III. MICROORGANISMS AND ENVIRONMENT

PARAMETERS OPTIMIZATION FOR INCREASED INTRACELLULAR INULINASE ACTIVITY OF A YEAST STRAIN

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Abstract: This study reveals the selection of a yeast strain, possessing inulinase activity and finding the optimal conditions of cultivation. Intra- and extracellular activity assay was performed after cultivation on media, containing inulin as a sole source of carbon. Optimization of the cultivation conditions was carried out for establishing the favorable conditions for biosynthesis of inulinase. Modifying the physicochemical and nutritional parameters of a cultivation process lead to major improvement of the enzyme activity. Highest intra- and extracellular inulinase activity was registered when 1.5% inulin was used, 5 % inoculum, temperature 28°C, pH=6.5 and agitation of 200rpm. The selected strain *Kluyveromyces sp. C* showed higher values for the intracellular inulinase activity, making it suitable for immobilization and further use.

Key words: *Kluyveromyces sp.*, inulinase activity, parameters optimization

INTRODUCTION

In recent years, attention has been given to issues concerning biotechnological processing of biomass from renewable raw materials. Inulin-containing plants - Jerusalem artichoke, chicory, dahlia, garlic and onion are of great industrial importance in the production of inulin, fructose-glucose syrups, animal feeds, pharmaceuticals [1, 2]. Fructans are naturally occurring reserve carbohydrates in plants, which depending on their degree of polymerization could be classified as fructooligosaccharides (degree of polymerization<10) and inulins (degree of polymerization>10) [3]. Fructose is preferred as sweetener due to fact that it's safer than sucrose which causes problems with diabetes, cariogenicity and atherosclerosis. Another advantage of fructose is its slow absorbance in the intestines in comparison with glucose. When acid hydrolysis of inulin is performed for fructose production an unwanted coloring of the hydrolysate appears. Also difructose anhydride is formed which has no sweetening properties [4]. Conventional production of fructose from starch is complicated process involving at least three enzymatic steps and the yield is less than 45%. Full inulin hydrolysis could be accomplished when inulinase (EC 3.2.1.7, β -2,1 – fructan fructanohydrolase) is used in one single step with final yield of 90 – 95% fructose [5, 6]. Inulinases are an important class of inducible enzymes, which act on the β -(2 $\rightarrow$ 1) linkage of inulin, resulting fructose, glucose and fructooligosaccharides as reaction products [7]. They are classified in endo- and exo-inulinases depending on their mode of action. A key point is the selection of microbial strains carrying out the enzymatic hydrolysis and biotransformation of inulin and the accompanying fructo-polysaccharides, fructose and glucose, applicable in the food industry. Among

yeasts the best-known producers are *Kluyveromyces marxianus*, *Candida kefir* and *Pichia polymorpha*, which produce only exo-inulinases, whereas most inulin-hydrolyzing moulds produce both types of the enzyme [8]. These enzymes can also be found in many plants and microbial species: *Aspergillus niger*, *A. ficum*, *Penicillium purpurogenum* and a number of *Lactobacillus* strains [9]. Although there is a big number of investigated microorganisms that produce inulinase, the enzyme is most often extracellular, being secreted in the cultural broth after submerged fermentation, less are the data about microorganisms which possess intracellular enzymatic activity.

The aim of this study was to find the most appropriate physicochemical and cultivation parameters for enhanced inulinase production by a newly isolated strain *Kluyveromyces sp. C*.

EXPERIMENTAL

Materials

All reagents in the present study were with analytical grade and were purchased from Merck KGaA. Inulin with plant origin was kindly provided by BeneoGmbH (Belgium).

Microorganism and culture conditions

Kluyveromyces sp. strain C was previously isolated from a fermented wheat beverage. The strain was maintained on media containing (g/L): yeast extract – 3; malt extract – 3; peptone – 5; glucose – 10; agar – 15; pH 6.5. After 24h at 30°C the strain was stored at 4°C for up to one month. Inoculum was prepared in medium containing (g/L): yeast extract – 3; malt extract – 3; peptone – 5; glucose – 10; pH 6.5 for 18 h on a shaker at 120 rpm.

Inulinase activity determination

Inulinase activity was analyzed based on the fructose liberated during the enzyme action using the PAHBAH method for reducing sugars [10]. For the extracellular enzyme assay 0.5 mL cultural broth were incubated with 0.5 mL 4% inulin solution (pH 7.0, 100 mM Tris-HCl buffer) on a 3-D rotary shaker at 30°C for 30 minutes. The reaction was stopped by boiling the samples at 100°C for 5 minutes, followed by cooling. Controls were developed as described with inactivated enzyme. For the intracellular inulinase activity determination 0.5 mL cell suspension (with known cell concentration) was incubated with 0.5 mL 4% inulin solution (pH 7.0, 100 mM Tris-HCl buffer) on a 3-D rotary shaker at 30°C for 30 minutes. The reaction was then stopped by boiling the samples, cooling and centrifugation at 10500 rpm for 5 minutes. The supernatant was taken for enzyme activity assay. Controls were performed as well with inactivated cell suspensions. One unit of inulinase activity is defined as the micrograms fructose liberated under the action of 1 mL cultural broth (1 g cells) for 1 minute under the assay conditions.

Medium optimization

The effect of inulin concentration in the medium on the inulinase activity was evaluated. Inulin was used as a sole carbon source in four different concentrations (0.5; 1.0; 1.5 and 2.0% w/v). The used medium contained also 0.5% peptone and pH 6.5.

Process parameters optimization

For the determination of the optimal parameters of the cultivation process media with 1.5% inulin and 0.5% peptone was used. The effect of pH,

temperature, duration of the cultivation process and the inoculum concentration on the enzyme activity was studied. The effect of pH was examined in the range 6.2 – 7.2, the temperatures were in the range 26 – 32, 5%, 10% and 15% inoculum was used and the cultivation duration was up to 72 h. Cultivation processes were carried out on a rotary shaker at 200 rpm.

Biomass concentration determination

The biomass concentration was determined using the dry weight method where the biomass was dried to constant mass. For the determination of the dry weight a RADWAG moisture meter was used.

RESULTS AND DISCUSSION

The strain selected for further studies was chosen due to its strong growth on inulin-based medium. Its growth was followed for 72 h. The exponential phase lasted until 36th hour and a decline of growth started after that time. Maximal inulinase production was registered also at the 36th hour (0.63 U/ml of extracellular activity and 9.5 U/mg of intracellular activity) before the onset of stationary phase (Fig.1 A, B).

As presented on Fig. 1 inulinase activity was growth associated. The same trend for the extracellular inulinase activity was established by Al-Dagal and Bazaraa [11] and Singh et al. [1] for strains *Kluyveromyces marxianus* NRRL 2415 and *Kluyveromyces marxianus* YS-1. The fact that the selected strain grew well on inulin improved it produced the enzyme of interest.

Fig. 1. Enzyme activity and growth curve as a function of time.
A - intracellular enzyme activity; B - extracellular enzyme activity.

The effect of the substrate quantity was evaluated by addition of concentrations from 0.5% to 2.5% of inulin in the fermentation medium (Fig. 2 A, B). It

was again proved that inulin induced inulinase production and was the best source of carbon for inulinase production [12].

Fig. 2. Effect of substrate concentration on the intracellular – A and extracellular inulinase activity – B.

A progressive increase in inulinase production was observed up to 1.5% (w/v) after which a decrease was established. This effect was more pronounced in the extracellular activity while the values for the intracellular one were nearer. It had been established and now again confirmed that increasing substrate concentration could lead to catabolic repression and duly lower enzyme activity [13]. Highest enzyme production was realized using 1.5% inulin in the fermentation

medium as a sole carbon source – 9.5 U/mg intracellular and 0.63 U/ml extracellular activity.

Inoculum concentration also affected inulinase production (Fig. 3 A, B). Most appropriate concentration appeared to be 10% (v/v). With the increase of its percent a decline in activity was registered. This was probably due to the increased viscosity by the abundant growth, mass transfer limitation and hence nutritional imbalance.

Fig. 3. Influence of the inoculum concentration on intracellular – A and extracellular inulinase activity - B.

Process optimization studies continued with evaluation of the pH of the medium and the temperature of cultivation. The obtained results showed an increase in enzyme activity changing the initial pH from 6.2 to 6.5. After this value the

activity started to decrease (Fig.4 A, B). Maximum enzyme production at pH 6.5 had been reported by Kalil et al., [14]. The effect of pH of the medium was more clearly demonstrated for the extracellular activity.

Fig. 4. Influence of pH on intracellular - A and extracellular inulinase activity – B.

The obtained results could be added to the main advancements achieved in the last decades in the study of the fructose production process by inulin enzymatic hydrolysis. The properties of inulin are important and appear relevant for the process with reference to its vegetable origin and availability. The characteristics of the inulinase enzyme that catalyzes inulin hydrolysis, accumulate relevant information for conveying a correct process design and its implementation.

Temperature of cultivation was another factor that affected the enzyme production of the studied strain. It was found to be 28 °C for both intra- and extracellular inulinase activity [Fig. 5 A, B]. Generally, temperature imposed its influence on the activity of enzymes. Thus Silva-Santisteban and Filho [15], used 30°C as optimal fermentation temperature for *Kluyveromyces marxianus* as well as 50°C and 60°C for some inulinase-producing marine yeasts [16], revealing it depended on the strain used.

Fig. 5. Influence of temperature on intracellular - A and extracellular inulinase activity – B.

The interaction effects of variables on the calculated responses (intracellular and extracellular inulinase) were studied by plotting 3D surface curves against any two independent variables, as shown in Figs. 6 and 7 [17, 6]. The specialized software package *Signal processing toolbox* for MATLAB 8.1 was used for processing the experimental data. Cubic spline data interpolation was applied. Figures represent the three dimensional plots as a function of the combined effect of pH-time

and temperature-time on intracellular and extracellular inulinase, respectively. pH value of the cultivation medium and temperature showed to be significant variables and should be maintained at their optimum. The surface curves show that the optimal fermentation conditions for maximum intracellular and extracellular inulinase production was achieved by the combined level of pH 6.5 and temperature 28°C at the 36th hour.

Fig. 6. Response surface for intracellular inulinase – A and extracellular inulinase - B productions as a function of pH and time.

Fig. 7. Response surface for intracellular inulinase - A and extracellular inulinase - B productions as a function of temperature and time.

The obtained results could be added to the main advancements achieved in the last decades in the study of the fructose production process by inulin enzymatic hydrolysis [18]. The properties of inulin are important and appear relevant for the process with reference to its vegetable origin and availability. The characteristics of the inulinase enzyme that catalyzes inulin hydrolysis, accumulate relevant information for a correct process design and its implementation.

CONCLUSIONS

A promising producer of both intra- and extracellular inulinase activity was selected. A significantly high intracellular inulinase activity yield was obtained after optimization of the process parameters, which makes it suitable for immobilization and further application. Experimental data and surface curves show that optimal fermentation conditions for maximum production of both intracellular and extracellular inulinase were achieved by the combined level of pH 6.5 and temperature 28°C at the 36th hour. The inulinase, obtained by the use of cheap renewable substrate from plants could be applied in a free or immobilized form for obtaining of fructooligosaccharides.

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ОПТИМИЗАЦИЯ НА ПАРАМЕТРИТЕ ЗА УВЕЛИЧАВАНЕ НА ВЪТРЕКЛЕТЪЧНАТА ИНУЛИНАЗНА АКТИВНОСТ НА ЩАМ ДРОЖДИ

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Резюме: Това изследване разкрива селекцията на дрождев щам, притежаващ инулиназна активност и намиране на оптималните условия на култивиране. Анализът на вътре- и извънклетъчната активност се извършва след култивиране върху среда, съдържаща инулин като единствен източник на въглерод. Проведена е оптимизация на условията за култивиране за установяване на благоприятни условия за биосинтеза на инулиназа. Модифицирането на физикохимичните и хранителните параметри на процеса на култивиране води до значително подобряване на ензимната активност. Най-високата интра- и екстрацелуларна инулиназна активност е регистрирана, когато е използван 1.5% инулин, 5% инокулум, температура 28 ° C, pH = 6.5 и разбъркване 200 rpm. Избраният щам *Kluyveromyces sp.* С показва по-високи стойности за вътреклетъчната инулиназна активност, което го прави подходящ за имобилизиране и по-нататъшна употреба.

Ключови думи: *Kluyveromyces sp.*, инулиназна активност, оптимизация на параметри

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