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Treatment of lactose by fermentation: Production process on β-galactosidase using *Kluyveromyces marxianus* isolated from kefir grains

*Ozarslan, S. and Kök Taş, T.

Department of Food Engineering, Faculty of Engineering, Suleyman Demirel University, Isparta, Turkey

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Abstract

β-galactosidase is an enzyme that catalyzes the hydrolysis of lactose, a disaccharide, into glucose and galactose. β-galactosidase is a commercially essential enzyme for its catalytic properties and is widely used in various biotechnology processes. In this study, optimum production conditions were studied to produce the highest level of β-galactosidase using *Kluyveromyces marxianus* isolated from kefir grains, and changes in some fermentation metabolites during enzyme production were determined under optimum conditions. The pH, temperature, and incubation time were optimised to produce of β-galactosidase. The pH (4.0, 7.0, 8.0), temperature (25 - 37°C), and incubation time (0 - 60 h) were evaluated in the ranges. The highest specific enzyme activity was 47.31 U/mL at the end of 48 hour-incubation at 200 rpm, at pH 8.0, and 30°C with 2% (v/v) inoculation rate. A high-performance liquid chromatography system determined the changes in lactose, glucose, galactose, lactic acid, and ethanol concentrations at the optimum fermentation conditions by taking samples from liquid fermentation medium during enzyme production under these conditions. The amount of glucose and galactose formed by the lactose fermentation metabolism of *K. marxianus* decreased during the fermentation. The ethanol concentration reached its highest concentration (18.44 g/L) at the 36th hour of fermentation. When fermentation metabolites were examined, β-galactosidase and *K. marxianus* metabolism treatment indicated remarkable findings.

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Introduction

Natural kefir is produced from kefir grains, a sophisticated collection of microorganisms embedded in a special polysaccharide structure (Koçak *et al.*, 2021). The structure contains numerous lactic acid bacteria, acetic acid bacteria, and yeasts (Demirci *et al.*, 2019; Kıvanç and Yapıcı, 2019). Kefir is a natural probiotic drink with antibacterial, anticarcinogenic and antistress properties, and antifungal effects (Guzel-Seydim *et al.*, 2011; Larosa *et al.*, 2020). Kefir consumption has many benefits for the digestive system and various health benefits (Erdoğan *et al.*, 2019; Mitra and Gosh, 2020). The microorganisms in the structure of kefir and the metabolites formed during fermentation supply easy digestion of kefir and increase the absorption of nutrients by the body (Ozarslan and Kok Tas, 2018). *Kluyveromyces marxianus*, which produces various enzymes, is a crucial yeast in kefir (Lim *et al.*, 2019). β-galactosidase (Lactase, EC 3.2.1.23) is the enzyme that catalyses the hydrolysis of lactose into glucose and galactose. In industrial practices, β-galactosidase is found in a wide variety of sources such as fungal, yeast, bacteria, plants, and animals; although, microorganisms are considered to be the most common source of β-D-galactosidase compared to other available sources because they are readily available and cheap (Panesar *et al.*, 2010; Kumar *et al.*, 2012).

Whey proteins are multifunctional food components with high nutritional value. They offer a wide range of functional properties that allow new products to be developed and existing products to be optimised at significantly lower cost. Also, whey is a major by-product of the dairy industry and constitutes a serious problem for use. In the food industry, hydrolysis of whey by-galactosidase is a significant practice for enzyme technology. Environmentally, research on membrane separation technology allows considerable new opportunities for lactose fractionation.

*Corresponding author.
Email: sedaozarslan1@gmail.com

β-galactosidase is widely used in the food industry to improve the sweetness, solubility, flavor, and digestibility of dairy products. Enzymatic hydrolysis of lactose with β-galactosidase is one of the most common technologies used in lactose-free milk and dairy products for people with lactose intolerance, and in the prevention of crystallisation during the production of ice cream and concentrated milk (Husain, 2010; Gupte and Nair, 2010). In a broader sense, the industrial demand for β-galactosidase has increased due to the need for more production methods by enzymatic hydrolysis. Thus, optimising the production of this enzyme is very important (Braga *et al.,* 2012). Several studies have been carried out on the optimisation of various culture conditions to produce of β-galactosidase by using *K. marxianus* (Fiedurek and Szczodrak, 1994; Furlan *et al.*, 2001; Manera *et al.,* 2008; Gupte and Nair, 2010; Braga *et al.*, 2012; Perini *et al.,* 2013).

Enzyme activity varies based on culture conditions such as source, pH, temperature, incubation time, agitation speed, and growth medium composition (Dagbaglı and Goksungur, 2008). In this study, optimum production conditions were studied to produce the highest level of β-galactosidase using *K. marxianus* isolated from kefir grain. Changes in some fermentation liquid medium metabolites were determined during enzyme production under optimum conditions.

Materials and methods

Organism and fermentation condition

The *K. marxianus* used in this study was obtained from kefir grains (Ataç *et al.,* 2021). This standard culture provided from Laboratory of Food Engineering, Suleyman Demirel University. *K. marxianus* was inoculated into a Yeast-Extract-Peptone-Dextrose broth medium (YPD in g/l = peptone, 20; yeast extract, 10; glucose, 20), and incubated for two days at 25°C. The grown yeast culture was stored at 4°C. The enzyme production medium was prepared to be 150 mL (4.5 g lactose; 0.15 g yeast extract; 0.3 g K_2HPO_4; 0.15 g $NH_4H_2PO_4$; 0.03 g $MgSO_4x7H_2O$) medium in 500 mL Erlenmeyer flasks and the pH of the medium was set to 4.0, 7.0, 8.0 at the beginning of the fermentation. The fresh grown culture was added to the enzyme production medium at a 2% (v/v) ratio and allowed to incubate at 200 rpm on a shaking incubator (SCILOGEX SK-0330-PRO, Berlin, USA). During the incubation at the various temperatures (25, 27, 30, 33, 35, 37°C), samples were taken at specific intervals and stored at 4°C for enzyme activity measurements.

Enzyme extraction

Since β-galactosidase is an intracellular enzyme, the cell suspension was centrifuged at 4,000 rpm for 15 min, after which the cell pellet was washed with a 0.03 M potassium phosphate buffer (pH 6.8) and suspended in the same buffer. It was then vortexed in plastic bottles with glass beads (1 mm Ø) for 5 min. At the end of the process, the mixture was centrifuged again at 4,000 rpm for 10 min at 4°C, and the supernatant was used in enzyme activity assays (Song and Jacques, 1997).

Enzyme activity assay

Each unit of β-galactosidase activity was defined as the amount of enzyme liberating 1 µmol of *o*-nitrophenol per minute. The number of enzyme units in a milligram (mg) protein is considered its specific activity. The method of Shah and Otieno (2007) was modified and used in the measurement of β-galactosidase activity. Activity was determined using *o*-nitrophenyl-β-D-galactopyranoside (*o*-NPG; Sigma, Germany) as the substrate. The reaction started by adding 1 mL enzyme extract into the reaction mixture containing 0.2 mL 15 mM *o*-NPG in 0.03 M potassium phosphate buffer (pH 6.8), incubated at 37°C for 15 min, and the reaction was then stopped by the addition of a 1 M 0.5 mL sodium carbonate (Merck) solution. Centrifugation was performed at 4,000 rpm for 10 min at 4°C, and the absorbance value was read at 420 nm with Biotek Synergy™ Multi-Detection Microplate Reader (Winooski, Vermont, USA). The same procedure was repeated with 1 mL 0.03 M potassium phosphate buffer (pH 6.8) instead of the crude extract, and a curve was prepared. The experiment was replicated three times using duplicate samples for each analysis.

Protein assay

Protein assay was performed by the Bradford method (Bradford, 1976). Bovine serum Albumin (Sigma, Germany) was used as a standard in this method.

Measurement of lactose, glucose, galactose, lactic acid, and ethanol concentrations in fermentation liquid

After the determination of optimum conditions to produce β-galactosidase by *K. marxianus*, fermentation took place to determine the fermentation metabolites. Lactose, glucose, galactose, lactic acid, and ethanol concentrations were determined by modifying the method of Sun *et al*. (2016). The fermentation liquid sample was taken at the 0th, 12th, 24th, 36th, 48th, and 60th hours. Then, the samples were centrifuged at 4,000 rpm for 10 min at 4°C, and the resulting supernatant was used after filtered through a 0.45 µm membrane filter. The amount of metabolites in the filtrate was determined using a high-performance liquid chromatography (HPLC; Shimadzu SCL-10A, Scientific Instruments, Inc., Tokyo, Japan). The HPLC consists of an RID (Refractive Index Detector), a system control unit (LC 20ADvp), a PUMP (LC 10ADvp), a degassing unit (DGU 20A), and a column oven (CTO 10Avp). For the sugar composition and the lactic acid, the HI-PLEX Na (Octo) column was used, with a flow rate of 0.8 mL/min, column temperature of 70°C, and 0.15% NaOH solution for the mobile phase. The amount of ethanol in the samples was determined with the Transgenomic COREGEL 87P column, using a flow rate of 0.8 mL/min, a column temperature of 50°C, and purified water for the mobile phase. The standard curve for each component was prepared and quantities were determined.

Results and discussion

The temperature, pH and fermentation time optimisation

The incubation temperature of the fermentation medium is an important factor since it has a significant effect on the metabolic activities of microorganisms. The temperature, pH, and fermentation time optimisation results for β-galactosidase production at the maximum level were given in Figure 1. The specific enzyme activity values for 25, 27, 30, 33, 35, and 37°C were 41.1, 22.82, 47.31, 24.10, 23.52, and 14.27 U/mg, respectively. The highest specific enzyme activity was 47.31 U/mL at 30°C. Several studies have been carried out for the optimisation of β-galactosidase production at 25 - 30°C (Artolozaga *et al*., 1998; Ramirez-Matheus and Rivas, 2003; Matthews, 2005; Panesar *et al*., 2006). Perini *et al*. (2013) found an optimum temperature of 31°C and a maximum specific activity of 21.99 U/mL for β-galactosidase production using *K. marxianus*. Panesar (2008) reported the optimum temperature and the enzyme activity for β-galactosidase production using *K. marxianus* NCIM 3465 to be at 30°C and 1490 IU/g dry weight, respectively.

The pH of growth medium plays a vital role by triggering morphological changes in the microorganism and enzyme production. The pH changes observed during the growth of the organism affect product stability in the medium (Gupta *et al*., 1994). In this study, at the end of the 48-hour fermentation, the maximum specific enzyme activity was 47.31 U/mL at pH 8.0, while the lowest specific enzyme activity was 5.12 U/mL at pH 4.0 and 37°C. For specific enzyme activity, the pH values showed differences depending on fermentation time and temperature. Gupte and Nair (2010) noted that the optimum pH was 5.0 for β-galactosidase production using *K. marxianus* NCIM 3551. Manera *et al*. (2008), on the other hand, reported that the optimum pH for 10.6 U/mL enzyme activity was 6.0 for β-galactosidase production using *K. marxianus* CCT 7082.

The highest specific enzyme activity was measured at the 48th hour. At all temperatures and pH values, enzyme activities decreased after the 48th hour. The unique intrinsic properties of different strains may cause variations in optimum conditions for the best enzyme activity.

Determination of lactose, glucose, galactose, lactic acid, and ethanol concentrations in fermentation liquid

Lactose is broken down to form some fermentation metabolites with fermentation, as shown in Table 1 and Figure 2. The amounts of galactose, glucose, lactose, and lactic acid of the samples were examined at the 0th, 12th, 24th, 36th, 48th, and 60th hours. Lactose began to break down; lactose content was 7.56 g/L at the 12th hour, and 2.48 g/L at the 60th hour. Different pathways are used in the metabolisms of glucose and galactose caused by the decomposition of lactose by β-galactosidase. Glucose is converted to glucose 6-phosphate, but galactose is converted to glucose 1-phosphate by the Leloir pathway. At this stage, lactose decreased gradually due to the presence of different enzymes originating from the yeast (Carvalho Silva and Spencer Martins, 1990). Glucose, which was not detected in the fermentation liquid at the beginning, was 0.39 g/L at the 12th hour, reaching the highest concentration (4.21 g/L) at the 36th hour. After that time, it began to decrease, and at

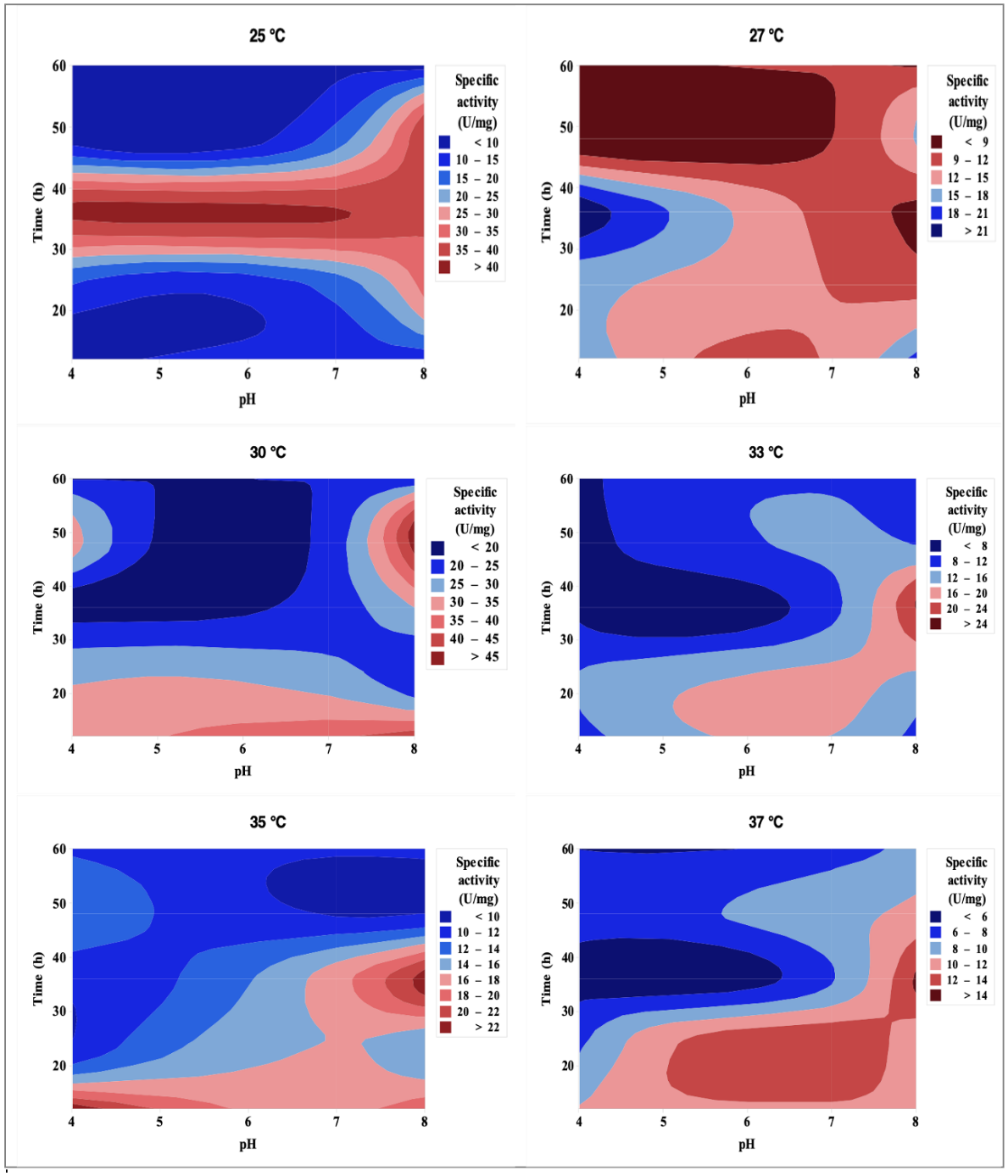

Figure 1. Specific activity values under different fermentation conditions.

Table 1. Amounts of lactose and extracellular metabolites during fermentation.

Time (h)	Lactose (g/L)	Glucose (g/L)	Galactose (g/L)	Ethanol (g/L)	Lactic acid (g/L)
0	30.00 ± 0.21	N.D.	N.D.	N.D.	N.D.
12	7.56 ± 0.25	0.39 ± 0.15	5.02 ± 0.23	12.28 ± 0.55	4.21 ± 0.05
24	3.38 ± 0.05	1.40 ± 0.08	1.06 ± 0.16	15.92 ± 0.04	5.25 ± 0.05
36	3.18 ± 0.09	4.21 ± 0.05	N.D.	18.44 ± 0.23	3.08 ± 0.06
48	2.95 ± 0.13	3.85 ± 0.28	N.D.	17.89 ± 0.38	2.56 ± 0.16
60	2.48 ± 0.05	3.51 ± 0.28	N.D.	13.13 ± 0.29	2.50 ± 0.06

N.D.: not detected.

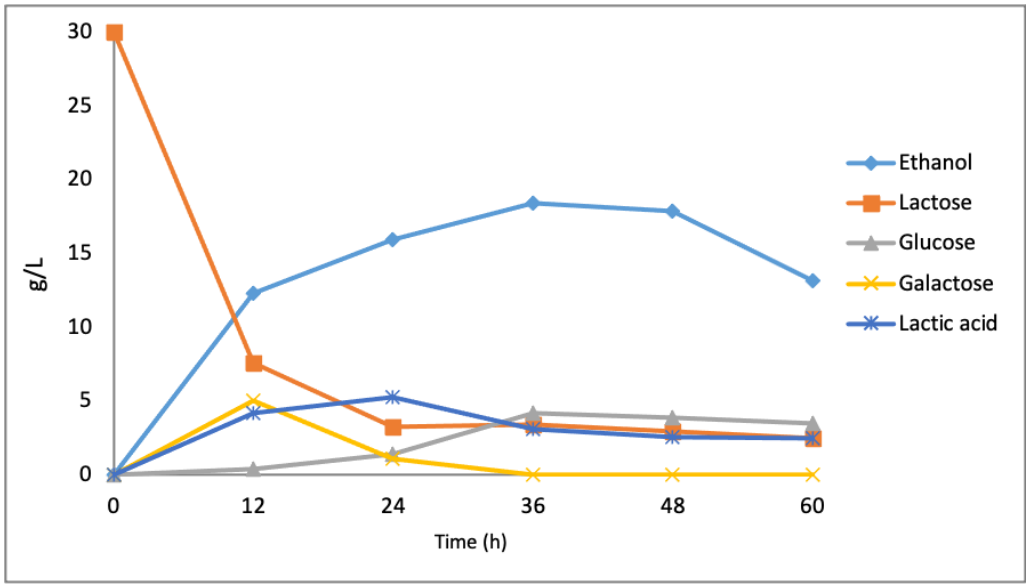

Figure 2. Variation of some fermentation component quantities

the 60th hour, it was found to be 3.51 g/L (Table 1). Because of the breakdown of lactose, glucose and galactose were released; however, they continued to decrease afterward due to the metabolism of *K. marxianus*.

During glycolysis, glucose is metabolised by *K. marxianus*, glucose content decreases, and lactic acid content increases; however, glucose was not depleted entirely until the 60th hour. This was interpreted as the conversion of galactose to glucose by the galactose metabolism and the Leloir pathway. Some enzymes in the pathway (galactose-1-phosphate uridyltransferase) and a uridine monophosphate (UMP) group catalyse the transfer from UDP-glucose to galactose 1-phosphate to produce glucose 1-phosphate and UDP-galactose. To complete the pathway, UDP-galactose is converted to UDP-glucose with UDP-galactose 4-epimerase. In most organisms, the conversion of β-D-galactose to the metabolically more beneficial glucose 1-phosphate is realised by the effect of four enzymes that form the Leloir pathway. Galactose was 5.02 g/L at the 12th hour. It decreased to 1.06 g/L at the 24th hour, and was not detected at the 36th hour. Considering the galactose metabolism, this decrease was interpreted as usual.

The resulting galactose is epimerised to α-D-galactose with galactose mutarotase. The next step involves ATP-dependent phosphorylation of α-D-galactose with galactokinase to obtain galactose 1-phosphate. The Leloir pathway enzymes are specified as galactose mutarotase, galactokinase, galactose-1-phosphate uridyltransferase, and UDP-galactose 4-epimerase (Holden *et al*., 2003). Lactic acid was 4.21 g/L at the 12th hour, and reached its highest concentration at the 24th hour. At the later hours of fermentation, the concentration continued to decrease. Glycolysis and galactose metabolism continued until the 60th hour. Ethanol formation started at the 12th hour, and was 13.13 g/L at the 60th hour. Considering the glucose metabolism of yeast, it was interpreted that sugar conversion to ethanol was achieved. Under conditions where the dissolved oxygen concentration in the medium is low and the substrate concentration is high, *K. lactis* and *K. marxianus* are capable of producing ethanol (Ornelas *et al*., 2008). As shown in Table 1, the ethanol concentration in the fermentation liquid medium reached its highest concentration (18.44 g/L) at the 36th hour; however, after this time, the ethanol in the medium gradually decreased. In this case, it is foreseen that previously produced ethanol was used instead of the lactose depleted in the medium, leading to reduced the ethanol in the medium.

Although glucose, galactose, and lactose were initially present in the medium fermented with *K. marxianus*, the lactose was used after near depletion of glucose and galactose after the start of the fermentation. It shows that lactose can induce the metabolism of *K. marxianus* (Cheng *et al*., 2006).

Many studies have been conducted on ethanol production from *K. marxianus* using different substrates under different production conditions. In this study, 18.44 g/L ethanol was obtained by *K. marxianus* using lactose as substrate. These results are similar to the studies presented so far (Zafar and

Owai, 2006; Ozmihci and Kargi, 2007; Limtong *et al*., 2007; Sansonetti *et al*., 2011; Garcia-Aparicio *et al*., 2011; Goshima *et al*., 2013; Hadiyanto *et al*., 2014; Gao *et al*., 2015; Galindo-Leva *et al.*, 2016; Sayed *et al*., 2018). According to the data obtained, whey powder and synthetic medium can be an attractive substrate for ethanol production, and therefore the problem of environmental pollution caused by waste whey can be solved (Das *et al*., 2016).

Conclusion

In the present work, the production of β-galactosidase was optimised using *K. marxianus*, a natural isolate, and the change in some fermentation metabolites during fermentation, giving optimum parameters for β-galactosidase production, was examined. The highest specific enzyme activity was found to be at 47.31 U/mL at the end of 48 hour-incubation at 200 rpm, pH 8.0, and 30°C with 2% (v/v) inoculation rate. Different optimum conditions have been reported in various studies due to factors such as microbial differences and various fermentation conditions. Aerobically assimilating yeasts are common, and fermenters such as *K. lactis*, *K. marxianus*, and *Candida pseudotropicalis* are quite rare. Fermentation of whey lactose to ethanol, especially using yeast, has been frequently mentioned in several publications. Ethanol fermentation may be an alternative to the bio-modification of permeate, the impurity remaining after separation of whey proteins. Examining the fermentation metabolites revealed essential findings. The research findings presented herein contributed to the literature. This could also lead to the investigation of different optimisation conditions to increase the production of both ethanol and fermentation metabolites**.** The enzymes obtained from microbial sources reduce production costs and provide higher efficiency as compared to animal and plant sources. Commercially produced enzymes are derived from safe sources, particularly yeasts (*K. marxianus*, *K. lactis*, and *K. fragilis*) and moulds (*A. niger* and *A. oryzae*). Yeasts have been viewed as a major source of β-galactosidases from a commercial aspect. Considering the high level of β-galactosidase production, *K. marxianus*, a natural yeast, can be used as a potentially useful industrial strain for β-galactosidase production.

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