Phenotypic regulation of the synthesis of carbohydrases by Kluyveromyces yeasts

O Korneeva, T Sviridova, G Shuvaeva, O Meshcheryakova, E Anokhina and E Motina

Voronezh State University of Engineering Technologies, 19, Revolution ave., Voronezh, 394036, Russia

E-mail:sviridovatv@yandex.ru

Abstract. The ability of Kluyveromyces marxianus Y-303 strain to synthesize highly active intracellular β-fructosidases (invertase and inulinase) was studied. The effect of separate sources of carbon, nitrogen, the initial pH of the nutrient medium, temperature and duration of cultivation process on the biosynthesis of enzymes were studied. The optimal composition of the nutrient medium and cultivation conditions that ensure the maximum production of the studied enzymes by K. marxianus yeast were selected. It was found out that the optimal medium for invertase synthesis contains 3% fructose and 0.6% NH₄H₂PO₄, and for inulinase - 0.5% inulin and 0.12% yeast extract of inulinase. Optimal cultivation conditions for the accumulation of invertase - pH 6.0, temperature - 30 °C, duration - 42 hours; and for inulinase, respectively: pH 5.0, 25 °C, 66 hours. The described cultivation conditions of K. marxianus led to 6-7 fold activity increase for invertase and 8-9 fold for inulinase.

1. Introduction

Dairy yeast of the genus Kluyveromyces is the second most important object of fundamental and applied research after the genus Saccharomyces. They are used in production of various heterologous proteins for medical and food purposes, as well as producers of bioethanol from lignocellulosic waste of agriculture and the wood processing industry [1, 2, 3].

K. lactis and K. marxianus belong to yeasts that produce β-galactosidase enzyme and assimilate lactose that allows them to be widely used as probiotic microorganisms in wide range of dairy products [1, 2, 4]. A characteristic feature of K. marxianus is a high growth rate - this is the fastest growing eukaryote [1], as well as heat resistance, which allows them to remain viable at a temperature of 52 °C, survive in human digestive system, preserving fermentation properties, and stimulate the growth of Bifidobacterium and suppression of pathogenic microflora [5]. Consequently, the study of biosynthetic ability of Kluyveromyces yeast, selection of optimal composition of nutrient medium, cultivation modes and methods for producing enzyme preparations based on them is an important task of modern biotechnology.

The analysis of scientific publications showed that the yeast of the genus K. marxianus is able to synthesize various hydrolases. For instance, in a medium with whey permeate as a carbon source, they synthesize β-galactosidase [4]; addition of sucrose and fructose induces synthesis by the producer of β-fructofuranosidase, and inulin and xylose induces inulinase [6, 7, 8]; addition of grape must into the nutrient medium promotes the formation of endo-polygalacturonase [9], pectinase [10].
Among hydrolases, researchers pay great attention to β-fructosidases due to their applicability in various sectors of food industry: baking, confectionery, non-alcoholic and distilled beverages [2, 11, 12].

β-Fructosidases are enzymes with β-D-O-fructofuranoside-fructohydrolase activity able to transfer β-D-fructofuranose residues from molecules of carbohydrate to molecules of water with formation of D-fructose [13]. Enzymes with β-fructosidase activity differ by substrate specificity and additional activities. β-Fructosidases can cleave sucrose, raffinose, stachyose, and various polyfructans [14, 15]. The most interesting ones are yeast β-fructosidases.

2. Objects of study and research methods

2.1 Objects of research
The main object of research is a pure culture of the yeast Kluyveromyces marxianus Y-303, obtained from the Russian National Collection of Industrial Microorganisms.

2.2 Research methods
Cultivation of the producer. The yeast culture K. marxianus was maintained on wort agar. Deep cultivation was carried out in 500 cm$^3$ flasks containing 250 cm$^3$ of the nutrient medium of the studied composition for 48-72 hours at a temperature of 29 ± 2°C. To study the biosynthetic ability of K. marxianus yeast, the following yeast medium was used,%: peptone - 1.0; yeast extract - 0.5; glucose - 2; pH of the medium was from 5.5 to 8.0.

An impact of components of carbon and nitrogen nutrition on the biosynthesis of invertase and inulinase was studied in experiments, by replacing the source of carbon and nitrogen of the yeast medium with the test substance in an equivalent ratio. Glucose, sucrose, fructose, lactose, rhamnose, starch, maltose, raffinose, inulin and xylose were examined as a carbon source. These components were added to the nutrient medium in an amount of 1% carbon. To choose the mineral source of nitrogen, we used sodium nitrate, potassium, and ammonium, ammonium sulfate, and di- and monosubstituted ammonium phosphate (at a concentration of 0.1% for nitrogen). They were malt, corn and yeast extracts (2% by weight of the medium), peptone (1%) as an organic source of nitrogen.

To study the effect of medium pH on the growth of K. marxianus Y-303 yeast and the biosynthesis of β-fructosidase, the initial pH was changed by adding 1 N HCl or NaOH.

Determination of the activity of β-fructosidases. Invertase and inulinase activities were determined in yeast biomass and supernatant. Biomass was separated from the nutrient medium by centrifugation at a rotation speed of 3000-3500 rpm for 10-15 minutes, with the precipitate washed several times with sodium chloride solution. Enzymatic activity was expressed in units/g of yeast biomass having a moisture content of 75% or units/cm$^3$ of supernatant. The amount of enzyme that hydrolyzes 1 mmol of glucose/fructose in 1 minute in 0.1M acetate buffer at pH 4.5 at 40 °C was taken as a unit of enzymatic activity. The activity of β-fructosidases was evaluated by the amount of formed reducing sugars, which were determined by the Somogy-Nelson method [16]. A 5% solution of sucrose in 0.1 M acetate buffer with pH 4.5 was used as a substrate for determining invertase activity, and inulin with a 5% mass fraction – for inulinase.

Determination of biomass. The biomass accumulation in a culture fluid was determined by counting yeast cells in hemocytometer [17].

The disintegration of yeast cells was carried out in the UZDN-2T disintegrator (power - 500W, frequency - 18-20 kHz, processing time - 1 min).
3. Results and discussion

3.1 Research of enzymatic activity localization

The known fact that Kluyveromyces yeast is able to synthesize both intracellular and extracellular enzymes [6, 7, 8, 9] was confirmed by our research. It was found that maximum amount of β-fructosidases (95–97%) is concentrated in the yeast cell, which corresponds to their highest activity in the biomass (Figure 1). Therefore, further studies were carried out with intracellular enzymes localized in producer cells.

3.2 The effect of different carbon sources on the biosynthesis of β-fructosidases

Monosaccharides, disaccharides, and polysaccharides were included one by one in the nutrient medium to determine the effect of various carbohydrates on the growth of *K. marxianus* Y-303 yeast and their synthesis of β-fructosidase.

Analysis of experimental data (Table 1) showed that invertase synthesis was observed for all carbohydrates. The biosynthesis of β-fructosidases in the medium containing xylitol and dulcite as a carbon source was at the lowest level. In a medium with lactose, significant culture growth was observed, but invertase synthesis was negligible, and no inulinase activity was detected. The addition of fructose and sucrose into the medium led to the induction of synthesis of both invertase and inulinase. However, the degree of invertase induction by sucrose was less than fructose, and vice versa for inulinase. It should be noted, that a strict correlation between an accumulation of yeast biomass and enzyme synthesis was not observed. Thus, fructose is the best carbon source providing the greatest synthesis of invertase, and inulin for inulinase. Our results are consistent with data from other research [18, 19].

![Graph of enzymatic activity localization](image)

**Figure 1.** β-fructosidase enzymatic activity localization for *K. marxianus*

| Carbon source | Activity, units/g of biomass | Biomass, cells/cm³ |
|---------------|------------------------------|--------------------|
| Glucose (control) | 490 | 350 | 870 |
| Fructose | 1180 | 850 | 945 |
| Ramnose | 130 | 100 | 900 |
| Xylose | 30 | 10 | 295 |
| Dulcite | 20 | 5 | 335 |
| Sucrose | 780 | 1150 | 860 |
| Lactose | 120 | 0 | 1100 |
| Maltose | 110 | 783 | 1027 |
| Raffinose | 230 | 380 | 560 |
| Insulin | 500 | 2500 | 1000 |

**Table 1.** The effect of different carbon sources on the biosynthesis of β-fructosidases by *K. marxianus*
The dependence of enzyme activity on concentration of selected carbon sources is shown in Figure 2. The maximum invertase activity was observed with a medium containing 3% fructose, inulinase - in a medium with 0.5% inulin. A further increase of sugar concentration led to enzymatic activity decrease. That is, fructose can act not only as an inducer, but also as a repressor of invertase biosynthesis. This is confirmed by the studies of Dynesen J. [20], who determined that fructose or glucose contained in the nutrient medium in an amount greater than 5g/dm$^3$ is equally capable of causing catabolite repression.

![Graph showing enzyme activity vs. carbon source concentration](image)

**Figure 2.** The effect of concentration of carbon source on the biosynthesis of β-fructosidases: a – synthesis of invertase on fructose carbon source; b – synthesis of inulinase on inulin carbon source; C – concentration of carbon source, %; A – activity of β-fructosidases, units/g

### 3.3 The effect of different nitrogen sources on the biosynthesis of β-fructosidases

Along with carbon, nitrogen sources play an important role in enzyme biosynthesis. To identify the best nitrogen sources for the biosynthesis of *K. marxianus* Y-303 β-fructosidases, various mineral salts and organic substrates were tested (Table 2). The source of carbon in the synthesis of invertase was fructose with a concentration of 3% by weight of the medium, inulin 0.5% - for inulinase.

**Table 2.** The effect of different nitrogen sources on the biosynthesis of β-fructosidases by *K. marxianus*

| Nitrogen source | Activity, units/g of yeast | Biomass, cells/cm$^3$ |
|-----------------|---------------------------|----------------------|
|                 | inverte | insuline | Fructose medium | Inulin medium |
| KNO$_3$         | 2260    | 286      | 1500          | 1300          |
| NaNO$_3$        | 800     | 334      | 1245          | 1450          |
| NH$_4$NO$_3$    | 1130    | 80       | 1155          | 1260          |
| (NH$_4$)$_2$HPO$_4$ | 1880   | 223      | 1125          | 1450          |
| NH$_4$H$_2$PO$_4$ | 3310   | 198      | 1300          | 1500          |
| (NH$_4$)$_2$SO$_4$ | 1570   | 719      | 1460          | 1425          |
| Yeast extract   | 2000    | 3050     | 1985          | 2050          |
| Corn extract    | 1190    | 670      | 1690          | 1800          |
| Malt extract    | 605     | 178      | 965           | 1300          |
It was found that all nitrogen sources led to a significant culture growth and the accumulation of β-fructosidases in yeast cells. The maximum synthesis of invertase was provided by ammonium dihydrogen phosphate, and inulinase was provided by yeast extract. These results are consistent with data from other authors [19, 21]. Other nitrogen sources suppressed inulinase synthesis. A high level of invertase biosynthesis on other salts was provided by potassium nitrate, whose nitrogen is in oxidized form. The least suitable is sodium nitrate, which suppressed enzyme synthesis by 80%. The decrease in invertase synthesis in a medium containing NaNO₃ instead of KNO₃ is explained by the absence of a K⁺ ion in the nutrient medium. The synthesis of inulinase on media with mineral salts was not significant.

A study of the influence of nitrogen sources concentration on the synthesis of enzymes showed (Figure 3) that the maximum accumulation of invertase is observed when ammonium dihydrogen phosphate is added in an amount of 0.086 g/100 cm³ for nitrogen or 0.6% of the medium volume, the maximum accumulation of inulinase - 0.12% yeast extract.

![Figure 3. The effect of ammonium dihydrogen phosphate content on the biosynthesis of invertase (a) and yeast extract on synthesis of inulinase (b): C – concentration of yeast extract, %; A – activity of β-fructosidases, units/g](image)

3.4 The effect of cultivation modes on the biosynthesis of β-fructosidases
A study of the influence of initial pH of the medium, temperature, and duration of cultivation on the growth of K. marxianus yeast and their biosynthesis of β-fructosidase demonstrated that this producer shows the ability to grow in a wide range of pH - from 3.0 to 8.0 (Fig. 4a). The maximum biosynthesis of invertase was observed at pH 6.0, inulinase – pH 5.0. Changing the pH to the acidic or alkaline side from the optimal value negatively affected the level of enzyme accumulation.

Figure 4b shows the effect of temperature on the biosynthesis of β-fructosidases by the K. marxianus Y-303 yeast. Cultivation was carried out at optimal pH values for their synthesis. Optimal temperature for invertase synthesis is 30 °C, for inulinase – 25 °C. An increase or decrease of the temperature of cultivation led to a decrease of the growth of yeast and enzyme activity by more than 50%. At 50 °C, culture growth was practically absent, which indicates the belonging of the yeast strain K. marxianus Y-303 to mesophiles.
A study of the synthesis of β-fructosidases during the cultivation of *K. marxianus* yeast (Figure 5) showed that the maximum invertase activity in yeast cells accumulated by 42 hours of cultivation, inulinase activity - by 66 hours. Reducing the cultivation time leads to a shorter, cost-effective production cycle.

**Figure 5.** The accumulation of β-fructosidases during cultivation of the yeast *K. marxianus*

**4. Results**

The high efficiency of the yeast *K. marxianus* Y-303 for the biosynthesis of intracellular β-fructosidases: invertase and inulinase was observed.

A study of the effect of various carbohydrates on the synthesis of β-fructosidases showed that they are inducible enzymes. The inductor of invertase synthesis is fructose in an amount of 3%, inulinase – inulin, 0.5%.

To ensure efficient biosynthesis of highly active intracellular invertase and inulinase by *K. marxianus* yeast, it is advisable to add fructose – 2% and inulin - 0.5%; nitrogen source - NH$_4$H$_2$PO$_4$ - 0.6% and yeast extract - 0.12%, as a carbon source. Rational cultivation modes for invertase: pH - 6.0, tem-
perature - 30 °C, duration 42 hours; for inulinase - pH 5.0, 25 °C, 66 hours. This allows one to increase the activity of inulinase 9-10 fold, invertase - 6-7 fold.

References
[1] Gombert AK, Madeira JV Jr, Cerdán ME and González-Siso MI 2016 *Kluyveromyces marxianus* as a host for heterologous protein synthesis *Appl Microbiol Biotechnol* 100(14) 6193-208
[2] Bannicina T, Kanarsky A, Scherbakov A, Chebotar B and Kipruskhina E 2016 Yeasts in modern biotechnology, *Journal of International Academy of Refrigeration* 124-29
[3] Du C, Li Y, Zhao X, Pei X, Yuan W, Bai F and Jiang Y 2019 The production of ethanol from lignocellulosic biomass by *Kluyveromyces marxianus* CICC 1727-5 and Spathaspora passalidarum ATCC MYA-4345 *Appl Microbiol Biotechnol*. 103(6) 2845-2855
[4] Yahtin I and RytchenkovaO 2011 A study of the growth of yeast *Kluyveromyces lactis* and *Kluyveromyces marxianus* on the waste of dairy plants *Russian Chemical Reviews* XXV10(126) 33-36
[5] Ceugniez A, Coucheney F, Jacques P, Daube G, Delcenserie V and Drider D 2017 Anti-Salmonella activity and probiotic trends of *Kluyveromyces marxianus* S-2-05 and *Kluyveromyces lactis* S-3-05 isolated from a French cheese, *Tommed' Orchies Res Microbiol*. 168(6) 575-582
[6] Hoshida H, Kidera K, Takishita R, Fujioka N, Fukagawa T and Akada R 2018 Enhanced production of extracellular inulinase by the yeast *Kluyveromyces marxianus* in xylose catabolic state *J Biosci Bioeng.* Jun. 125(6) 676-681
[7] Jiaoqi Gao, Lijie Chen, Wenjie Yuan and Jiaoqi Gao 2012 Effects of carbon sources, oxygenation and ethanol on the production of inulinase by *Kluyveromyces marxianus* YX01 *J. BioSci. Biotech.* 1(2) 155-161
[8] Hoshida H, Kidera K, Takishita R, Fujioka N, Fukagawa T and Akada R 2018 Enhanced production of extracellular inulinase by the yeast *Kluyveromyces marxianus* in xylose catabolic state *J Biosci Bioeng.* 125(6)676-681
[9] Mansoldo FRP, Neves Junior A, Cardoso VDS, Rosa MDSS and Vermelho AB 2019 Evaluation of *Kluyveromyces marxianus* endo-polygalacturonase activity through ATR-FTIR *Analyst*. 144(13) 4111-4120
[10] Rollero S, Zietsman AJJ, Buffetto F, Schückel J, Ortáz-Julien A and Divol B2018 *Kluyveromyces marxianus* Secretes a Pectinase in Shiraz Grape Must That Impacts Technological Properties and Aroma Profile of Wine *J Agric Food Chem*. 66(44) 11739-11747.
[11] Vagabov M, Kerimova Z, Malceva T and Korneeva O 2006 Intensification of the process of hydrolysis of inulin-containing raw materials in ethanol production *Storage and Processing of Farm Products* 43-45
[12] Rawat HK, Soni H, Treichel H and Kango N 2017 Biotechnological potential of microbial inulinas: Recent perspective *Crit Rev Food Sci* 57(18) 3818-3829
[13] Naumova D and Doroshenko V 1998 β-Fructosidas: The New Superfamily of Glycosyl Hydrolases *Molecular biology* 32(5) 902-907
[14] Shuvaeva G, Korneeva O and Rutkovskaya T 2010 Inulinase from yeast S. cerevisiae VGS-2. Preparative production and some physicochemical properties *Fundamental research* 10 17-25
[15] Nagem RA, Rojas AL, Golubev AM, Korneeva OS, Eneyskaya EV, Kulminskaya AA, Neustroev K N and Polikarpov I 2004 Crystal structure of exo-inulinase from Aspergillus awamori: the enzyme fold and structural determinants of substrate recognition *J Mol Biol.* 344(2) 471-80
[16] Somogyi MJ 1952 Determination of reducing sugar *J. Biol. Chem.* 195(1) 19-28
[17] Slyusarenko T 1984 *Laboratory Workshop on Microbiology of Food Production*(Moscow: Light Industry)
[18] Pandey A, Soccol CR, Selvakumar P, Soccol VT, Krieger N and Fortana JD 1999 Recent developments in microbial inulinas. Its production, properties, and industrial applications *Appl. Biochemistry and Biotechnology* 81 35-52
[19] Singh RS, Chauhan K and Kennedy JF 2017 A panorama of bacterial inulinases: Production, purification, characterization and industrial applications *Int J Biol Macromol.* 96 312-322.
[20] Dynesen J, Smits HP, Olsson L and Nielsen J 1998 Carbon catabolite repression of invertase during batch cultivations of *Saccharomyces cerevisiae*: the role of glucose, fructose, and mannose *Appl Microbiol Biotechnol.* 50(5) 579-582
[21] Pandey A, Soccol CR, Selvakumar P, Soccol V T, Krieger N and Fortana JD 1999 Recent developments in microbial inulinases. Its production, properties, and industrial applications *Appl. Biochemistry and Biotechnology* 81 35-52