Effective technological pectinase and cellulase by Saccharomyces cervisiae utilizing food wastes for citric acid production

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Abstract: The production of a notable and highly effective pectinase and cellulase, by the commercial baker’s yeast Saccharomyces cervisiae utilizing potato processing wastes, was achieved in 5-day solid state fermentation (SSF) cultures, at temperature 25 °C, pH range 4.0-5.0 and additive of ferric chloride. Pectinase and cellulase activities were stimulated by using potato wastes supplemented with urea, as the sole carbon and nitrogen sources, resulted in 70.20 and 98.85 % reduction of viscosity. It is concluded that citric acid production from pectinolytic and cellulolytic Saccharomyces cervisiae optimization with Aspergillus niger MAF3, maximally 46.67 and 68.44 g/kilogram solid potato wastes.

Keywords: Saccharomyces cervisiae, Potato wastes, Solid state fermentation (SSF), Pectinas and cellulase, Citric acid.

1. Introduction:
At the present, the fundamental exploitation of food waste, which participate in pollution, is the controlled biological degradation of the wastes by microorganisms for the production of valuable compounds such as enzymes, citric acid and others as raw materials for medical and industrial uses. In Egypt potato is one of the most important crops grown for local consumption, export and processing. The area cultivated with potatoes about 212,000 acres producing about 2.2 million tons with an average of 10.5 tones per acre (Hegazy, 2009). In 2002, the world production of starch amounted to approximately 58 million tons (roughly 69% from corn, 10% from cassava, 9% from sweet potatoes, 6% from wheat, 6% from potatoes, and less than 1% from other sources) (Peters, 2007). Different methods created for potato wastes utilization were reported by many authors (Mahmood et al., 1998; Huang et al., 2003; Parawira et al., 2005 and Darwish et al., 2009). In addition, enzymatic hydrolysates exploited as substrate for the production of organic acids (Kuhad and Singh, 1993; Khare et al., 1995; Sarangbin and Watanapokasin, 1999; Saber et al., 2010).

Pectinases have widespread applications in the food and textile industries (Henriksen et al., 1999), and in addition to plant tissue maceration, wastewater treatment and degumming of natural fibers (Baracat-Pereira et al., 1994). Cellulases have diverse applications in environmental, food and agricultural industries (Deshpande et al., 1992), also, they act synergistically (Kim et al., 2003), and used to modify the surface properties of cellulosic fibers and fabric in order to achieve a desired surface effect (Kotchoni et al., 2003). Moreover, citric acid is of industrial importance because, it is widely used in dairy, medicine and biochemical industries (Wang and Liu, 1996; Tongwen and Weihua, 2002). Yeasts like Saccharomyces cerevisiae and Candida sp. have been reported to produce pectinolytic enzymes (pectin lyase, polygalacturonases, and pectinesterases) (Gainvors et al., 1994). Also, cellulase and pectinase were produced from some aquatic hyphomycetes by Osman et al. (2008).

This work aims at biodegradation of potato waste of potato processing industry in Egypt for production of pectinase and cellulase enzymes by a commercial bakery yeast and using their optimizations in prefermentation and mixed fermentation with the highly citric acid producer (Aspergillus niger MAF3). This methodology has two benefits, the first one is environmentally safe, the second is the utilization of low cost production of the enzymes pectinase and cellulase as well as citric acid.

2. Materials and Methods:
Materials:
Organisms and inoculums
The commercial live bakers' yeast, Saccharomyces cerevisiae was obtained, in the forms of active dry yeast, from the Egyptian Company for Advanced Foodstuff Industries. Active dry yeast (5g) was dispersed in 99 mL of 0.1% sterile peptone water prewarmed at 38°C for 20 min. The yeast solution contained 2×10^5 viable cells/mL and used as
inoculum for fermentation medium (Wang et al., 1997).

The fungal species A. niger MAF3 was obtained from Botany and Microbiology Department, Faculty of Science, Al-Azhar University, Assiut, Egypt. A. niger MAF3 spores were produced in Czapek-Dox Broth (50 ml) in a 250 ml Erlenmeyer flask, incubated at 28°C for eight days. A spore suspension was prepared by adding 25 ml distilled water with Tween-80 (0.1%) and was stored at 4°C for a maximum of two weeks. It contained 10⁷ spores/ml according to the method described by Vandenberghe et al. (2000).

**Fermentation medium**

Solid potato wastes were obtained from potato processing industry (chips), Assiut, Egypt. The other components of the SSF culture media were obtained from Merck and Sigma in the highest purity. Ten grams of solid potato wastes were taken in Erlenmeyer flask (250 ml), mixed with 10 ml of Czapek-Dox mineral solution, and sterilized at 121 °C for 30 min. Cultivation was carried out by adding 1.0 ml of the inoculum then the flasks were incubated at 30°C statically.

**Preparation of crude enzyme extract**

Culture media and cells were harvested after incubation for 7 days by filling to 100 ml of sterilized distilled water. The mixture was vigorously stirred for about 15 min and filtered through Whatman No. 1 filter paper on Büchner funnel, and centrifuged at 5000 rpm and 4 °C for 15 min. The supernatant thus obtained was used as crude enzyme preparation.

**Methods:**

**Assay for pectinase activity**

Pectinase activity was measured as reduction percentage in pectin solution viscosity using Ostwald viscometer according to White and Fabian (1953).

**Assay for CM-cellulase activity**

CM-cellulase was measured viscometrically as described by (Eriksson and Hollmark, 1969; Child et al., 1973, El-Sheekh et al., 2009).

**Pre-fermentation and mixed fermentation**

This experiment was performed as the method of Khare et al. (1995). In this set of experiments, SPW was pre-fermented with the optimization of SSF of pectinolytic and celluolytic Saccharomyces cerevisiae, prior to inoculation with the citric acid-producing A. niger MAF3. In one set pre-fermented SPW samples were sterilized after the fifth day to kill the pre-fermenting Saccharomyces cerevisiae and then inoculated with A. niger MAF3 (PF-1). In a second set, A. niger MAF3 was inoculated on the fifth day without killing Saccharomyces cerevisiae (PF-2), and the third set was a mixed fermentation created by inoculating both the organisms simultaneously at the start (PF-3). Other fermentation conditions were kept as described above in Section 2.2, unless, the supernatant fluid thus obtained was used for citric acid estimation.

**Analysis for citric acid**

The concentration of citric acid in culture filtrate was measured by titration with 0.1 N NaOH as described by (Imandi et al. 2007; Khosravi-Darani and Zoghi, 2008). After titration, citric acid was determined spectrophotometrically at 420 nm by the acetic anhydride-pyridine method according to (Marrier and Boulet, 1958; Imandi et al., 2008).

**3. Results and Discussion**

Several created methods for utilizing potato wastes in order to support the growth and extracellular hydrolytic enzyme production have been described (Mahmood et al., 1998; Parawira et al., 2005; Darwish et al., 2009). In addition, (Kuhad and Singh, 1993; Khare et al., 1995; Sarangbin and Watanapokasin, 1999; Saber et al., 2010) pointed out that the enzymatic hydrolysates exploited as substrate for the production of organic acids which strongly pronounced them for multienzyme complexes’ production through microbial biodegradation. This therefore justified this study for the perfect utilization of SPW, excluded in the potato processing factories in Egypt (chips), as a substrate material suitable for a commercial baker’s yeast strain capable of the production of pectinase and cellulase enzymes, as well as utilization of their hydrolysates for production of citric acid, essential for numerous applications.

When the commercial bakers yeast (Saccharomyces cerevisiae) was grown on SPW as a solid support in SSF, could produce pectinase and cellulase as high as 17.45 and 49.20 % reduction of viscosity after 5 days incubation (Fig. 1). Similarly, Aspergillus carneus NRC1 produced the highest yield of pectinase, cellulase and high hemicellulase after 5 days (El-Sheekh et al., 2009). Also, the optimum production of pectic enzymes by Trichoderma lignorum attained after 5-day (Abdel-Fattah et al., 1977). In addition, the maximum filter paperase (FPase) activity was 19.5 IU g⁻¹ in 4 days, while, the highest CMCase activity was concurrently obtained after 5-6 days of fermentation when A. niger KK2 was grown on rice straw alone as a solid support in SSF (Kang et al., 2004).

In relation to these results, the cellulose (CEL) culture were statistically similar to those from the 3rd and 4th days of the untreated sugar cane bagasse (SCB) using Penicillium echinulatum 9A02S1 culture, and the 5th and 6th days gave the greatest filter paper activity (FPA) (Camassola and Dillon, 2009). On contrast, the maximum cellulase secretion from A. niger using maize straw was after 3
days of incubation (Milala et al., 2005), moreover, the maximum of FPase, carboxy methyl cellulase (CMCase) and xylanase activity was obtained after 7 days incubation of *Aspergillus oryzae* MTCC 1846 at 28 ± 0.5 (Chandel et al., 2009).

The addition of different mono- and disaccharides in equal carbon basis in the production medium led to low or high enzyme activities. The control culture characterized by the highest levels of enzymes activity (58.50 and 65.0 % reduction of viscosity) for pectinase and cellulase, respectively. This distinctly reflects the effect of inducible substrate type of the enzymes involved by the commercial bakery yeast (Fig. 2). The inducible nature of the aforementioned fungal enzymes was previously reported (Ismail, 1996; Silva et al., 2002; Bai et al., 2004). In addition, the control culture using *Penicillium echinulatum* 9A02S1, gave the greatest FPA (Camassola and Dillon, 2009), while, the cellulase production from *A. niger* NRRL 2001 and *A. niger* NRRL 2007 reporting 5 and 6 U/ml of cellulase, consequently, using de-starched corn fiber as a carbon source (Dien et al., 2006).

The initial pH of the basal medium ranged from 3.0 to 11.0 (Fig. 3) and that was suitable for pectinase and cellulase enzymes produced by *Saccharomyces cerevisiae*. The marked effect of initial pH 4 and 5 was mainly on pectinase and cellulase enzyme productivities which reached their maximal value of 52.12 and 69.50 % reduction of viscosity, consequently. An increase in initial pH of fermentation above 4 and 5 shows a decrease in pectinase and cellulase enzymes productivity, consequently. Similarly, Foda et al. (1984) reported the optimum initial pH values for polygalacturonase (PG) enzyme production were 4.0–5.0 for *A. aculeatus*. Birgisson et al. (2003) concluded the production of PG by *Cystofilobasidium larimarini* (55,000 U/I) and *C. capitatum* (32,000 U/I) was maximum at an optimum pH of 3.2 and 3.9, respectively. CMCase showed optimal activity at pH 5.0, while xylanase, pectinase and FPase activities were optimal at pH 6.0 as reported by Parawira et al. (2005).

In contrast to these results, Gummadi and Kumar (2008) reported the optimum pH for pectin lyase (PL) and pectate lyase (PGL) production by *Debaryomyces nepalensis* was found to be 7.0. From one hand, while PL production was achieved at an initial pH 6.5 on the other (Manachini et al., 1988). In addition, the optimum pH for pectin lyase activity was 8.0 (Solís et al., 2009). Hence, in all subsequent experiments, initial pH of the medium was maintained at 4 and 5 for pectinase and cellulase enzyme productivities.

Pectinase and cellulase production was investigated in a temperature range of 15–60 °C. The highest production of pectinase and cellulase activities (66.0 and 98.85 % Reduction of viscosity), respectively, were obtained at 25 °C and at higher temperatures their production decreased gradually (Fig. 3). The incubation temperature in previous range is favored and denoting good mesophilicity of pectinase and cellulase and their producer *Saccharomyces cerevisiae*. This adds good applicable advantage to the crude *Saccharomyces cerevisiae* pectinase and cellulase. Thus, *Saccharomyces cerevisiae* was, a little similar to, *Aspergillus foetidus* (NRRL 341, ATCC 16878) which optimally produced pectinases at 30 °C (Hours et al., 1988), and *A. niger* A-20 was optimally produced multi-enzyme systems of pectinases, cellulases and xylanase at 30 °C (Ismail, 1996) from one hand, and differ to, *A. carneus* NRCl which favored pectinases production at 50 °C (El-Sheekh et al., 2009). In respect to enzyme activity, the simultaneous addition of glucoamylase and yeast (*Saccharomyces cerevisiae*) was performed at 30 °C (Srichuwong et al. (2009), while, and the optimal activities was at 50 and 60 °C for pectinase and CMCase, respectively (Parawira et al., 2005).

Study of the effect of the SPW quantity in the SSF culture medium using pectinases and cellulase enzymes, indicated that their productivities increased parallely with the added SPW till 25 and 80g which represented the most proper concentrations for obtaining the highest productivities (69.3 and 96 % reduction of viscosity), respectively (Fig. 5). Break down of cell-wall materials causing by the hydrolytic activities of enzymes was clearly observed. According to the results of these studies, orange bagasse 50% (w/w) and wheat bran mixture was the most proper for the maximal pectin lyase productivity in SSF cultures of *Penicillium viridicatum* Rfe3 (Silva et al., 2002). Taking the pectin content of orange peels and pulps (OPP) into consideration, the proper percentage 6% (w/v) equals 1.44% (w/v) pectin (El-Sheekh et al., 2009) and also this was near to that concluded for apple pectin by Abdel-Fattah et al. (1977) for optimum production of pectic enzymes by *Trichoderma lignorum*. Moreover, the maximum cellulase secretion from *A. niger* obtained using 6% maize straw concentration (Milala et al., 2005).
Table 1. Effect of pre-fermentation and mixed fermentation in the optimized medium pectinase and cellulase enzymes productivity by Saccharomyces cervisiae cultures for citric acid production.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Optimized pectinase medium</th>
<th>Optimized cellulase medium</th>
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<tbody>
<tr>
<td></td>
<td>Citric acid (g/kg SPW)</td>
<td>Final pH</td>
</tr>
<tr>
<td></td>
<td>Citric acid (g/kg SPW)</td>
<td>Final pH</td>
</tr>
<tr>
<td>OM</td>
<td>15.86</td>
<td>2.98</td>
</tr>
<tr>
<td>PF-1</td>
<td>17.61</td>
<td>2.34</td>
</tr>
<tr>
<td>PF-2</td>
<td>6.17</td>
<td>6.52</td>
</tr>
<tr>
<td>PF-3</td>
<td>46.67</td>
<td>2.02</td>
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</table>

OM: Optimized medium of each enzyme produced by Saccharomyces cervisiae.
PF-1: Prefermented with Saccharomyces cervisiae for 5 days and sterilized followed by A. niger MAF3.
PF-2: Prefermented with Saccharomyces cervisiae for 5 days followed by inoculation with A. niger MAF3.
PF-3: Mixed fermentation with both fungi inoculated at 0 day. Citric acid measured at 5 days after inoculation of A. niger MAF3.

Fig. 1. The pectinase and cellulase enzymes activities (% reduction in viscosity) of the SSF culture filtrates of static Saccharomyces cerevisiae cultures during different periods of incubation.

Fig. 2. Effect of different carbon sources on pectinase and cellulase enzymes productivity by Saccharomyces cervisiae in 5-day static cultures.
Fig. 3. Influence of initial pH value on pectinase and cellulase enzymes productivities by S. cerevisiae grown for 5 days in static cultures.

Fig. 4. Effect of incubation temperatures on pectinase and cellulase productivities by Saccharomyces cervisiae in 5-day SSF cultures.
Fig. 5. Effect of solid potato waste (SPW) quantity in SSF cultures on pectinase and cellulase productivity by Saccharomyces cervisiae in 5-day static cultures.

Fig. 6. Effect of different Nitrogen sources on pectinase and cellulase enzymes productivities by Saccharomyces cervisiae grown for 5 days in static SSF cultures.

Fig. 7. Effect of different additives (1% w/w) to the optimizes medium pectinase and cellulase enzymes productivity by Saccharomyces cervisiae grown in 5- day static SSF cultures.
The effect of different nitrogen sources was investigated by replacement of NaNO₃ in equal nitrogen basis by any of N sources (peptide, urea, ammonium phosphate, ammonium sulphate, casein, gelatin or glysine) to verify their suitability for pectinase and cellulase enzymes production by S. cerevisiae. As shown in Fig. 6, the replacement of NaNO₃ in the SSF culture medium by any of the above-mentioned N sources led to many dissimilar effects on the productivity of the pectinase and cellulase enzymes. Generally, some N sources tested led to moderate or good pectinase (except peptone, gelatin and glysine show a low effect) and cellulase productivities, particularly urea by which the highest pectinase and cellulase yield was attained, and resulted in 70.20 and 98.85 (% reduction of viscosity), respectively. Yeast cells require sufficient nutrients to survive the osmotic stress and maintain their metabolic functions. Nitrogen limitation for protein synthesis and yeast growth is particularly observed in very high gravity (VHG) fermentation, which can be remedied by the addition of assimilable nitrogen sources such as yeast extract, urea and ammonium salts (D’Amore et al., 1988; Jones and Ingledew, 1994; Blateyron and Sablayrolles, 2001).

The effect of some additives on pectinase and cellulase enzymes productivities by Saccharomyces cerevisiae was graphically illustrated in Fig. 7.

These additives included, some polyalcohols (glycerol, mannitol and sorbitol), polysaccharides (starch, pectin and cellulose), and metal ions (FeCl₃, ZnSO₄, and FeCl₃ + ZnSO₄ 0.5+0.5% w/w) and added in equal weight basis (1%, w/w).

Among all the additives above-mentioned FeCl₃, highly stimulated cellulase and moderately stimulated pectinase, productivity (95 and 60% reduction of viscosity), respectively, while, the others had varied stimulatory effects. Each of glycerol, manitol and Zn²⁺ had completed inhibitory effects. Fungal pectinases production media formulated by many authors and principally contained orange bagasse or peels were devoid of the metal ions Zn²⁺ and Fe³⁺ (Ismail, 1996; Silva et al., 2002).

Study of the effects of the prefermentation and mixed fermentation pointed out that a maximum of a many-folds increase in citric acid yield was obtained in the case of mixed fermentation with both Saccharomyces cerevisiae and A. niger MAF3 inoculated at 0 day (PF-3), and resulted in the production of 46.67 and 68.44 g citric acid/kilogram potato waste, consequently, which is quite, similar to the condition recorded by Khare et al., 1995, and comparable to the yields obtained by fermentation of other agro-wastes (Panda et al., 1984; El-Abayad et al., 1992; Khare et al., 1995; Pramod and Lingappa, 2008).

5. Conclusion:
In conclusion, using a pectinolytic and celluololytic Saccharomyces cerevisiae utilizing solid potato wastes, as sole carbon source at a concentration of 15 and 85g at pH 4 and 5, supplemented with urea and FeCl₃, and incubated at 25 °C for 5 days, represented the optimal conditions to attain the highest pectinase and cellulase yield (70.2 and 98.85 % reduction of viscosity), consequently. These conditions were, used in pre and mixed fermentation with the highly citric acid producer A. niger MAF 3, maximally produced 46.67 and 68.44 g citric acid/kilogram solid potato waste in pectinase and cellulase medium optimization, respectively. This offers an opportunity to recover potato chips by-products and used as substrate for producing the cheapest with the highest productivities of cellulase and pectinase, as well as citric acid to be used in food and biochemical industries.

References:


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