Development of an ideal starch saccharification process using amylolytic enzymes from thermophiles

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Abstract

The extensive efforts to screen thermophilic fungi and bacteria, isolated from various environmental samples, have resulted in the selection of Thermomucor indicae-seudaticae, Geobacillus thermoleovorans NP33 and G. thermoleovorans NP54 for the production of glucoamylase, amylpullulanase and α-amylase, respectively. Submerged and solid-state fermentation processes were optimized for maximizing the secretion of glucoamylase by T. indicae-seudaticae. The production of amylpullulanase and α-amylase by NP33 and NP54 in submerged fermentation was also optimized. Glucoamylase was optimally active at pH 7.0 and 60°C and was shown to saccharify soluble as well as raw starches. Amylopullulanase and α-amylase exhibited optima at pH 7.0 and 100°C and saccharified starch efficiently. Differential inhibition and action on mixed substrates clearly suggested that there are two separate active sites for α-amylase and pullulanase activities of amylpullulanase. Both α-amylase and amylpullulanase are high maltose-forming and Ca²⁺-independent. These amylolytic enzymes have been shown to be useful in starch saccharification alone and in combination.

Introduction

Starch is a polysaccharide composed of α-glucose units that are linked by α-1,4- and α-1,6-glycosidic bonds, forming two high-molecular-mass molecules: amylose (15-25%), a linear polymer composed of α-1,4-linked glucopyranose residues, and amylopectin (75-85%), a branched polymer containing α-1,6-glycosidic linkages at the branch points. Starch is used in the food industry as a thickener, binder, stabilizer, emulsifier and as a suspending and gelling agent. Furthermore, it is the primary source of various sugar syrups, which provide the basis of several pharmaceutical and confectionery industries.

Industrial processes for starch hydrolysis to glucose rely on inorganic acids or enzyme catalysis. The use of enzymes is preferred as it offers a number of advantages including improved yields and favourable economics. Enzymic hydrolysis allows greater control over amyolysis, the specificity of the reaction, and the stability of the generated products. The milder reaction conditions involve lower temperatures and near-neutral pH, thus reducing unwanted reactions. Enzymic methods are favoured because they also lower energy requirements and eliminate neutralization steps [1]. The enzymes employed in the industrial starch hydrolysis market are estimated to occupy 10-15% of the total world enzyme market. This industrial sector is the second biggest consumer of enzymes.

In the conventional enzymic starch saccharification process, variation of parameters in different steps causes many handicaps in the industry. Due to pH variation, large amounts of salts have to be removed by ion exchangers. Besides being time consuming, these steps lead to reverse reactions and lower yields. Undesirable products like branched oligosaccharides, panose, isopanose and isomaltose are formed. Improvement of the starch-conversion process by finding new efficient and suitable enzymes with high thermo-stabilities, functioning in the acidic to neutral pH range, and independent of cations such as Ca²⁺ for stability/activity would significantly lower the cost of sugar syrup production [2-4]. Increasing the starch-saccharification process temperature would result in several benefits such as higher substrate concentrations, decreased viscosity and lower pumping costs, limited risk of bacterial contamination, increased reaction rates and decrease of operation time, lower costs of enzyme purification, and longer catalyst half-life, due to increased enzyme thermostability [4].

In order to find novel enzymes, we have screened a large number of thermophilic bacterial and fungal strains, and selected Geobacillus thermoleovorans NP33, G. thermoleovorans NP54 and Thermomucor indicae-seudaticae for the production of thermostable and Ca²⁺-independent amylpullulanase, α-amylase and neutral glucoamylase. The enzymes were tested for their potential application in starch saccharification.

Key words: α-amylase, amylpullulanase, glucoamylase, starch saccharification, thermophile

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Materials and methods

Source of microbial strains

*G. thermoleovorans* NP54 and NP33 were isolated from water samples of a hot water spring of the Waimangu volcanic valley, New Zealand. The thermophilic mould *T. indicae-seudaticae* was procured from Professor A. Subramanyam (Department of Botany & Microbiology, Kakatiya University, Warangal, India).

Enzyme production

α-Amylase was produced by cultivating *G. thermoleovorans* NP54 in shake flasks containing SYT (starch/yeast extract/trypptone) broth (20.0 g l⁻¹ soluble starch, 3.0 g l⁻¹ yeast extract, 3.0 g l⁻¹ tryptone, 0.2 g l⁻¹ MgSO₄, 1.0 g l⁻¹ NaCl and 1.0 g l⁻¹ K₂HPO₄, pH 7) at 70°C and 200 rev/min.

Amylopullulanase of *G. thermoleovorans* NP33 was produced in a synthetic complex medium (30.6 g l⁻¹ starch, 4.2 g l⁻¹ yeast extract, 1.0 g l⁻¹ (NH₄)₂SO₄, 0.3 g l⁻¹ MgSO₄, 1.0 g l⁻¹ NaCl, 2.3 g l⁻¹ K₂HPO₄ and 1.0 g l⁻¹ maltose, pH 7.0) at 70°C and 200 rev./min in flasks/fermentor.

Glucoamylase production was carried out in sucrose/yeast extract broth (20.0 g l⁻¹ sucrose, 1.0 g l⁻¹ yeast extract, 3.0 g l⁻¹ asparagine, 1.3 g l⁻¹ MgSO₄, 0.5 g l⁻¹ K₂HPO₄ and 1 ml of microelement solution, pH 7.0) at 40°C and 250 rev./min, and also in solid-state fermentation using wheat bran as the substrate.

Characterization of enzymes

Amylolytic enzymes were purified by general protein-purification techniques, and characterized.

Enzyme assays

Amylopullulanase was assayed by determining reducing sugars liberated from starch (α-amylase) and pullulan (pullulullanase) at 80°C. The activities of α-amylase of *G. thermoleovorans* NP54 and glucoamylase of *T. indicae-seudaticae* were determined by estimating reducing sugars released from starch at 100 and 60°C, respectively.

One i.u. of enzyme is defined as the amount of enzyme required for the liberation of 1 μmol of reducing sugar·ml⁻¹·min⁻¹ under the assay conditions.

Application of amylolytic enzymes in starch saccharification

The applicability of enzymes in starch saccharification was tested by allowing α-amylase/amylopullulanase/glucamylase to act alone or in different combinations after gelatinizing raw starch/soluble starch at 105°C for 10 min and estimating reducing sugars liberated at the desired intervals.

Results

*G. thermoleovorans* NP33 and NP54 are aerobic and extremely thermophilic bacteria, which grow in the temperature range between 45 and 85°C, with optima of 70°C. The thermophilic mould *T. indicae-seudaticae* grows optimally at 40–45°C.

In the medium formulated by the ‘one-variable-at-a-time’ approach, containing cholic acid, the highest α-amylase secretion (48 000 units l⁻¹) was attained in *G. thermoleovorans* NP54. Surfactants such as cholic acid are known to solubilize membrane proteins, which leads to an increase in cell membrane permeability, thereby enhancing the secretion of biomolecules such as enzymes. Cholic acid also stabilized α-amylase activity during preservation at 4°C (Table 1). The enzyme was high maltose-forming, hyperthermostable and Ca²⁺-independent (Table 2).

Amylopullulanase titre was the highest (α-amylase, 12 300 units l⁻¹; pullulanase, 5200 units l⁻¹) when *G. thermoleovorans* NP33 was cultivated in a synthetic complex medium formulated by one-variable-at-a-time and response-surface methodology approaches. The enzyme had a molecular mass of 48 kDa, and it was optimally active at 80°C, with half-life values of 2 h (for α-amylase) and 3.5 h (pullulanase) at 100°C. The differential inhibition behaviour and activities in mixed substrates suggested that both enzyme activities are carried out by two separate active sites of amylopullulanase (Table 3). The enzyme was hyperthermostable as well as Ca²⁺-independent. The enzyme was stabilized by poly(ethylene glycol), and the substrates starch and pullulan.

A thermophilic mould *T. indicae-seudaticae* secreted glucoamylase in submerged (46 000 units l⁻¹) as well as solid state (475 units g⁻¹ of dry mouldy bran) fermentations. The molecular mass of the pure enzyme was 42 kDa, and it was
Table 3 | Reaction rates in the presence of mixed substrates

<table>
<thead>
<tr>
<th>Substrate (mg·ml⁻¹)</th>
<th>Enzyme activity (units·ml⁻¹)</th>
<th>Total of individual enzyme activities</th>
<th>Activity in the presence of both substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starch</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.25</td>
<td>0.18 (a)</td>
<td></td>
<td>a - b</td>
</tr>
<tr>
<td>Pullulan</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>0.035 (b1)</td>
<td>0.215 (a + b1)</td>
<td>0.233</td>
</tr>
<tr>
<td>1.0</td>
<td>0.043 (b2)</td>
<td>0.223 (a + b2)</td>
<td>0.246</td>
</tr>
<tr>
<td>1.5</td>
<td>0.048 (b3)</td>
<td>0.228 (a + b3)</td>
<td>0.259</td>
</tr>
<tr>
<td>2.0</td>
<td>0.060 (b4)</td>
<td>0.240 (a + b4)</td>
<td>0.271</td>
</tr>
<tr>
<td>2.5</td>
<td>0.071 (b5)</td>
<td>0.252 (a + b5)</td>
<td>0.292</td>
</tr>
</tbody>
</table>

Table 4 | Starch saccharification (%) by amylolytic enzymes

<table>
<thead>
<tr>
<th>Glucoamylase concentration (units/g)</th>
<th>Two step</th>
<th>Single step</th>
<th>24 h</th>
<th>48 h</th>
<th>24 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α-Amylase pretreatment</td>
<td>Amylopullulanase pretreatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.15</td>
<td>30</td>
<td>48</td>
<td>58</td>
<td>65</td>
<td>69</td>
<td></td>
</tr>
<tr>
<td>0.6</td>
<td>35</td>
<td>51</td>
<td>65</td>
<td>69</td>
<td>76</td>
<td></td>
</tr>
<tr>
<td>1.2</td>
<td>37</td>
<td>63</td>
<td>75</td>
<td>73</td>
<td>81</td>
<td></td>
</tr>
<tr>
<td>2.4</td>
<td>39</td>
<td>65</td>
<td>79</td>
<td>76</td>
<td>91</td>
<td></td>
</tr>
</tbody>
</table>

Active in the pH and temperature ranges of 5–9 and 40–90°C, with optima at 7.0 and 60°C. The enzyme exhibited activity on raw starch as well as soluble starch. The inhibition of enzyme activity by n-ethylmaleimide and n-bromosuccinimide suggested a critical role of cysteine and tryptophan in the catalysis.

All three enzymes exhibited starch saccharification, individually and also in combination, to a varied extent (Table 4). When starch was hydrolysed with amylpopullulanase, the major product was maltose. Starch pre-treated with amylopullulanase was very efficiently saccharified by glucoamylase in comparison with that of α-amylase.

Conclusions

All the three enzymes are active around neutrality, Ca²⁺ independent and thermostable. Application of a statistical method, response-surface methodology, allowed us to enhance the enzyme titres. α-Amylase-/amylopullulanase-pretreated starch was very efficiently saccharified to glucose by glucoamylase.

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References

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