Influence of variable agitation and aeration on inulinase production from *Kluyveromyces marxianus*

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**Abstract**
Fructose and Fructo-oligosaccharides (FOS) are emerging fast as important ingredients in the food and pharmaceutical industry and inulinase constitute an important class of enzymes for producing these products from inulin. With the ever increasing need of inulinase, it is necessary to enhance the yield of inulinase in the already available organisms. In our study fermentation parameters were optimized for increase in production of inulinase by yeast *Kluyveromyces marxianus* MTCC 3995. Variable agitation and Variable aeration parameters were shown to be significant on production of inulinase because the activity of inulinase was higher using variable agitation and aeration compared with fixed agitation and aeration. There was 17.7% increase in enzyme activity with variable agitation and aeration as compare to activity with fixed agitation and aeration. Inulinase Enzyme activity was observed 55.1 IU/ml with combination of variable agitation and aeration compare to 46.8 IU/ml with fixed agitation as 200rpm and fixed aeration as 0.75 vvm during fermentation in laboratory scale fermenter. Agitation combination of 175 rpm for 0-24 hrs, 225 rpm for 24-48 hrs, 200 for 48-72 hrs and aeration at a combination of 0.75 vvm for initial 24 hrs, 1.0 vvm for 24-48 hrs and 0.75 vvm for 48-72 hrs supports to achieve highest inulinase activity (55.1 IU/ml) in 72 hrs at 27° C. Higher biomass buildup with variable agitation and aeration influence the increase in enzyme activity. Results suggest that the variable agitation and aeration combination can be applied for cost effective inulinase production from dahlia extract.

**Citation:**

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oligosaccharides etc. (Singh et al., 2006; Pandey et al., 1999).

1.2 Effect of different parameters on inulinase production

Owing to the cost of pure inulin, search for inulin sources have recently received a great deal of attention as they represent a renewable, low-cost and abundant raw material. Production of inulinase is affected by medium components, operational parameters and type of the organism used for fermentation. Reports in favor of these arguments can be summarized with the work of (Vandamme et al., 1983) in which various carbon sources like fructose, sucrose, and purified inulin have been examined for production of this enzyme. Major improvements in the productivity of a fermentation process can be achieved by modifying parameters like physiochemical and nutritional environments to which the organism is exposed (Singh et al., 2006). Investigation was performed for the effects of sucrose concentration, pH, temperature and aeration rate on the production of biomass and inulinase (Cazetta et al., 2008). Effect of inulinase activity per unit biomass under different aeration rates was found favorable for inulinase production (Jiaoqi et al., 2012). Dahlia is a flowering plant of family asteraceae commonly grown for ornamental purpose. The tubers of dahlia contain about 12.5% of inulin as a storage polysaccharide (Cruz et al., 1998). Among the microbial strains used for inulinase production, those of K. marxianus and A. niger are the most commonly used ones (Pandey et al., 1999; Kango et al., 2011). Taking into consideration the utmost need of commercial production of inulinase, this study was carried to find out the influence of the variable agitation and aeration and surfactant concentration on the production of inulinase by K. marxianus.

2. Objective of Research

To date, to the best of our knowledge, no systematic studies have been conducted that clearly outline the effect of variable agitation and aeration on inulinase production. Objective of our study is to evaluate influence of variable agitation and aeration on inulinase production in laboratory scale reactor and Scaling-up feasibility for commercial production. Successful completion of this study will support the low cost enzyme production by using single step process in place of current multistep production process of inulinase.

3. Materials and Methods

Inulinase enzyme production on commercial scale is challenge today because of low yield and high cost. Improvement in process and enzyme activity by using simultaneous saccharification and fermentation can increase the feasibility of inulinase commercial production with low cost. Research plan designed to select the cheap raw material as inulin source, optimization of media and various process parameters for maximum yield of inulinase enzyme.

3.1 Microorganism

The yeast Kluyveromyces marxianus MTCC 3995 was obtained from Microbial Type Culture Collection (MTCC), Chandigarh, India. The culture was grown on agar medium incubated at 26-30°C and maintained at 2-8°C on the slants of the same media.

3.2 Substrates and chemicals

All the chemicals and reagents used in the present study were of analytical grade and procured from GE Healthcare Bio-Sciences Ltd., Glaxo India Ltd., Hi-media, Merck, Qualigens Fine Chemicals, Ranbaxy Labs Ltd., SD Fine Chemicals and Sigma Labs Pvt. Ltd., etc. Mature dahlia tubers were obtained from local area.

3.3 Preparation of inulin source extract

Cichorium roots and Dahlia tubers were used as inulin source. After washing, these were sliced and dried in an oven at 70°C till a constant weight was achieved. Dried roots/tubers were then ground to a homogenous powder. To optimize the inulin extraction various extracts were prepared taking 3.5% of root powder in distilled water under shaking at 100 rpm, boiling water and under pressure at 10 psi and 15 psi, each for 10 min. Thereafter, extracts were filtered through double layered muslin cloth, characterized for total sugars, reducing sugars and inulin. All the extracts were tested for inulinase production.

Taking into consideration the utmost prevailing need of commercial production of inulinase, this study was carried to find out the influence of surfactant concentration in initial basal volume of fermenter media, Variable agitation and Variable aeration on the production of inulinase by K. marxianus. Increase in inulinase production by optimization of process parameters and efforts to reduce the fermentation cycle time will make commercial production of inulinase enzyme more feasible.
To convert inulin into fructose, the most rational and economic option is its enzymatic hydrolysis. Microbial inulinase are an important class of enzymes that catalyzes the hydrolysis process for the production of fructose. Currently 45% yield of fructose is feasible with multienzymatic starch hydrolysis. Simultaneous saccharification and fermentation for inulinase production is more cost effective to get the 90% yield of fructose.

3.4 Preparation of inoculum
The cultures were grown in glucose yeast extract medium containing peptone (0.5%, w/v), yeast extract (0.3%, w/v), dextrose (2.0%, w/v) with a pH of 6.0±0.2. The flasks were incubated at 26-30° C for 18 h. Cell suspension was used as an Inoculum at a concentration of 10% (v/v).

3.5 Enzyme production
The minimal fermentation medium was prepared with raw inulin extracts. Inulin in the medium was adjusted to 1% (w/v) using each raw inulin extract, supplemented with peptone (0.5%, w/v) and adjusted to pH 6.5±0.2. Flasks (250 ml) containing 50 ml of each type of medium were autoclaved (121-124° C, 20 min) and inoculated with 18 h old inoculum (10%, v/v). Fermentation was carried out at 28±2° C for 72 h under agitation (200 rpm). Flasks were withdrawn at regular interval of 24 h and clear culture filtrate was obtained by filtering the broth through whatman filter paper no. 1.

3.6 Effect of surfactants
Different surfactants like PEG-400, PEG-600, Brij-35 and SAG Antifoam were supplemented independently at a concentration of 0.002% in the medium, to investigate their effect on enzyme production.

3.7 Effect of variable agitation and aeration
As the production of several important metabolites in the fermentation is influenced by the agitation and aeration, sets of experiments were performed to study inulinase production by varying these parameters. Agitation rate of the lab scale bioreactor was studied in terms of revolutions per min (rpm). Fermentations were carried out under stationary rpm and air volume (fixed during complete fermentation cycle) and variable agitation and aeration (speed and air volume changes after every 24 h). Agitation was varied from 100-250 rpm, to study its effect on enzyme production. Air was sterilized by passing through a hydrophobic membrane filter (0.2 µm) and it was measured by a rotameter in terms of volume of air per volume of medium per min (vvm). The medium in the fermenter was aerated at 0.50, 0.75 and 1.00 vvm, to study its effect on enzyme production.

4. Analytical Methods
Inulinase activity was measured as described by Miller (1959). Culture broth samples were centrifuged at 6000 g for 10 min. The supernatant was then collected and diluted appropriately with distilled water, and subjected to inulinase activity assay. Briefly, 0.5 ml culture supernatant was incubated with 2% (w/v) inulin prepared in 0.02 M sodium acetate buffer (pH 4.6) at 55° C for 10 min, and the reducing sugar was analyzed by the dinitrosalicylic acid method Miller (1959). One enzyme unit was defined as the amount of fructose (µmol) hydrolyzed per min under the above conditions (Parekh, et al., 1985). Fructose was used as the standard substance to plot a standard curve. Cell mass concentration was determined using optical density at 620 nm.

5. Results and Discussion
5.1 Inulin substrate
Among various substrates employed as carbon source for inulinase production, inulin containing plant materials offer advantage in comparison to purified substrates in terms of lower cost and high productivity. Complex substrates from agro-industrial wastes have been of wider interest for production of microbial enzymes (Mazutti et al., 2010) and (Park et al., 2001). The substrates prepared by different extraction methods showed that Dahlia extract prepared at 15 Kg/cm² pressure was the most favorable inducers of inulinase with maximum activity of 9.8 IU/ml (Table 1).

Table 1: Effect of inulin extraction methods on inulinase activity

<table>
<thead>
<tr>
<th>Method</th>
<th>Enzyme Activity (IU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dahlia inulin</td>
<td>Cichorium inulin</td>
</tr>
<tr>
<td>Cold water</td>
<td>3.4</td>
</tr>
<tr>
<td>Boiling Water</td>
<td>7.1</td>
</tr>
<tr>
<td>10 Kg/cm²</td>
<td>8.8</td>
</tr>
<tr>
<td>15 Kg/cm²</td>
<td>9.8</td>
</tr>
</tbody>
</table>

Highest inulinase activity (25.3 nkat ml⁻¹) reported with Dahlia extract as carbon source which was 1.4 folds higher than that observed in media containing pure chicory inulin (Jain et al., 2012). Tubercules of yacon (Polymnia sanchifolia), also a member of Asteraceae, have been have been reported as an inexpensive substrate for inulinase production from Kluyveromyces marxianus (Cazetta et al., 2005) and garlic bulbs (Allium sativum) have
been used for inulinase production from Streptomyces sp. (Sharma et al., 2006).

5.2 Effect of different surfactant on inulinase production

It is well known that surfactants dilate the pores by interaction with phospholipids located in cell membrane, thus they affect cell permeability (Tukmachev et al., 1977). Surfactants can increase the secretion of extracellular protein, while on other hand, may causes the lethality of living cells. To study the effect of surfactants on inulinase production from K. marxianus, different surfactants were supplemented in the medium at a concentration of 0.002% (w/v) and the results are presented in Fig. 1. An increase in enzyme production from 21.6 to 25.7 IU/ml has been observed on addition of SAG-Antifoam in the fermentation medium. PEG-600 showed no significant influence on enzyme production, while negative impact was seen on addition of PEG-800 & Brij-35 in the medium.

The concentration of SAG Antifoam was varied from 0.002% to 0.01%, to investigate its effect on inulinase production. There was an increase in inulinase activity (27.9 IU/ml) with an increase in SAG Antifoam concentration up to 0.006% (Fig. 2). Above this concentration, SAG Antifoam has shown an inhibitory effect on the inulinase production. The higher concentration may have proved lethal for the microorganism due to solubilization of membrane bound proteins and phospholipids, which causes release of these components from the cells. High SAG concentration causes denaturation of inulinase, but low concentration (0.002-0.006%) of SAG in fermenter broth may cause only reversible unfolding of proteins as well as dilates pores in cell membrane enhancing secretion of extracellular enzymes including inulinase.

Figure 1: Effect of different surfactants

Figure 2: Effect of surfactant concentration

5.3 Effect of agitation on inulinase production

Two types of experiments were performed to evaluate the effect of agitation on inulinase production. First experiment set was performed with different agitation speed and speed was kept constant throughout the fermentation cycle for particular set of experiment. Second experiment set was performed with variable speed after every 24hrs during each fermentation cycle. Aeration rate was 0.75 vvm for all sets of experiment performed for evaluation of agitation effect. For fixed agitation experiment the inulinase production was maximum 42.4 IU/ml with 200rpm (Fig.3) supported by maximum biomass 7.5 g/l build up with fixed agitation of 200 rpm (Fig.4). When the agitation was further increased to 250 rpm both inulinase activity and biomass concentration was not obviously improved. This is attributed to the fact that agitation is related to better dispersion of the substrate and oxygen and further its corresponding availability to the cells. Highest inulinase production by Geotrichum candidum using Jerusalem artichoke as carbon source reported at 200 rpm (Serkan et al., 2011). Inulinase activity and biomass concentration improved with variable agitation during each fermentation cycle. The present study at variable rate denoted highest inulinase production (53.8 IU/ml) at agitation combination of 175 for 0-24hrs, 225 rpm for 24-48 hrs, 200 for 48-72hrs and aeration 0.75 vvm (Fig.5) compare to inulinase activity as 42.4 IU/ml with fixed 200 rpm. There was 26.8 % increase in inulinase activity with variable agitation compare to maximum inulinase activity with fixed agitation speed as 200rpm. Biomass build up with variable agitation was 9.6 g/l (Fig.6). There was 28% increase in biomass build up with variable agitation as compare to biomass build up with fixed agitation. In another study, Kluyveromyces marxianus showed maximum inulinase production of 176 IU ml⁻¹ at 450 rev min⁻¹ and
aeration at the rate of 1 vvm (Silva et al., 2005).

**Figure 3: Effect of fixed agitation on Inulinase**

**Figure 4: Effect of fixed agitation on biomass**

**Figure 5: Effect of variable agitation on Inulinase activity**

**Figure 6: Effect of variable agitation on biomass**

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5.4 Effect of aeration on inulinase production

Two types of experiments were performed to evaluate the effect of aeration on inulinase production. First experiment set was performed with different aeration (0.50, 0.75, 1.0 & 1.5 vvm) and was kept constant throughout the fermentation cycle of particular set of experiment. Second experiment set was performed with variable aeration after 24 hrs in each fermentation cycle. Variable agitation as optimized in pervious experiment of present study was used during each experiment of effect of aeration on inulinase production. Inulinase activity was observed maximum 46.8 IU/ml with aeration 0.75 vvm (Fig.7). Maximum biomass concentration build up was 8.3 g/l with aeration of 0.75 vvm (Fig.8). When the aeration was further increased to 1.0 or 1.5 vvm both inulinase activity and biomass concentration was not obviously improved and was at a level similar to that observed when aeration was 0.5 vvm. Less production of inulinase at higher aeration rate may be due to decrease in oxygen transfer rate and change in rheological properties of the medium. It was observed that the inulinase enzyme activity decreased with the increase in aeration during working with Kluyveromyces marxianus var. bulgaricus in a stirred batch reactor (Cazetta et al., 2010). Inulinase production and biomass concentration improved if the aeration rate was increased to 0.5 vvm (Jiaoqi et al., 2012). In the present report, it was found that at variable rate of aeration, inulinase production was maximum (55.1 IU/ml) with aeration.
combination of 0.75 vvm for initial 24 hrs, 1.0 vvm for 24-48 hrs and 0.75 vvm for 48-72 hrs (Fig.9) compare to inulinase activity as 46.8 IU/ml with fixed aeration 0.75 vvm. Biomass concentration was marginally improved and observed as 10 g/l with variable aeration (Fig.10). This in agreement with findings that the enzyme production from *Kluyveromyces marxianus* is strongly influenced by mixing conditions (Silva et al., 2005). It has also been established by them that the death rate of cells increases with the increase in agitation rate due to shear stress caused by blade tips of the impeller.

**Research Highlights**

Different substrates were characterized for inulin source and observed that inulinase activity is more with Dahlia tubers as compare to *Chichorium* roots.

Various process parameters were optimized for maximum production of inulinase enzyme in laboratory scale bioreactor.

There was significant increase in enzyme activity was observed if variable agitation and aeration approach being applied during fermentation cycle of inulinase production.

Application of recommendations of this study could be significant cost effective process for commercial production of inulinase.

**Limitations**

This research was performed in laboratory fermenter and could not be further tested on pilot scale reactors. Factors like impeller size distance between impellers and type of impellers could not be studied during our research work. Further study can be planned with fed batch fermentation process for more cost reduction in inulinase production.

**Recommendations**

On the basis of our study it is recommended that variable agitation and aeration approach should be applied for increase in yield of inulinase from *Kluyveromyces marxianus* using Dahlia tubers as source of inulin.
Funding and Policy Aspects

Government should make collaboration with industry for research on large scale (Pilot Plant) to make more appropriate evaluation for commercialization of product based on research recommendations.

Justification of Research

Taking into consideration the utmost prevailing need of commercial production of inulinase, this study was carried to find out the influence of surfactant concentration in initial basal volume of fermenter media, Variable agitation and Variable aeration on the production of inulinase by *K. marxianus*. Increase in inulinase production by optimization of process parameters and efforts to reduce the fermentation cycle time will make commercial production of inulinase enzyme more feasible. To convert inulin into fructose, the most rational and economic option is its enzymatic hydrolysis. Microbial inulinase are an important class of enzymes that catalyzes the hydrolysis process for the production of fructose. Currently 45% yield of fructose is feasible with multienzymatic starch hydrolysis. Simultaneous saccharification and fermentation for inulinase production is more cost effective to get the 90% yield of fructose.

Conclusion

It is concluded from the present study that inulin extraction at 15 kg/cm² pressure produces highest inulinase from Dahlia tubers with SAG Antifoam concentration 0.006% (w/v). Variable agitation and aeration during fermentation cycle further influence the increase in inulinase activity. Highest inulinase production (55.1 IU/ml) was observed at agitation combination of 175 for 0-24 hrs, 225 rpm for 24-48 hrs, 200 for 48-72 hrs and aeration at a combination of 0.75 vvm for initial 24 hrs, 1.0 vvm for 24-48 hrs and 0.75 vvm for 48-72 hrs.

Author’s Contribution and Competing Interests

Dr. Sanjiv Maheshwari provided guidance to design the experiments and technical inputs for trouble shooting encountered during the research programme. Dr. Manjinder Kaur Bedi provided support to collect literature and microbiological aspects of research programme. Gurjeet Singh Bedi performed all experiments of the present study for the award of Ph.D degree.

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