Veana, F.; Aguilar, C. N.; Rodríguez Herrera, R.
KINETIC STUDIES OF INVERTASE PRODUCTION BY XEROPHILIC ASPERGILLUS AND PENICILLIUM STRAINS UNDER SUBMERGED CULTURE
Colegio de Postgraduados
Puebla, México

Available in: http://www.redalyc.org/articulo.oa?id=68519085001
Kinetic studies of invertase production by xerophilic Aspergillus and Penicillium strains under submerged culture

F. Veana, C. N. Aguilar and R. Rodríguez Herrera*

Department of Food Science and Technology, School of Chemistry, Universidad Autónoma de Coahuila, Boulevard Venustiano Carranza and José Cárdenas s/n, República Oriente, Saltillo 25280, Coahuila, Mexico.

Accepted for publication June 27, 2011

ABSTRACT

Invertase catalyzes the hydrolysis of sucrose into glucose and fructose. This enzyme is important in the food industry. Several fungal species have been used for invertase production, most of them isolated from tropical areas. However, there are xerophilic strains capable of increasing the amount of enzyme produced. In this study, four xerophilic fungal strains (Penicillium pinophilum EH2, P. purpurogenum GH2, P. citrinum ESS, Aspergillus niger GH1) isolated from a Mexican semi-desert were used for invertase production in submerged culture. The strain GH1 of A. niger showed higher specific growth rate than Penicillium strains. This strain produced extracellular invertase with the highest enzymatic activity (8,625 U/L) after 48 h. The strain ESS of P. citrinum showed the maximum invertase activity (2,308 U/L) after 72 h. Xerophilic strains tolerated pH changes during submerged culture. The specific rate of substrate absorption, specific rate of product formation, productivity and enzyme yield of the strain GH1 of A. niger were higher than those observed for Penicillium strains. Results suggested that this xerophilic strain is an alternative for invertase production.

Key words: β-fructofuranosidase, invertase activity, kinetic parameters, radial growth, submerged culture.

* Corresponding author: R. Rodríguez Herrera. Tel.: +52 (844) 4169213. Fax: +52 (844) 415-95-34. E-mail: rrh961@hotmail.com
INTRODUCTION

Invertase enzyme or β-fructofuranosidase (EC 3.2.1.26) is produced by several microorganisms. The enzyme catalyses the hydrolysis of sucrose into glucose and fructose, recognizing the fructose site in sucrose. Invertase acts on non-reducing fructofuranoside terminal residues of β-fructofuranosides. This enzyme is of great importance in the food industry, particularly in confectioneries as a catalytic agent to obtain an artificial sweetener². Apart from hydrolyzing sucrose, the enzyme may also have fructosyltransferase activity for the synthesis of short-chain fructo-oligosaccharides⁹.

Invertase is produced by Saccharomyces cerevisiae and S. carlsbergensis¹⁸, Penicillium¹² and Aspergillus¹⁹. Most enzymes including invertase are produced using submerged cultures (SmC). In recent years, there have been studies to identify high yielding strains of invertase, which will facilitate high level of enzyme purification¹⁵,²⁰.

The xerophilic fungal strains isolated from the Mexican semi-desert tolerate extreme conditions typical of this region (45 to -15 C). These strains have an efficient
enzymatic machinery capable of overproducing several enzymes with potential industrial applications\textsuperscript{4,5,6}. This study was carried out to evaluate invertase production by four xerophilic fungal strains isolated from a Mexican semi-desert under submerged culture.

**MATERIALS AND METHODS**

*Microorganisms and culture conditions.* Four xerophilic fungal strains were studied, namely: *Penicillium pinophilum* Thom (EH2), *P. purpurogenum* Stoll (GH2), *P. citrinum* Thom (ESS), and *Aspergillus niger* Tiegh. (GH1). These strains are deposited at the culture collection of the Department of Food Science and Technology, University of Coahuila. All strains were grown on potato dextrose agar (PDA) at 30 C for 4 days. Fungal spores were harvested using 0.1\% Tween 80. Modified Czapek-Dox medium was used, having the following composition (g/L): NaNO\textsubscript{3} (7.65), KH\textsubscript{2}PO\textsubscript{4} (3.04), MgSO\textsubscript{4} \cdot 7H\textsubscript{2}O (1.52), and KCl (1.52). The medium was added with sucrose as substrate. In the kinetic analysis of radial growth and invertase production, the sucrose concentrations were 25 g/L and 12.5 g/L, respectively.

*Kinetic analysis of radial growth.* In order to evaluate the best condition for submerged fermentation, three levels of pH and temperature were used. Treatments were studied following a completely randomized design with factorial arrangement. Levels used for each factor were: strain (GH1, EH2, GH2, ESS), temperature (25 C, 30 C, 35 C), and pH (4, 4.5, 5). A Petri dish was inoculated at the centre with 10 \mu L of a spore suspension (1 \times 10\textsuperscript{6} spores/mL) of one fungal strain and incubated at different temperatures. The pH of the culture medium was adjusted with either 1.0 M HCl solution or 1.0 M NaOH, according to levels studied. Kinetics of mycelial growth from the multisporic colony was monitored every 12 h for 120 h, starting from the inoculation point. The measurements were performed using a vernier caliper, and the maximum growth rate was expressed as mm/h. Each treatment was performed in triplicate. Data were analyzed using an analysis of variance (ANOVA) for the experimental design studied. The Tukey range test was used when necessary in order to compare the means of treatments.

*Submerged culture.* Erlenmeyer flasks (250 mL) were used as reactors. Each flask contained 25 mL of modified Czapek-Dox medium added with sucrose (12.5 g/L of distilled water). The best pH conditions previously determined were used (EH2 and GH2: pH 4; GH1: pH 4.5; ESS: pH 5). Each flask was inoculated with 2 \times 10\textsuperscript{6} spores/mL, and incubated under the following conditions: shaking conditions (300 rpm), at the best temperature level determined by radial growth analysis for 120 h (EH2: 35 C, GH2: 30 C, ESS: 25 C, GH1: 35 C). Kinetics of fermentation were monitored every 24 h for 120 h. All experiments were carried out in duplicate to determine enzyme activity, total sugars, biomass production, pH changes and kinetic parameters.

*Enzymatic assay.* The invertase activity was determined by measuring the amount of reducing sugars released during the sucrose hydrolysis. The released sugars in the reaction mix were determined by the spectrophotometric method described by Ashokkumar et al.\textsuperscript{2}, using DNS (dinitro-salicylic acid) reagent. The absorbance was read at 540 nm in Thermo Spectronic Biomate 3. The calibration curve was car-
ried out using glucose (500 mg/L) as standard. One unit of invertase (U) was defined as the amount of enzyme required to liberate 1 μmol equivalent of reducing sugars per minute. The enzymatic extract was previously dialyzed for 24 h using a dialysis tubing cellulose membrane (Sigma) and 0.1 M sucrose in acetate buffer pH 4.6.

**Total sugars.** The sugars were determined by the phenol-sulfuric acid method, considering 12.5 g/L of sucrose as 100% of substrate and reading the absorbance at 480 nm in the spectrophotometer.

**Biomass production and pH changes.** Fungal biomass was determined at t sampling time. The mycelial biomass of the flask was filtered through Whatman filter paper no. 41, and dried for 24 h at 60 C. The biomass weight was determined gravimetrically. The pH changes were monitored during submerged fermentation using a pH meter (Mettler Toledo).

**Kinetics parameters.** Major metabolic parameters including enzyme yield \(Y_{P/X}\), specific rate of substrate absorption \(qs\), and productivity \(P\) were calculated using equations, which are defined in Table 1. The \(\mu\) was obtained from growth curves fitted by the Solver-Excel, Microsoft, software. The algorithm minimized the sum of the least square errors, comparing experimental data with the theoretical values given by the Verhulst-Pearl equation. The \(Y_{XS}\) and \(Y_{P/X}\) were obtained by Pirt and Luedeking and Piret equations, respectively.

**RESULTS AND DISCUSSION**

The fungal growth was evaluated by a kinetic analysis of radial growth. The effect of temperature and pH on mycelial growth is shown in Figures 1-2. The assay tested the invasiveness of the strains. The strain GH1 of *Aspergillus niger* showed the highest specific growth rate at pH 4.5 and 35 C. This strain had the capacity to consume the substrate at a specific growth rate of \(\mu=0.4053\ mm/h\). Total colonization of petri dishes was achieved in 120 h by this strain. The rest of the strains were capable of consuming the substrate, but at a lower specific growth rate. The best conditions for each strain were: *Penicillium pinophilum* (EH2), \(\mu=0.1167\) (pH 4, 35 C); *P. purpurogenum* (GH2), \(\mu=0.108\) (pH 4, 30 C); and *P. citrinum* (ESS), \(\mu=0.133\) (pH 5, 25 C). The specific growth rate \(\mu\) was significantly different for each strain and temperature.

Invertase production in filamentous fungi is regulated by gene expression...
Invertase production by xerophilic *Aspergillus* and *Penicillium*

Table 1. Definition of kinetic parameters for submerged cultures (SmC) studied, according to previous studies (Aranda *et al.*).

<table>
<thead>
<tr>
<th>Name</th>
<th>Symbol</th>
<th>Definition</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yield (biomass/substrate)</td>
<td>(Y_{XS})</td>
<td>(Y_{XS} = \frac{\Delta X}{\Delta S} = \frac{X_{max} - X_0}{S_0 - S})</td>
<td>gX/gS</td>
</tr>
<tr>
<td>Enzyme yield</td>
<td>(Y_{PX})</td>
<td>(Y_{PX} = \frac{\Delta E}{\Delta X} = \frac{E_{max} - E_0}{X_0 - X})</td>
<td>U/gX</td>
</tr>
<tr>
<td>Specific rate of substrate absorption</td>
<td>(q_s)</td>
<td>(q_s = \frac{\mu}{Y_{XS}})</td>
<td>gS/gXh</td>
</tr>
<tr>
<td>Specific rate of product formation</td>
<td>(q_p)</td>
<td>(q_p = \frac{Y_p}{\mu})</td>
<td>U/gXh</td>
</tr>
<tr>
<td>Productivity</td>
<td>(P)</td>
<td>(P = \frac{P_{max}}{t})</td>
<td>U/h</td>
</tr>
</tbody>
</table>

\(X=\) Mycelial biomass (g/L) produced at time \(t\). \(X_0=\) Initial mycelial biomass at sampling time \((t_0)\). \(X_{\text{max}}=\) Final biomass at the end of fermentation \((t\to\infty)\). \(S_0=\) Substrate concentration at sampling time \((t_0)\). \(S=\) Substrate concentration at \(t\to\infty\). \(E_0=\) International units of invertase activity in the extract at \(t_0\). \(E_{\text{max}}=\) International units of invertase activity in the extract at \(t\to\infty\). \(\mu=\) Maximum growth rate. \(Y_{XS}=\) The amount of biomass produced per gram of sugar utilized. \(Y_p=\) The invertase activity produced per gram of biomass. \(P_{\text{max}}=\) The amount of enzyme produced at \(t\to\infty\).

control. They synthesize and secrete only the hydrolytic enzymes needed for substrate degradation. In this case, the strain GH1 showed the highest enzymatic activity of 8,625 U/L (Fig. 3). This amount is 1 to 8.5 times higher than those previously reported in similar studies for different strains of *A. niger*\(^2,14,15,16\), which may be relevant for the food industry (Table 2). In fact, other enzymes from *A. niger* also have industrial applications, such as tannase, elagitannase, pectinase, proteases, and endo-polygalacturonase\(^5,11\). Invertase from the strain GH1 is produced at 48 h in submerged culture. A decrease in enzyme activity was recorded later due to insufficient substrate availability. It is possible that the strain GH1 began the secretion of proteases into the culture medium, degrading protein and reducing the culture yield. If this was the case, then *A. niger* could consume amino acids, which would explain the increase of enzyme activity at 120 h (10,933 U/L).
Strains required an inducer for invertase production (sucrose). In the exponential phase, 80% of this substrate was consumed by the strain GH1 of *A. niger* in 48 h, when the highest enzyme activity was recorded. The strain ESS of *Penicillium citrinum* showed similar pattern as GH1, but the exponential phase was reached at 72 h (Fig. 4).

Biomass production is shown in the Figure 5. The highest biomass production was obtained by the strain EH2 of *Penicillium pinophilum* (7.24 g/L). The kinetic pattern of biomass production by the strain GH1 of *A. niger* could be explained by some limitation of the oxygen required for growth. An alternative explanation may be that this strain produces higher amounts of proteases thereby reducing the amount of available nutrients.

The pH level is important in submerged fermentation. The enzyme activity is best expressed when this factor is optimum in the culture medium. The invertase is tolerant to pH changes; however, drastic pH changes may alter enzyme structure and/or the substrate properties. The pH stability of invertase ranges from 2.6-5.5\(^{17}\), showing the maximum activity at pH 4.5\(^{3}\). In this study, the pH value measured during submerged fermentation is shown in Figure 6. At 48 h, the strain GH1 of *A. niger* decreased the pH to 3.4, which fits within limits of enzyme tolerance.

Kinetic parameters evaluated are shown in Table 3. The strain EH2 of *Penicillium pinophilum* had a higher biomass/substrate yield \((Y_{X/S} = 0.62 \text{ gX/gS})\) than that recorded for the rest of the strains studied. This indicated that most consumed substrate was used for fungal growth (Fig. 5), a rather undesirable effect on enzyme production. Furthermore, the specific rate of substrate
Table 2. Production of invertase by *Aspergillus niger* (GH1) in submerged culture for this study, in comparison with previous reports.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Invertase (U/L)</th>
<th>Production time (h)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. niger</em> GH1</td>
<td>8,625</td>
<td>48</td>
<td>-</td>
</tr>
<tr>
<td><em>A. niger</em> C28B25</td>
<td>1,258</td>
<td>60</td>
<td>Romero-Gómez <em>et al.</em>(^{15})</td>
</tr>
<tr>
<td><em>A. niger</em> N-402</td>
<td>1,020</td>
<td>60</td>
<td>Romero-Gómez <em>et al.</em>(^{15})</td>
</tr>
<tr>
<td><em>A. niger</em> Aa20</td>
<td>1,262</td>
<td>60</td>
<td>Romero-Gómez <em>et al.</em>(^{15})</td>
</tr>
<tr>
<td><em>A. niger</em> NRRL 330</td>
<td>2,196(^{a})</td>
<td>120</td>
<td>Ashokkumar <em>et al.</em>(^{2})</td>
</tr>
<tr>
<td><em>A. niger</em></td>
<td>6,996(^{b})</td>
<td></td>
<td>Ashokkumar <em>et al.</em>(^{2})</td>
</tr>
<tr>
<td><em>A. niger</em> Aa 20</td>
<td>2,900</td>
<td>48</td>
<td>Rubio and Navarro(^{16})</td>
</tr>
<tr>
<td><em>A. niger</em></td>
<td>3,873</td>
<td>48</td>
<td>Robledo-Olivo <em>et al.</em>(^{14})</td>
</tr>
<tr>
<td><em>A. niger</em></td>
<td>2,582</td>
<td>48</td>
<td>Robledo-Olivo <em>et al.</em>(^{14})</td>
</tr>
</tbody>
</table>

\(^{a}\) Unoptimized value.  
\(^{b}\) Optimized value.

Fig. 4. Substrate consumption during submerged fermentation by the following fungal strains: *Aspergillus niger* (GH1: ♦), *Penicillium pinophilum* (EH2: ■), *P. purpurogenum* (GH2: ▲), and *P. citrinum* (ESS: ×).

Fig. 5. Biomass production during submerged fermentation by the following fungal strains: *Aspergillus niger* (GH1: ♦), *Penicillium pinophilum* (EH2: ■), *P. purpurogenum* (GH2: ▲), and *P. citrinum* (ESS: ×).
absorption \((q_s)\) and the specific rate of product formation \((q_p)\) were both assessed for better understanding the enzyme synthesis by fungal strains studied. The strain GH1 of \(A. niger\) showed the highest enzyme synthesis \((q_s = 0.16 \text{ gS/gXh}; q_p = 110.42 \text{ U/gXh})\). The productivity \((P)\) and enzyme yield \((Y_{P/X})\) produced by the strain GH1 were around 82 and 100 times higher than those recorded for \(Penicillium\) strains \((P= 179.68 \text{ U/h}; Y_{P/X} = 1,447.10 \text{ U/gX})\). These kinetic parameters were 7-13 times higher than those previously reported for different strains (Aa20, N-402, C28B25) of \(A. niger\). Therefore, the invertase production by xerophilic fungal strains is considerably higher. The strain GH1 of \(A. niger\) showed efficient enzymatic yield and fermentation time, deserving further analysis for possible industrial applications.

**Table 3.** Kinetic parameters obtained during submerged fermentation involving the following fungal strains: \(Aspergillus niger\) (GH1), \(Penicillium pinophilum\) (EH2), \(P. purpurogenum\) (GH2), and \(P. citrinum\) (ESS).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>GH1</th>
<th>EH2</th>
<th>GH2</th>
<th>ESS</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Y_{XS})</td>
<td>0.48</td>
<td>0.62</td>
<td>0.43</td>
<td>0.58</td>
</tr>
<tr>
<td>(q_s)</td>
<td>0.16</td>
<td>0.13</td>
<td>0.12</td>
<td>0.13</td>
</tr>
<tr>
<td>(Y_{P/X})</td>
<td>1,447.10</td>
<td>14.31</td>
<td>50.90</td>
<td>387.30</td>
</tr>
<tr>
<td>(q_p)</td>
<td>110.42</td>
<td>105.97</td>
<td>2.69</td>
<td>29.55</td>
</tr>
<tr>
<td>(P)</td>
<td>179.68</td>
<td>2.19</td>
<td>2.77</td>
<td>32.06</td>
</tr>
</tbody>
</table>

\(Y_{XS}\) (gX/gS) is the amount of biomass produced per gram of sugar utilized. \(q_s\) (gS/gXh) is the amount of sugar consumed per gram of biomass per hour. \(Y_{P/X}\) (U/gX) is the invertase activity produced per gram of biomass of fungal cells. \(q_p\) (U/gXh) is the amount of enzyme produced per gram of biomass per hour. \(P\) (U/h) is the amount of enzyme produced per hour.

**ACKNOWLEDGEMENTS**

This study was made possible by the financial support of The Universidad Autonoma de Coahuila. FV acknowledges the Mexican Council of Science and Technology (CONACYT) for financial support during her postgraduate studies.

**LITERATURE CITED**


