

Determination of Proper Conditions for the Production of Crude Beta-galactosidase Using *Lactobacillus delbrueckii* ssp. *bulgaricus*.

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ABSTRACT

Proper conditions for producing crude beta-galactosidase from waste materials were determined. This enzyme is to be used in the production of lactose-hydrolyzed milk. Whey permeate was used as a basic medium. Twenty seven treatments were developed by 3 varying factors of: yeast extract (1, 2, and 3 %), wheat steep liquor (1, 2, and 3 %), and whey powder (0.5, 1, and 1.5 %). Crude enzyme extract was obtained by sonication of the cells collected from cultivation of *Lactobacillus delbrueckii* ssp. *bulgaricus* in various media at 43°C. The beta-galactosidase activity was assessed using Ortho-Nitro-Phenyl-beta-D-galactopyranoside (ONPG). Yeast extract and whey powder had both significant effects ($P < 0.01$), while wheat steep liquor proved to be ineffective. Yeast extract had the most pronounced effect on the production of beta-galactosidase. The effect of the interactions of yeast extract-whey powder and wheat steep liquor-whey powder were significant at 5 % level ($P < 0.05$), while the effect of the interaction of yeast extract-wheat steep liquor was significant at 1% level ($P < 0.01$). Interaction effect of the 3 factors on the production of beta-galactosidase was significant ($P < 0.01$). The best combination for production of beta-galactosidase (4.924 U ml^{-1}) was 3% yeast extract, 1.5% whey powder and 2% wheat steep liquor.

Keywords: Beta-galactosidase, Crude enzymatic extract, *Lactobacillus bulgaricus*, Lactose hydrolysis, Wheat steep liquor, Whey permeate.

INTRODUCTION

Lactose is a disaccharide found in mammalian's milk. Cow's milk and its various products form a part of man's main daily food. Therefore, lactose forms a main part of one's daily intake of carbohydrates. Beta-galactosidase hydrolyzes lactose into glucose and galactose, so it is commercially referred to as lactase [13]. The main industrial application of beta-galactosidase is converting lactose to glucose and galactose. There are several advantages embodied in lactose hydrolysis: (1) rapid fermentation of glucose, (2) a higher degree of sweetness of the liquid in which lactose has been hydrolyzed, (3) higher solubility of glucose and

galactose, (4) higher stability of frozen condensed milk, in which lactose has been hydrolyzed, (5) application of lactose-hydrolyzed milk in cheese making results in rapid fall of pH and as a consequence rapid development of cheese flavor and texture takes place [14, 19], and (6) use of beta-galactosidase in whey eliminates technological problems (such as sandiness in whey powder and ice cream) improving the nutritional quality of whey and whey powder. It also leads to the development of novel products and the production of new sweeteners [7]. Some people cannot tolerate and digest lactose due to a lack of beta-galactosidase in their intestines. A consumption of milk and dairy products by these people leads to cramp, flatulence, vomiting, etc [9]. So one

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valuable source of nutrition would be unavailable for more than half of the people in the world due to lactose intolerance [17]. Since lactose intolerance is affecting a large proportion of the people (up to 50 million in USA), a cheap source of beta-galactosidase for the effective production of lactose-hydrolyzed dairy products is of a substantial potential [1].

Optimum pH and temperature for beta-galactosidase production from *Streptococcus thermophilus* were 6.6-7, and 57°C respectively [11]. Maximum activity of beta-galactosidase from *Streptococcus thermophilus* was achieved in the presence of K⁺, Mn⁺⁺ ions [4]. Effective factors in producing beta-galactosidase from *Streptococcus cremoris* in whey were 36 hours incubating at 30°C and the best nutrient complements for producing the enzyme were yeast and meat extracts [15]. Vasiljevic and Jelen (2001) reported that the highest activity of beta-galactosidase was obtained when NH₄OH was used for adjusting pH of the medium in contrast with NaOH and KOH.

Lactic acid bacteria are generally recognized as safe, so their enzymes can be used directly in foodstuff without any need for purification [17]. Crude enzyme extract of thermophilic bacteria can be used in milk at high temperatures without causing any complications [17, 18].

Half life time of beta-galactosidase obtained from thermophilic bacteria is relatively high. Half life time of beta-galactosidase from *Streptococcus salivarius* ssp. *thermophilus* is about 146 minutes at 60°C [2].

In the present research, *Lactobacillus delbrueckii* ssp. *bulgaricus* has been used to obtain beta-galactosidase. This thermophilic bacterium, is widely found in yoghurt along with *Streptococcus thermophilus* (almost 3.2×10^8 cfu ml⁻¹ at pH= 3.9) [3]. So, the obtained beta-galactosidase can be directly used in milk without any need for purification.

The effects of yeast extract, whey powder, and wheat steep liquor as well as their inter-

actions on production of beta-galactosidase were evaluated.

MATERIALS AND METHODS

Materials

Lactobacillus delbrueckii ssp. *bulgaricus* (CHR Hansen Lb-12) which is a thermophilic strain of common yoghurt microorganisms was obtained from Hansen Company (Denmark). Whey permeate was obtained from Pegah Dairy Plant, Shiraz, Iran. Wheat steep liquor was prepared in laboratory. Skim milk powder and sweet whey powder were obtained from Ramak Dairy Plant, Shiraz, Iran. Orthonitrophenyl beta-D-galactopyranoside (ONPG) was purchased from sigma (N1127-5G, 120K5307, Sigma-Aldrich, Canada). KM medium which is a suitable medium for growing lactic acid bacteria was prepared according to the method of Schwab *et al.* (1984). KM medium contains 1% skim milk powder, 0.5% glucose and 2.5% yeast extract. All other analytical grade chemicals were obtained from Merck Company.

Methods

In order to determine proper conditions for production of beta-galactosidase by *Lactobacillus delbrueckii* ssp. *bulgaricus*, whey permeate was used as a basic medium. Lactose, protein, fat and total solid contents of whey permeate were determined according to the methods of Michel *et al.* (8), AOAC, Gerber and dry oven drying at 105±2°C, respectively.

Experimental Design

The effects and interaction effects of three nutrients on the production of beta-galactosidase were investigated. Each nutrient was applied in three different proportions. The three nutrients were: yeast extract (1, 2 and 3% W/V), sweet whey powder

(0.5, 1 and 1.5% W/V) and wheat steep liquor (1, 2 and 3% V/V). All experiments were carried out according to a completely randomized factorial design. Three factors with 3 levels of each, in total 27 treatments obtained were evaluated in 4 replicates.

Below is a presentation of the experimental design:

YE= Yeast extract WSL= Wheat steep liquor WP= Whey powder

YE 1= 1% WSL 1= 1% WP 1= 0.5% YE 2= 2% WSL 2= 2% WP 2= 1%

YE 3= 3% WSL 3= 3% WP 3= 1.5%

1= YE 1 WSL 1 WP 1 2= YE 1 WSL 1 WP 2 3= YE 1 WSL 1 WP 3

4= YE 1 WSL 2 WP 1 5= YE 1 WSL 2 WP 2 6= YE 1 WSL 2 WP 3

7= YE 1 WSL 3 WP 1 8= YE 1 WSL 3 WP 2 9= YE 1 WSL 3 WP 3

10= YE 2 WSL 1 WP 1 11= YE 2 WSL 1 WP 2 12= YE 2 WSL 1 WP 3

13= YE 2 WSL 2 WP 1 14= YE 2 WSL 2 WP 2 15= YE 2 WSL 2 WP 3

16= YE 2 WSL 3 WP 1 17= YE 2 WSL 3 WP 2 18= YE 2 WSL 3 WP 3

19= YE 3 WSL 1 WP 1 20= YE 3 WSL 1 WP 2 21= YE 3 WSL 1 WP 3

22= YE 3 WSL 2 WP 1 23= YE 3 WSL 2 WP 2 24= YE 3 WSL 2 WP 3

25= YE 3 WSL 3 WP 1 26= YE 3 WSL 3 WP 2 27= YE 3 WSL 3 WP 3

Preparations of Growth Medium

Exact amounts of the three nutrients were added to 50 ml of whey permeate to develop a medium for each treatment. After mild heating and making nutrient agents soluble in whey permeate, pH was adjusted to 6.8 by phosphate buffer (pH= 12, 0.15 M). The developed medium was then sterilized at 121°C for 15 minutes. It was immediately used after being cooled.

Propagation of Organism

Lactobacillus delbrueckii ssp. *bulgaricus* was propagated daily in sterile KM culture.

Preparation of Enzyme

Approximately 1 ml of propagated *Lactobacillus delbrueckii* ssp. *bulgaricus* was used to inoculate 50 ml of developed medium to be then incubated at 43°C for 12-13 hours. Microorganisms were then collected by centrifugation (Sorvall Rc-5 super speed refrigerated centrifuge, USA) of the medium at 5°C and 4000g for 10 minutes. Forty ml phosphate buffer (pH= 7.2, 0.15 M) was added to the precipitant. Bacterial cells were lysed by ultra sonication (Schoeller and Co Frankfurt am main-sud TG125) at 65% intensity for 10 minutes. Samples were cooled using ice water bath to prevent loss of activity during sonification. The obtained extract was called crude enzyme extract (CEE). Enzyme activity was determined immediately after sonication according to the method of Hestrin (1976). According to this method, 0.1 ml of CEE was diluted by 3 ml of phosphate buffer (pH=7.2, 0.15 M), then 1 ml of ONPG solution (M=0.01) was added to the tubes. Enzymatic reactions were immediately stopped by adding 1 ml of NaCO₃ 1M after 5 minutes and at 40°C. The concentration of orthonitrophenyl (ONP) released was determined from the absorbance at 420 nm, using the standard calibration curve (Jenway 6405 UV/VIS Spectrophotometer).

A unit of activity was defined as the amount of the enzyme, which could hydrolyze 1 μ mole of ONPG in 1 minute. Enzyme unit activity was calculated per 1 ml of growth medium. All experiments and analyses were carried out in duplicate. Statistical significance of differences was determined by ANOVA and *F-test* wherever appropriate. All given values were means of 4 replicates. Statistical analyses were done through Mstatc software.

RESULTS AND DISCUSSION

Lactobacillus delbrueckii ssp. *bulgaricus* is one of the best bacteria which can grow well in whey permeate [10]. Whey permeate ingredients were as follows: protein 0.35% (w/v), lactose 4.9% (w/v), fat 0% (w/v), and dry material 5.5% (w/v).

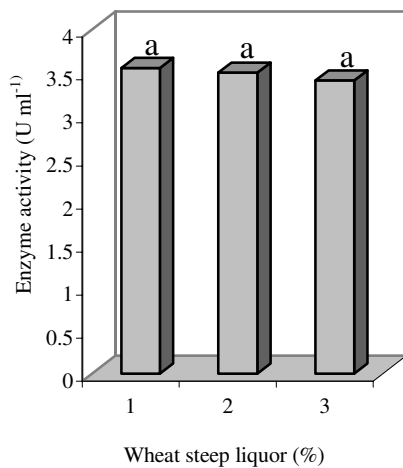


Figure 1. Effect of different amounts of yeast extract on CEE production.

Effects and interaction effects of yeast extract, wheat steep liquor and sweet whey powder on production of CEE from *Lactobacillus delbrueckii* ssp. *bulgaricus* were inspected.

According to ANOVA, the effects of different amounts of sweet whey powder and yeast extracts were significant ($P < 0.01$), while the effect of different amounts of wheat steep liquor were not significant ($P < 0.05$). The interaction effects of YE-SWP-WSL were significant ($P < 0.05$).

As illustrated in Figure 1, 3% yeast extract had the highest effect on producing CEE. Its effect decreased as its concentration depleted in growth media. Thomas *et al.* (1984) showed that 0.5% yeast extract had the most desirable effect on producing beta-galactosidase. They opined that the effect of lactose on producing beta galactosidase from *Kluyveromyces fragilis* was more significant than yeast extract and K_2HPO_4 . They could obtain 35 unit activity of enzyme by using 12.5% lactose. In the present research, whey permeate (as a basic medium) and sweet whey powder (as a nutrient) were used which contained around 5 and 70% of lactose, respectively. Around 6% lactose was obtained by using 1.5% sweet whey powder in growth media in the present research. So, it is rea-

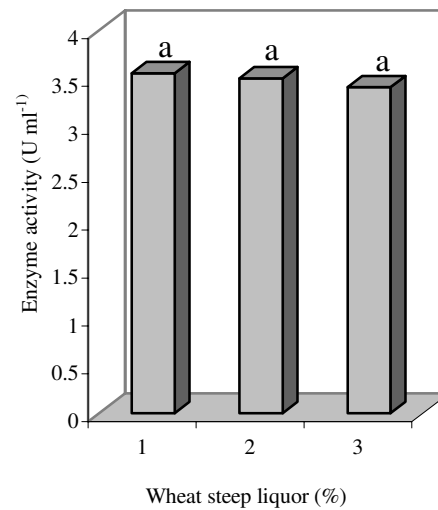


Figure 2. Effect of different amounts of wheat steep liquor on CEE production.

sonable that they got to 35 unit activity per 1 ml of media. It seems that if sweet whey powder content in medium is increased, one can obtain more CEE.

Wheat steep liquor had no positive effect on producing CEE (Figure 2.). Greenberg and Mahoney [4] used corn steep liquor as growth factor and reported that it had the highest ef-

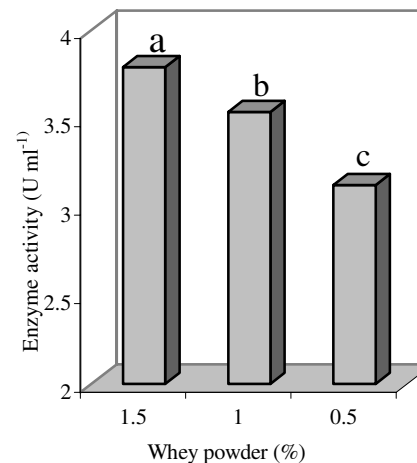


Figure 3. Effect of different amounts of whey powder on CEE production.

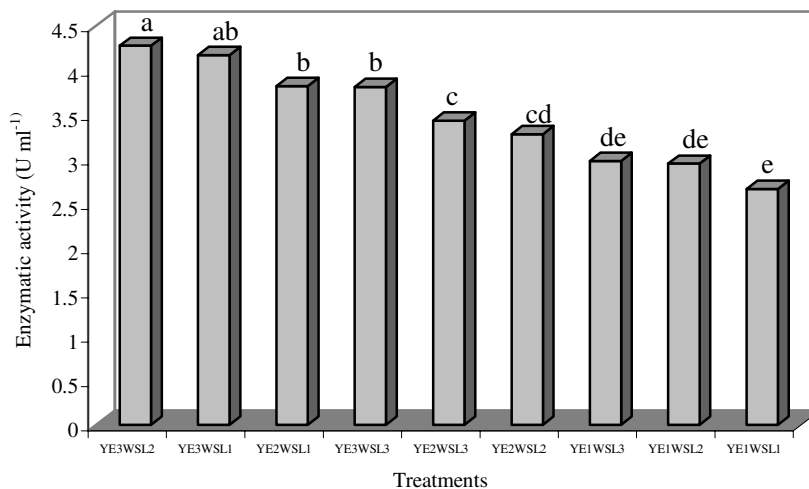


Figure 4. Interaction effect of yeast extract and wheat steep liquor on CEE production.

fect on producing beta-galactosidase. Wheat steep liquor was used in our work because we have plenty of it in our food industry units which produce starch from wheat. But, it was ineffective.

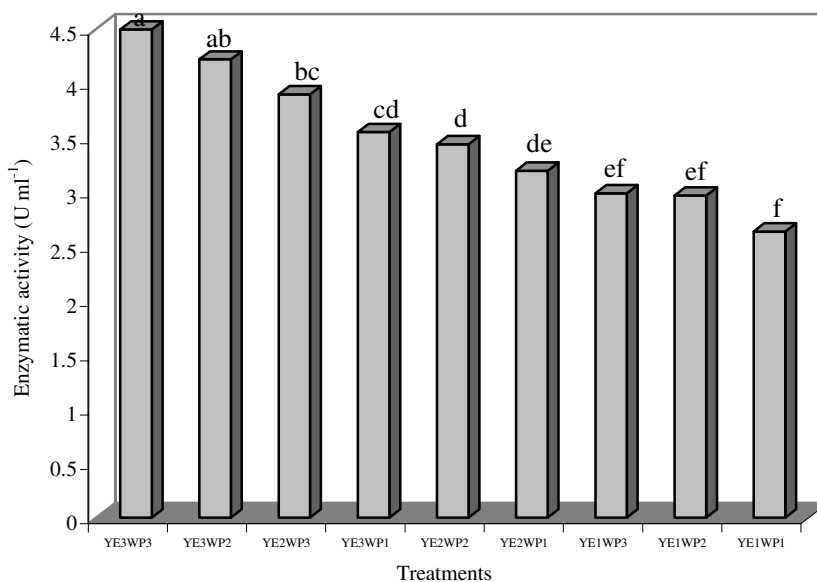
Since sweet whey powder contains a high amount of lactose (70%), its effect was significant (Figure 3).

Below is a model which illustrates correlation between yeast extract and sweet whey

powder. One can obtain more reliable multiple regression if wheat steep liquor eliminated.

$$\text{Enzyme activity} = 1.837 + 0.735 \text{ YE} + 0.397 \text{ SWP}, R^2 = 0.672$$

Interaction effects of YE-WSL, YE-SWP, WSL-SWP, and WSL-SWP-YE are shown in Figures 4, 5, 6, and 7 respectively. These figures approve the effect of each nutrient individually. WSL had a somehow preventive



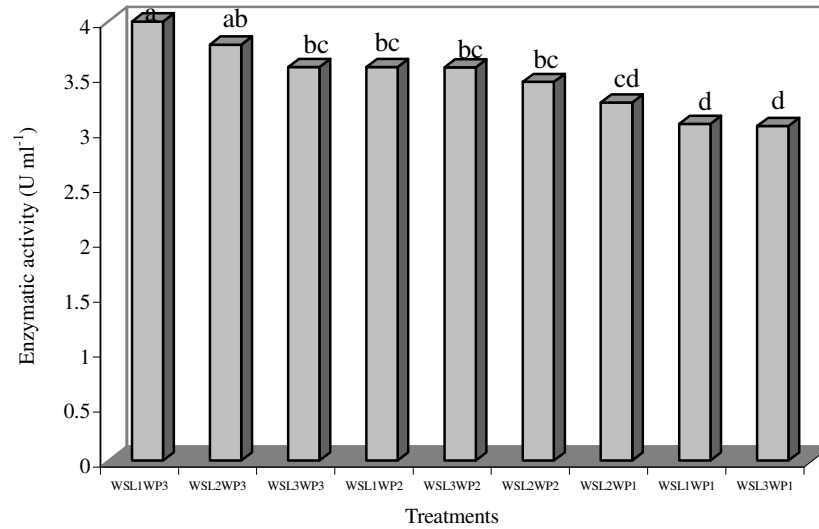


Figure 6. Interaction effect of wheat steep liquor and whey powder on CEE production.

effect on the growth of *Lactobacillus delbrueckii* ssp. *bulgaricus*. So corn steep liquor is suggested to be used instead of wheat steep liquor.

Figures 1, 2, 3, 4, 5, 6, and 7 show the effects of different amounts of nutrients on CEE production. As shown in Figure 7, the

best conditions for producing CEE were 3% YE, 1.5% SWP and 2% WSL. By applying this treatment, we obtained 4.924 unit activity of CEE per 1 ml of growth medium. Vasiljevic and Jelen (2001) obtained one unit activity of CEE per one ml of medium by applying whey permeate along with 1.2% MRS

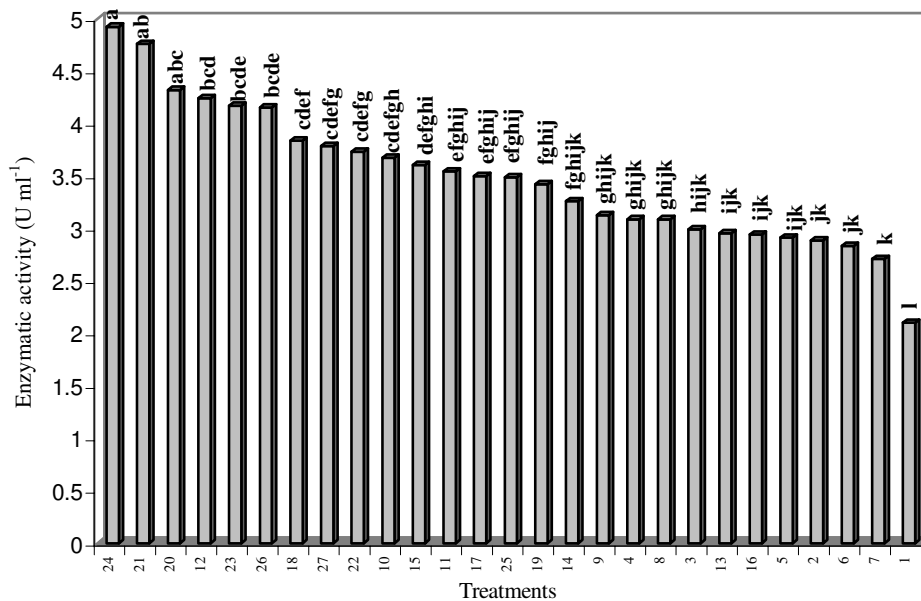


Figure 7. Interaction effects of yeast extract, whey powder, and wheat steep liquor on CEE production.

culture. They reported that 5.491 unit activity was obtained when they used skim milk as growth medium. It seems that the effect of applying 3% YE, 2% WSL and 1.5% SWP as in the present research was much more significantly pronounced than 1.2% MRS. In the present research, 5.87 unit activity of CEE was obtained by using skim milk as growth medium.

So the effect of any ingredients in growth medium must be taken into consideration. Phosphate buffer (pH=12, 0.15 M) was used to adjust the pH of the growth medium in the present research. Vasiljevic and Jelen (2002) showed that the best agent for adjusting pH was NH₄OH. They obtained the highest CEE (118.47±14.97 unit activity per gram of milled bacteria) by using NH₄OH, and bead milling as the method for breaking bacterial cell walls.

Whey or whey permeate without an addition of nutrients can not be a suitable medium for growing lactic acid bacteria. Sridhar and Dutta [15] used fresh deproteinized whey as a medium for growing *Streptococcus cremoris*, and obtained 2.892 unit activity of enzyme per ml of medium which is relatively low. By using yeast extract and sweet whey powder results of more interest might be obtained.

Jokar and Karbassi (2003) used CEE for producing lactose-hydrolyzed milk. They could hydrolyze nearly 80% lactose content of milk without any serious problems confronted with. They used 2% CEE and kept the milk in 52°C for 6 hours. They used high temperature to prevent proteinases from CEE. Quality of lactose-hydrolyzed milk was acceptable [6].

CONCLUSIONS

Lactobacillus delbrueckii ssp. *bulgaricus* is an appropriate bacterium for producing CEE and applying it in milk to produce lactose-hydrolysed milk. The highest amount of CEE (4.9 unit activity) can be obtained by adding 3%YE, 2%WSL and 1.5%SWP to whey permeate, which is a waste and cheap material of dairy plants. The effects of YE and SWP on the production of CEE were signifi-

cant (P< 0.01). Lactose-hydrolyzed milk which was produced by CEE was of an acceptable quality.

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تعیین شرایط مناسب تولید آنزیم بتا-گالاکتوسیداز ناخالص با استفاده از *Lactobacillus delbrueckii ssp. bulgaricus*

۱. جوکار و ا. کرباسی

چکیده

در این تحقیق شرایط بهینه تولید آنزیم بتا-گالاکتوسیداز ناخالص توسط *Lactobacillus bulgaricus* با استفاده از مواد زائد تعیین شد. این آنزیم در تولید شیر با لاکتوز هیدرولیز شده کاربرد دارد. تراوه شیر فرآیند به عنوان محیط پایه در نظر گرفته شد. با استفاده از مقادیر مختلف عصاره مخمر (۱، ۲، و ۳ درصد)، پودر آب پنیر شیرین (۱/۵، ۱، و ۱/۵ درصد)، و محلول حاصل از نرم شدن گندم (۱، ۲، و ۳ درصد) ۲۷ تیمار مختلف به دست آمد. باکتری *Lactobacillus bulgaricus* در محیطهای مختلف به دست آمده در دمای 43° سلسیوس به مدت ۱۲ ساعت رشد کرده و سپس با شکستن دیواره سلولی آنها با استفاده از امواج مافوق صوت آنزیم ناخالص به دست آمد. با به کار گرفتن ارتونیترونیل بتا دی گالاکتوپیرانوساید، فعالیت آنزیمی تعیین شد. تأثیر عصاره مخمر و پودر آب پنیر قابل ملاحظه بود ($P < 0.01$)، در حالی که محلول حاصل از نرم شدن گندم تأثیری بر تولید آنزیم نداشت. بیشترین تأثیر بر تولید آنزیم مربوط به عصاره مخمر بود. آثار متقابل عصاره مخمر-پودر آب پنیر، محلول حاصل از نرم شدن گندم-پودر آب پنیر، و عصاره مخمر-محلول حاصل از نرم شدن گندم به ترتیب در سطح ۵ و ۱ درصد قابل ملاحظه بود. تأثیر متقابل هر ۳ فاکتور عصاره مخمر-محلول حاصل از نرم شدن گندم-پودر آب پنیر قابل ملاحظه بود ($P < 0.01$). با به کار گرفتن ۳ درصد عصاره مخمر، ۱/۵ درصد پودر آب پنیر، و ۲ درصد محلول حاصل از نرم شدن گندم بالاترین فعالیت آنزیمی ($4/924 \text{ U ml}^{-1}$) به دست آمد.