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Effect of feeding strategies on inulinase production analyzed in a biocalorimeter

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ABSTRACT

Kluyveromyces marxianus exoinulinase production in shaker and bio reaction calorimeter has been studied. Biocalorimetric studies revealed enhanced inulinase production due to the linear and exponential feeding of sucrose. Linear fed-batch inulinase production (355 IU/mL) was seventeen-fold greater than batch mode (20.98 IU/mL). Further controlling the specific growth rate by exponential feeding of sucrose gave highest inulinase activity of 728 IU/mL. Exponential feeding strategy yielded highest inulinase activity in the synthetic medium with a reduction in batch time to half that of the linear feeding. This has been substantiated by metabolic and physiological aspects of inulinase production. A Michaelis-Menten type empirical model was developed for fed-batch that can be adopted for controlling specific growth rate based on metabolic heat responses, towards achieving maximum enzyme production and release.

1. Introduction

Inulinase (β-2, 1-D-fructan fructanohydrolase, EC 3.2.1.7) hydrolyzes inulin into fructose and fructooligosaccharides. Fructooligosaccharides have great potential in the food industry because they can be used as calorie-reducing compounds and noncariogenic sweeteners as well as soluble fiber, prebiotic compounds for stimulation of Bifidus and as a source of dietary fiber in food preparations. One of the proven methods for high fructose syrup production is by isomerization of glucose involving several enzymes. Fructose is produced in a single step by inulinase and the process is economical and simple [1,2]. Due to low yield, extracting inulinase from plants involves high costs. Using microorganisms to produce enzymes is an alternative step to increase productivity and has the potential for industrial application. Production of inulinase from various types of microorganisms and yield improvements thereof has been investigated in the recent past [3,4]. Publications deal with three different types of research: the first is that in which the accent is on direct production of inulinase from microbial sources and by recombinant DNA technology [5–8]. The second concentrates on conversion of inulin to fructose, glucose, and other bioconversion products (e.g., bioethanol). This has been done by using inulinase as a catalyst and also by immobilizing different types of microorganisms and inulinase [9–12]. The third approach deals with the purification of crude inulinase by different methodologies [13,14].

Interestingly, microorganisms such as *Kluyveromyces marxianus, Bacillus* sp., *Aspergillus* sp., marine yeast and others seem to produce inulinase under varying conditions; pH 3–7 [15,16] temperatures 25–45 °C, and carbon sources like sucrose, inulin, agricultural source materials—Jerusalem artichoke, chicory, kuth and dahlia [6,17]. Attempts were made to produce inulinase by solid state fermentation [18], even though the process had its own inherent problems like heat and mass transfer. The literature concentrates [3,4,6,17,19] on employing different types of carbon sources, such as the conventional ones (sucrose, inulin, fructose, glucose) and agricultural feed stocks such as Jerusalem artichoke, chicory, kuth, and dahlia.

In all these studies, increments in the production of inulinase have been demonstrated with little applicability for commercial production. So far the maximum activity (1294 U/mL) with agro-industrial residues as a substrate for inulinase production was demonstrated by Treichel et al. with molasses (which has sucrose in it) and corn steep liquor. One of the problems in employing the agro-industrial wastes are the insoluble components and

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Nomenclature

Q	Heat generated (kJ)
MTCC	The microbial type culture collection and gene bank, Institute of Microbial Technology (IMTECH), Chandigarh, India
K. marxianus	Kluyveromyces marxianus
OUR	Oxygen uptake rate (g/L s)
CER	Carbon dioxide evolution rate (g/L s)
DCW	Biomass dry cell weight (gdcw/L)
Bio RC1	Bio reaction calorimeter
q_{bl}	Baseline heat (W)
q_j	Heat flow through the reactor wall to the jacket oil (W/L)
q_r	Heat generated by the reaction (W/L)
T_j	Temperature of jacket oil (°C)
T_r	Temperature of reactor contents (°C)
LPM	Volumetric flow of air-liter per minute (L/min)
$Y_{q/x}$	Heat yield coefficient with respect to biomass (kJ/g)
$Y_{q/o}$	Heat generated due to oxygen consumed (kJ/mol)
$Y_{q/s}$	Heat yield due to substrate depletion (kJ/g)
S	Substrate (g)
X	Biomass (g)
°C	Degree celsius
F_s	Feed rate required to maintain the specific growth rate (L/h)
$Y_{s/x}$	Biomass yield on substrate
μ	Specific growth rate (h^{-1})
m_s	Substrate requirement for maintenance (g)
V_0	Initial volume (L)
C_{x0}	Initial biomass concentration (g/L)
C_{si}	Initial sucrose concentration (g/L)
T	Time (h)
r_p	Product concentration in the fermenter (IU/mL)
q_p	Specific rate of product formation (IU/gdcw)
C_p	Product concentration (g/L)

substances that may affect the purification process and enzyme recovery. This will necessitate the incorporation of unit processes for purification, thereby may increase the cost of the enzyme by 50–80% [19]. Furthermore, there may be difficulty in estimating the consumption profiles of substrate and inability to further characterize the upsets in bioprocess.

To our best knowledge, the viable strategy would be to concentrate on manipulating engineering factors such as mode of operation and substrate feeding strategies, to achieve a phenomenal increase in inulinase activity and production. The argument that employing agro-industrial waste as a potential carbon source appears uneconomical, while consolidated bioprocessing (CBP) and/or simultaneous saccharification fermentation (SSF) process of combined carbon conversion (agro, bioprocess and microbial conversions) to commercial products is appreciated [20,21]. There is no literature available on the current status of industrial production of inulinase. All these studies prove that synthesis of inulinase is very important and productivity has to be improved.

Biocalorimetric measurement of heat flows to and from the reactor allows the determination of the correlation between different process variables [22]. Online monitoring and control are vital for achieving good productivity, reproducibility of the bioprocess as well as to provide opportunities for interventions if necessary, in the case of bioprocess upsets such as failures in exponential growth, poor utilization of substrate and O_2, contamination of culture and broth. Heat generation is a by-product of all the metabolic processes taking place in the biological system. Generally, the heat generation in the biological system is ascribed to the energy transformations during biochemical conversions and formation of intermediate metabolites. This motivated us to undertake the present studies using biocalorimetry.

The objectives of the present study are multi-fold. (i) To use inulinase production in *K. marxianus* as a model system to study heterologous protein synthesis and associated metabolic heat measurement; (ii) To improve the yield of inulinase production;

(iii) To evaluate the outcomes in batch versus fed-batch modes; (iv) to formulate an empirical model between the specific growth rate and inulinase yield and (v) to validate the empirical model with experimental data.

2. Materials and methods

2.1. Growth medium

K. marxianus strain (MTCC 4139) was maintained on yeast malt agar (YM Agar) slants containing yeast extract (3 g/L), malt extract (3 g/L), peptone (5 g/L), glucose (10 g/L), and agar (20 g/L) at 4 °C. Sub-cultured at 15–21 days intervals in seed culture media containing yeast extract (10 g/L), peptone (20 g/L), sucrose (20 g/L) at 30 °C, 150 rpm pH 3.5 for 16 h. 30 mL seed culture served as 2% inoculum for 1500 mL production medium (0.3 g of dry cell weight per liter (DCW g/L)).

2.2. Shaker flask studies

2.2.1. Batch medium selection

From the available literature, four different suitable media were selected (Table 1). Media A [23], Media B [16], Media C [24], Media D [25]. Experiments were carried out for 72 h on a rotary shaker at 30 °C, 150 rpm with a uniform inoculum size of 2%. Tukey's High Significant Difference (HSD) statistical test was performed for the determination of significant medium among the four mediums. Response surface methodology central composite design was employed for the carbon and nitrogen source optimization in the medium (Supplementary file II).

2.2.2. Fed-batch medium selection

Three fed-batch media (P, Q, and R) were formulated from medium D in order to study the effect of carbon and nitrogen source on enzyme activity (Table 2). The three media were inoculated with 2% seed culture (0.3 DCW g/L) and fermentation was carried out in a 500 mL conical flask containing 125 mL of the media, for 63 h at pH 5, temperature 30 °C and with 150 rpm.

2.2.3. Optimization of feeding rate

In order to optimize the feeding rate, two methods were followed for the concentrated feeding of the carbon, nitrogen, and mixed feeding (Table 2). In the first method (P1, Q1, R1) 0.5 mL/h feed rate and a dilution rate of $D = 0.004\ h^{-1}$ was maintained. In the second method (P2, Q2, R2) 0.25 mL/h feeding with a dilution rate of $D = 0.002\ h^{-1}$ was maintained. Feed solutions for P, Q, and R contained sucrose 1 g/mL, ammonium sulphate 1 g/mL, sucrose and ammonium sulphate each 0.5 g/mL respectively. From 8th to 23rd h continuous feeds were given at the above dilution rate to the respective media. Media P1 and P2 were subjected to carbon source feeding while media Q1 and Q2 were subjected to nitrogen source feeding [4]. A combination of carbon and nitrogen sources was fed to media R. Enzyme Activity (IU/mL) and DCW (g/L) at the end of 24, 36 and 63 h were estimated. These feed solution concentrations were optimized by several pilot studies (results not given here).