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Comparative study for obtaining inulinase and invertase by yeasts

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Abstract

The aim of this comparative study was to obtain a model for production of inulinase and invertase by species *Saccharomyces*, *Candida* and *Hansenula*, strains from culture collection of INCDCF-ICCF, using submerged fermentation in a medium containing inulin as source of C. This model explained the data variation and the actual relationships between the parameters and responses. The dry biomass content as well as the production of inulinase and invertase in the bioprocess medium was influenced by inulin concentration and microelement composition. The main parameters for bioprocesses were: inoculum size 2% (v/v), pH 6, temperature 28°C and 220 rpm agitation speed. Following comparative study for production of extracellular inulinase (exo and endo inulinase) and invertase were obtained for *Candida arborea* the best results, invertase production having significantly higher concentrations than inulinase (35.92 U/mL invertase activity vs. 8.01 U/mL inulinase activity), on M5 medium. These results could be useful for industrial applications such as food industry, pharmaceutical.

Keywords Inulin, inulinase, invertase, yeasts, bioprocess

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Introduction

Inulinases (EC 3.2.1.7) are enzymes that catalyze hydrolysis of inulin to fructose and fructooligosaccharides. Inulinases are enzymes encountered in nature, found in the digestive tract of some animals [1] and in the storage tissues of plants rich in inulin [2]. They are also produced by various microorganisms. Their properties differ depending on the source and the cultivation environment. A number of fungal, bacterial and yeast strains have been reported as potent producers of inulinase [3,4].

Among the producing microorganisms are bacteria (e.g., *Bacillus* sp., *Pseudomonas* sp., and *Streptomyces* sp.), yeasts (*Saccharomyces* sp., *Candida* sp., *Hansenula* sp.) and fungi (*Aspergillus* sp., *Penicillium* sp.). Yeasts are one of the most versatile sources of inulinases. Unicellularity, easy cultivation, high growth rate and good inulinase yield within a short span are some of the advantages of using yeast strains. Among the yeasts, *Kluyveromyces* spp, *Pichia* spp and *Candida* spp have a high potential for inulinase production with high yields and activities [5]

Kluyveromyces spp strains are the most widely used yeasts for obtaining inulinase. A number of researchers have optimized the fermentation process by investigating inulinase production factors (agitation, aeration, carbon source, composition of the fermentation medium, duration of fermentation). The inulinase activity of yeasts has been primarily characterized in *K. fragilis* and *K. marxianus*. The partially purified inulinase reported in *K. fragilis* was optimally active at 45°C and pH=5.0 [6].

Studies accomplished on effects of carbon and nitrogen sources, and oxygenation of inulinase production led to an inulinase activity of 208 IU / ml using 20 g / l sucrose as carbon source [7].

Regarding, the purification of inulinase from *Kluyveromyces marxianus*, studies were performed using the precipitation method with ethanol followed by ultrafiltration reaching a specific activity of 262.9 U / mg [8].

Inulinases are an important class of enzymes used in many areas, especially in the food and pharmaceutical industry, to produce fructose syrups. Thus, the production of high purity fructose syrup has been studied by enzymatic hydrolysis of inulin to D-fructose with inulinase. The properties of fructose, such as low cariogenicity and favoring increased iron abundance in children, have led to an increase in interest in research to obtain and use it. Besides the interest of researchers for inulinases as a biocatalyst of inulin hydrolyzate to fructose syrup, it is currently being studied to involve these enzymes in other applications such as ethanol, acetone and butanol, pullulan, gluconic acid and sorbitol.[9, 10].

Invertases (EC 3.2.1.26), also called beta-fructofuranosidases break the terminal non-reducing beta-fructofuranosides residues. Invertase is widespread in the biosphere. It is found mainly in plants and microorganisms. *Saccharomyces cerevisiae*, commonly called Baker's yeast, is the main strain used to produce invertase. Although plants such as Japanese fruit pear (*Pyrus pyrifolia*), peas

(*Pisum sativum*), oats (*Avena sativa*) can also be used in obtaining invertase, microorganisms such as *S. cerevisiae*, *Candida utilis*, *A. niger* are considered ideal for its study [11].

Invertases are glycoside hydrolases and occur mostly in microorganisms. Among microbial strains, for many decades yeast species have been extensively researched for invertase production, characterization, and applications in industries. Besides, limited literature is available on invertases from bacterial strains. The enzymic and molecular biological reports from bacterial invertases are scarce [12].

Invertase has a role in the metabolism, for sucrose storage, as catalytic in the conversion of sucrose into glucose and fructose, produces inverted syrup (which contains glucose and fructose at equimolar concentrations). The invert syrup is used in food and beverage industries as a humectant in the preparation of candies, noncrystallizing creams, jams and artificial honey [11]. Invertases are also important for the prevention of diseases (ulcers, intestinal ailments, reduce colds etc.) in humans, in anti-aging process and physical rejuvenation. Their ability to convert glucose into hydrogen per oxide categorize them as powerful antimicrobial and antioxidant agents, hence, assist in defense reaction against bacterial infections and gut fermentation by oxidation. Microbial invertases have been extensively studied during last decades [13,14].

These enzymes have important applications in the food and pharmaceutical industry [15-19].

This report presents the production of extracellular inulinase and invertase, in submerged fermentation by species *Saccharomyces*, *Candida* and *Hansenula* in similar conditions.

Materials and Methods

Biological Material

Ten strains of yeast (*Candida albicans* ICCF 91, *Candida arborea* ICCF 193, *Hansenula anomala* ICCF 217, *Hansenula subpelliculosa* ICCF 187, *Hansenula polymorfa* ICCF 218, *Saccharomyces boulardii* ICCF 224, *Saccharomyces cerevisiae* ICCF 192, *Saccharomyces cerevisiae* ICCF 349, *Saccharomyces cerevisiae* ICCF 386, *Saccharomyces chevalieri* ICCF 312) from the Culture Collection of Industrial Importance Microorganisms of the National Institute for Chemical & Pharmaceutical Research and Development, ICCF Bucharest, were investigated.

Media used in bioprocesses

The strains were maintained in agar slant tubes and in Petri dishes at 4°C in their respective maintenance media: YMPG. The medium contained: glucose 1%, malt extract 0.3%, yeast extract 0.3%, peptone 0.5% and agar 1.7%. The inoculum on liquid YMPG medium contained: 1% glucose, 0.3% malt extract, 0.3% yeast extract and 0.5% peptone, final pH was adjusted to 6.5 with 1N NaOH. The medium was dispensed into flaks (100ml / 500ml flaks) and sterilized at 115°C for 20 minutes. After checking the sterility, the medium was inoculated with 2% pre-inoculum

culture. The inoculum culture was developed for 48 hours at 28°C and 220 rpm.

In bioprocess have been tested several media to establish optimal conditions for the cultivation of selected strains. These are presented in table 1.

The media were inoculated with 2% inoculum culture. The bioprocess lasted 5-7 days and monitored (starting with T⁰ - the moment of inoculation) of the microbial development parameters (pH, O.D., content of reducing sugar)

Cultivation

Each one of the isolates, positive for inulinase and invertase activity, was further transferred from the pre-inoculum slant tubes to 100 ml of YMPG (in 500 ml Erlenmeyer flasks) and cultivated aerobically for 48 hours to obtain the inoculum culture.

Each inoculum was used at 2% concentration to inoculate 500 ml Erlenmeyer flasks containing 100 ml of the fermentative medium (composition specified in table 1) supplemented with inulin or fructose, to induce enzyme production. The bioprocesses were conducted for five or seven days at 28°C and 220 rpm.

Table 1. The composition of the fermentation media used to establish the experimental model.

Media	Component	Concentration (% g/v)
M2 / M3 tested on the 5 strains of yeast	Inulin / Fructose	2
	Yeast Extract	1.4
	(NH ₄) ₂ SO ₄	0.21
	KH ₂ PO ₄	1
	MgSO ₄ ·7H ₂ O	0.03
	CaCl ₂ ·2H ₂ O	0.03
M4 tested on the 5 strains of yeast	Inulin	1.5
	Yeast Extract	1.4
	(NH ₄) ₂ SO ₄	0.21
	KH ₂ PO ₄	1
	MgSO ₄ ·7H ₂ O	0.03
	MnCl ₂ ·4H ₂ O	0.03
M5 tested on <i>Candida arborea</i>	Inulin	2
	Yeast Extract	1.4
	(NH ₄) ₂ SO ₄	0.21
	KH ₂ PO ₄	1
	MgSO ₄ ·7H ₂ O	0.03
	MnCl ₂ ·4H ₂ O	0.03
	CoSO ₄ ·7H ₂ O	0.015

Enzymatic screening

The capacity of the 10 strains of yeast to metabolize inulin as a carbon source was evaluated by using Durham

test tubes [20]. Small test tubes are placed in an inverted position within the ordinary test tubes used for cultivations. The larger tube is then filled with medium and sterilized. The medium used, contained 0.5% yeast extract and 1% inulin. The evaluation of the degraded inulin was observed as accumulation of gas in the small inverted tube.

Enzyme Purification

The fermentation medium was centrifugate at 8000 rpm, for 20 minutes at 4°C and the obtained supernatant was treated with ethanol for partial purification. The processing of purification consists in fractionated precipitation with ethanol in two steps. To 560 ml of supernatant 162 ml of 29% ethanol was added, mixed for 60 minutes at 4°C. After the first step, the formed precipitate without enzymatic activity was discarded. At the second step of purification over the supernatant (672 ml) obtained following centrifugation, 267 ml of 40% ethanol was added, mixed and refrigerated overnight. The next day, centrifugation was done at 8000 rpm, for 10 minutes at 4°C and the supernatant was discarded. The precipitate was collected and dissolved in sodium acetate buffer 50 mM, pH 4.7 [21].

Analytical Methods

Cell growth was determined by spectrophotometric method, in the visible UV-Vis range at λ=540 nm.

The substrate consumed (g/100mL) was analyzed by the iodometric method, Schoorl, determination of the total sugar from the fermentation medium.

The inulinase / invertase activity was assayed as follows: 0.1 ml of enzyme solution was mixed with 0.9 ml of 2% inulin / sucrose in 0.1M acetate buffer, pH 5.5. The sample was incubated at 50°C for 15 min.

The reducing sugars were determined by the dinitrosalicylic acid (DNSA) method [22]. For this, 2 ml of DNSA reagent was added to each tube were placed in boiling water for 5 min to stop the enzyme activity. Each sample was cooled to room temperature. Absorbance was determined in a spectrophotometer at 540 nm. One activity unit is defined as the amount of enzyme required to produce one micromole of reducing sugar per minute, under assay conditions.

The protein content was determined by Lowry method using BSA as a standard [23].

Results and Discussions

Cell growth, inulinase and invertase microbial biosynthesis in submerged cultivation

The strains that exhibited the highest accumulation of gas produced by the metabolism of inulin from the medium were chosen for further experiments.

In this respect, five strains of yeast (*Candida arborea* ICCF 193, *Hansenula anomala* ICCF 217, *Saccharomyces cerevisiae* ICCF 192, *Saccharomyces cerevisiae* ICCF 349, and *Saccharomyces chevalieri* ICCF 312) were investigated.

The strains studied for obtaining inulinase and invertase were grown on fructose and inulin 2% (M2 / M3) as carbon

sources. Results of this comparative study, made in triplicates, are presented, as an average, in table 2.

As can be seen, for best results regarding enzymes biosynthesis, inoculum optical density had values up to 14, which led to cell development.

At the end of fermentation, significant differences appeared for the production of inulinase vs invertase, although regarding biomass development had similar values. In the case of strain *C. arborea*, the production of invertase was predominant, 3.968 and 4.156U / ml of invertase activity, respectively, compared to 0.01 and 0.005U / ml of inulinase activity, respectively. The higher production of invertase vs inulinase is also observed in the case strains *Saccharomyces* and *H. anomala*, but not with a large accumulation.

Following to this studies were obtained different amounts of inulinase versus invertase, which led us to the following experiments, that consisted in improving the composition of trace elements and the concentration of carbon source (inulin 1.5 vs 2%, M4), the results being represented in Figure 1. As can be seen *Candida arborea*

ICCF 193 it has developed significantly better in submerged fermentation; in the fifth day, a larger amount of biomass has accumulated. Enzymatic activity was higher for invertase accumulation.

Optimizing of the bioprocess to obtain inulinase and invertase on *Candida arborea*

As *C. arborea* ICCF 193 (figure 2) obtained best results in these studies, our next researches were conducted with this strain.

In order to optimize enzyme biosynthesis conditions, research has been conducted on different inulin concentrations. Both, the cellular growth (by monitoring the development of biomass and final pH) as well as enzymatic activity for inulinase and invertase, were studied (figure 3).

The strain *Candida arborea* ICCF 193 chosen for the study had a relatively uniform distribution, in terms of biomass development on the tested media (M2, M4 and M5). The enzymatic activity was higher in M5 medium, invertase production having significantly higher concentrations than inulinase (35.92 U/mL invertase activity vs. 8.01 U/mL inulinase activity).

Table 2. Comparative study on inulinase and invertase production

Microorganism	Inoculum		Precursor	Final Fermentation (7 days)			
	pH	O.D.		pH	O.D.	Inulinase Activity (U/ml)	Invertase Activity (U/ml)
<i>Candida arborea</i> ICCF 193	5.63	21.25	Inulin	6.61	13.95	0.01	3.968
			Fructose	5.99	23.03	0.005	4.156
<i>Hansenula anomala</i> ICCF 217	4.79	16.5	Inulin	5.37	22.23	0.003	2.008
			Fructose	4.62	18.98	0.001	0.979
<i>Saccharomyces cerevisiae</i> ICCF 192	4.5	15.1	Inulin	5.66	21.78	0.002	1.426
			Fructose	4.71	18.25	0.001	0.346
<i>Saccharomyces cerevisiae</i> ICCF 349	4.49	17.08	Inulin	5.26	23.08	0.002	1.066
			Fructose	4.66	23.75	0.006	0.093
<i>Saccharomyces chevalieri</i> ICCF 312	4.63	14.23	Inulin	5.09	20.83	0.003	1.052
			Fructose	4.57	17.58	0.001	0.35

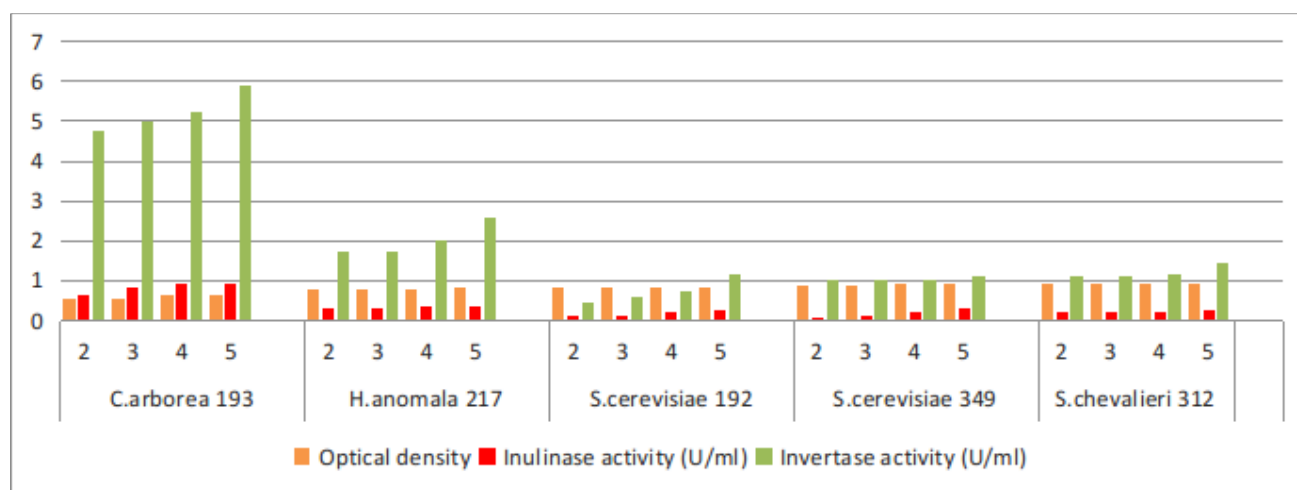


Figure 1. Biomass development and enzymatic activities of yeasts strains

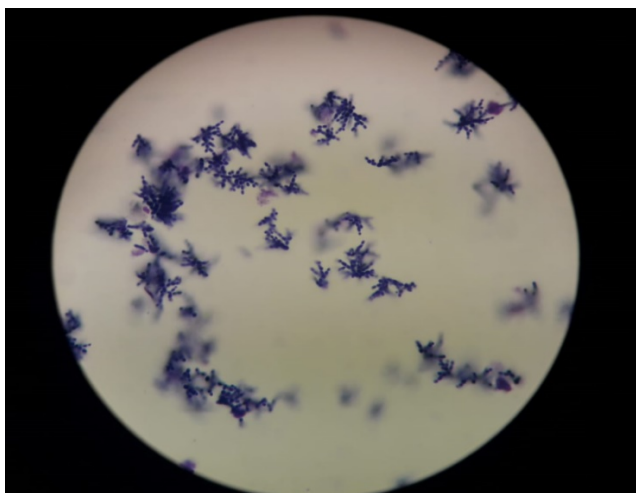


Figure 2. Microscopic view of *Candida arborea* ICCF 193

Inulinase purification

The supernatant obtained by centrifugation of *Candida arborea* ICCF 193 culture, showing inulinase activity, was purified by two successive steps: fractionated precipitation with ethanol (Table 3). By adding ethanol in supernatant with enzymatic content, to obtain a final 29% ethanol saturation, a first purification step is produced. The precipitate obtained has no enzymatic activity, while the enzymes are in the liquid phase. The enzymes precipitate, separated from other impurities, supplementing liquid phase ethanol content until 40% saturation.

This final precipitate has an inulinase activity of 19.7% and invertase activity of 50.3% of the initial activity. It is observed that in the case precipitation with ethanol, inulinase activity retrieved is 10 times lower than in the case of the invertase. In this study is remarkable a greater specific activity for invertase versus inulinase, 503.42U/mg protein versus 43.88 U/mg protein.

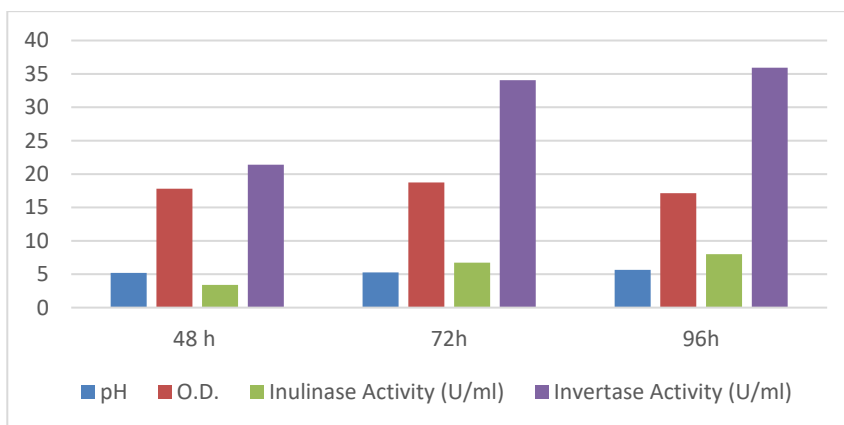


Figure 3. Microbial growth of *Candida arborea* ICCF 193

Table 3. Isolation of compounds with enzymatic activity

Stages	Volume (ml)	Inulinase Activity (U/ml)	Invertase Activity (U/ml)	Protein Concentration (mg/ml)	Specific Activity Inulinase (U/mg protein)	Specific Activity Invertase (U/mg protein)
Fermentation Supernatant	560	8.01	35.92	1.09	7.35	32.95
Precipitation	30	29.40	337.29	0.67	43.88	503.42

Conclusions

From the five strains of yeast tested as inulinase and invertase producers, *Candida arborea* strain was selected as the most important in terms of biomass growth and enzymes production.

Based on the studies performed, it was observed that the bioprocessing media induced predominantly the production of invertase, according to enzymatic activities obtained.

Due to the worldwide concern for the production of microbial inulinase and invertase, and their use in order to obtain from inulin, a renewable raw material, cheap and abundant for industry, the optimization of biosynthesis conditions can be done by diversifying carbon sources using agri-food products.

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Conflict of interest

The authors have no conflict of interest to declare.

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