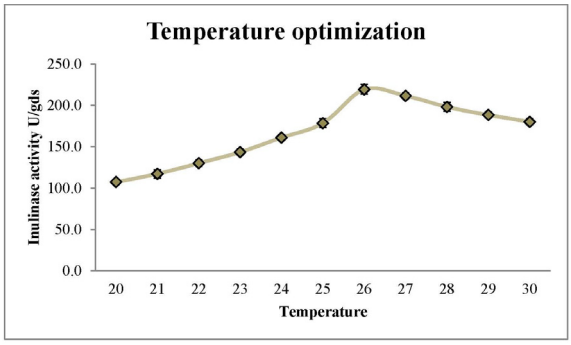


**Fig. 3:** Temperature optimization studies

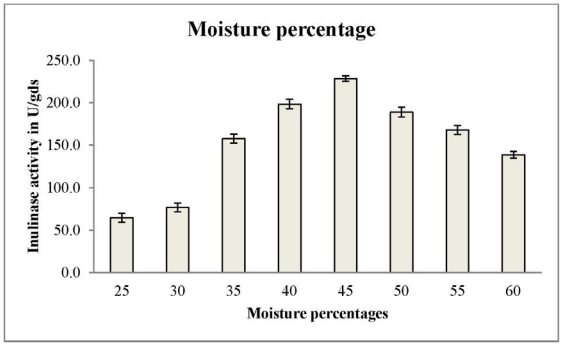


**Fig. 2:** Moisture percentage optimization

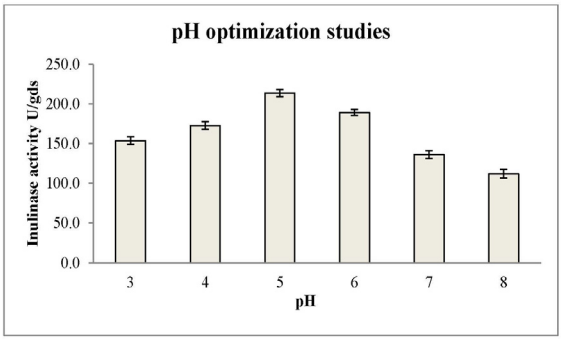


**Fig. 4:** pH optimization studies

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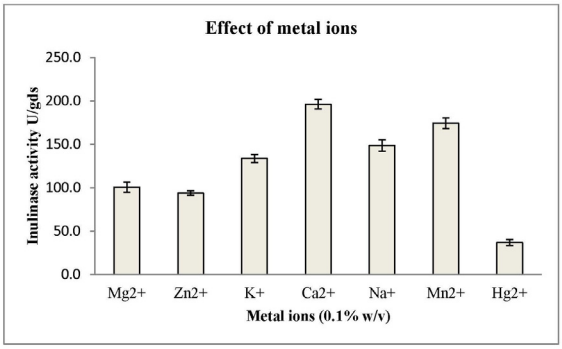


**Fig. 5** Effect of metal ions on inulinase production

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**Conflict of interest**

Authors have no conflict of interest.

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