

## **Determination of Proper Conditions for The Production of Crude Beta-galactosidase**

### **Using *Lactobacillus delbrueckii* ssp. *bulgaricus*.**

A. JOKAR\* and A. KARBASSI

Dept. of Food Science, Agricultural Collage, Shiraz University, Iran.

### **ABSTRACT**

In this research, 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 factors as follows: 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 measured using Ortho-Nitro-Phenyl-beta-D-galactopyranoside (ONPG). Both yeast extract and whey powder had significant effect ( $p < 0.01$ ), while wheat steep liquor proved to be ineffective. Yeast extract had the highest 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 condition for production beta-galactosidase (4.924 U/ml) was 3% yeast extract, 1.5 % whey powder and 2% wheat steep liquor.

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

\* Marvdasht Azad University E-mail: [akbarjokar121@yahoo.com](mailto:akbarjokar121@yahoo.com)

## 1. INTRODUCTION

Lactose is a disaccharide, which is found in mammalian's milk. Cow's milk and various products, which are produced from, are parts of the men's main foods. Therefore, lactose is the main part of the daily intake carbohydrates. Beta-galactosidase hydrolyzes lactose into glucose and galactose, so it is commercially called lactase (13). The main industrial application of beta-galactosidase is converting lactose to glucose and galactose. Lactose hydrolysis has several advantages: 1) rapid fermentation of glucose 2) higher 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 in consequence rapid development of cheese flavor and texture (14,19). 6) using beta-galactosidase in whey eliminates technological problems (such as sandiness in whey powder and ice cream) and improves 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 the lack of beta-galactosidase in their intestine. Consuming milk and dairy products by these people leads to cramp, flatulence, vomiting, etc (9). So one valuable source of food 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 portion of the people (up to 50 million in USA), a cheap source of beta-galactosidase for effective production of lactose-hydrolyzed dairy products has a substantial potential (1).

Optimum pH and temperature for beta-galactosidase from *Streptococcus thermophilus* were 6.6-7, and 57 °C respectively (11). Maximum activity of beta-galactosidase from *Streptococcus thermophilus* achieved in the presence of K<sup>+</sup>, Mn<sup>++</sup> ions (4). Effective factors in producing beta-galactosidase from *Streptococcus cremoris* in whey were 36 hours incubating in 30 °C and the best nutrient complements for producing the enzyme were yeast and meat extracts (15). Vasiljevic and Jelen reported that the highest activity of beta-galactosidase obtained when NH<sub>4</sub>OH was used for adjusting pH of the medium in comparison with NaOH and KOH(17).

Lactic acid bacteria are generally recognized as safe, so their enzymes can be used directly in foods without any purification (17). Crude enzyme extract of thermophilic bacteria can be used in milk at high temperatures without causing any problems (17, 18).

Half life time of beta-galactosidase which 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 present research, we have used *Lactobacillus delbrueckii* ssp. *bulgaricus*, to obtain beta-galactosidase. This thermophilic bacterium, is widely found in yoghurt along with *Streptococcus thermophilus* (almost  $3.2 \times 10^8$  cfu/ml at pH = 3.9) (3). So obtained beta-galactosidase can be used directly in milk without any purification.

The effects of yeast extract, whey powder, and wheat steep liquor and their interactions on production of beta-galactosidase were evaluated.

## 2. MATERIAL AND METHODS

### 2.1. 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 and others (12). KM culture contains 1% skim milk powder, 0.5% glucose and 2.5% yeast extract. All other analytical grade chemicals obtained from Merck Company.

### 2.2. 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 at  $105 \pm 2$  °C, respectively.

### 2.3. 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 amounts. 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 done according to a completely randomized factorial design. We had 3 factors with 3 levels of each one, so 27 treatments obtained and were evaluated in 4 replicates.

Here is an explanation of 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

## 2.4. PREPERATION OF GROWTH MEDIUM

Exact amounts of three nutrients were added to 50 ml of whey permeate in order 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). Then, the developed medium was sterilized at 121 °C for 15 minutes, after cooling it was immediately used.

## 2.5. PROPAGATION OF ORGANISM

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

## 2.6. PREPARATION OF ENZYME

Approximately 1 ml of propagated *Lactobacillus delbrueckii* ssp. *bulgaricus* was used to inoculate 50 ml of developed medium and incubated at 43 °C for 12-13 hours. Then, microorganisms were collected by centrifugation (Sorvall Rc- 5 super speed refrigerated centrifuge USA) of the medium at 5 °C and 4000 × g for 10 minutes. Forty ml phosphate buffer (pH = 7.2, 0.15 M) was added to the precipitant. Bacterial cell were lysed by ultra sonication (Schoeller & Co Frankfurt am main-sud TG125) at 65% intensity for 10 minutes. Samples were cooled using ice water bath to prevent activity loss during sonification. Obtained extract was called crude enzyme extract (CEE). Enzyme activity was determined immediately after sonication according to the method of Hestrin (5). According to this method, 0.1 ml CEE diluted by 3 ml 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 NaCO<sub>3</sub> 1M after 5 minutes 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 hydrolyze 1 μmole 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 where appropriate. All given values were means of 4 replicates. Statistical analyses were done by Mstac software.

## 3. RESULTS AND DISCCUSION

*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).

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 ( $P < 0.01$ ), but 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 it is 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 and others (16) showed that 0.5% yeast extract had the best effect on producing beta-galactosidase. They announced 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 present research, whey permeate (as a basic medium) and sweet whey powder (as a nutrient) were used which had around 5 and 70% lactose content, respectively. We obtained around 6% lactose by using 1.5% sweet whey powder in growth media. So, it is reasonable that they got to 35 unit activity per 1 ml of media. It seems If we increase sweet whey powder content in medium, we 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 announced that it had the highest effect on producing beta-galactosidase. We used wheat steep liquor because we had plenty of it in our companies which produce starch just by wheat. But, it was ineffective. Since sweet whey powder has high amount of lactose (70%), its effect was significant (Figure 3).

Here is a model which illustrates correlation between yeast extract and sweet whey powder. We can obtain more reliable multiple regression If we eliminate wheat steep liquor.

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

Interaction effects of YE-WSL, YE-SWP, WSL-SWP, and WSL-SWP-YE are shown in Figures 4, 5, 6, and 7. These Figures approve the effect of each nutrient individually. WSL had somehow prevention effect on the growth of *Lactobacillus delbrueckii* ssp. *bulgaricus*. So we suggest corn steep liquor instead of wheat steep liquor.

Figures 1, 2, 3, 4, 5, 6, and 7 show the effects of different amounts of nutrients on producing CEE. As It has been 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 (17) obtained one unit activity of CEE per one ml of medium by applying whey permeate with 1.2% MRS culture. They announced 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 in present research was much more significant than 1.2% MRS. In 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 present research. Vasiljevic and Jelen (18) showed that the best agent for adjusting pH was  $NH_4OH$ . They obtained the highest amount of CEE ( $118.47 \pm 14.97$  unit activity per gram of milled bacteria) by using  $NH_4OH$ , and bead milling as a method for breaking bacterial cell walls.

Whey or whey permeate without adding 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 more interesting results might be obtained.

Jokar and Karbassi used CEE for producing lactose-hydrolyzed milk. They could hydrolyze nearly 80% lactose content of milk without serious problems. 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).

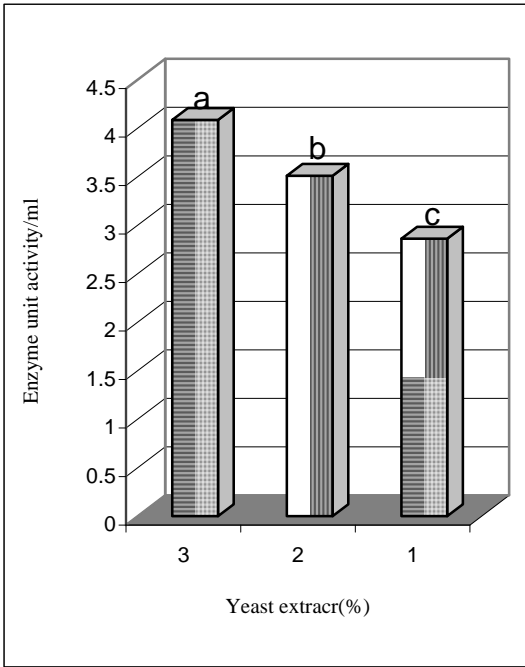
#### 4. CONCLUSIONS

*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 in dairy plants. The effects of YE and SWP on the production of CEE were significant ( $p < 0.01$ ). Lactose-hydrolyzed milk which was produced by CEE had acceptable quality.

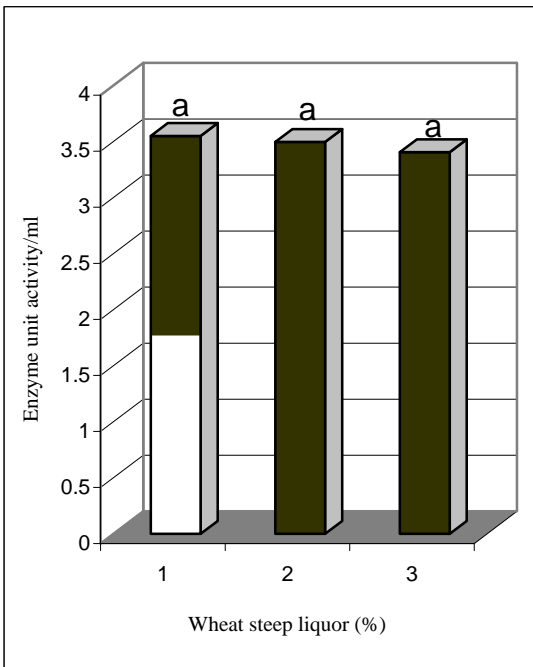
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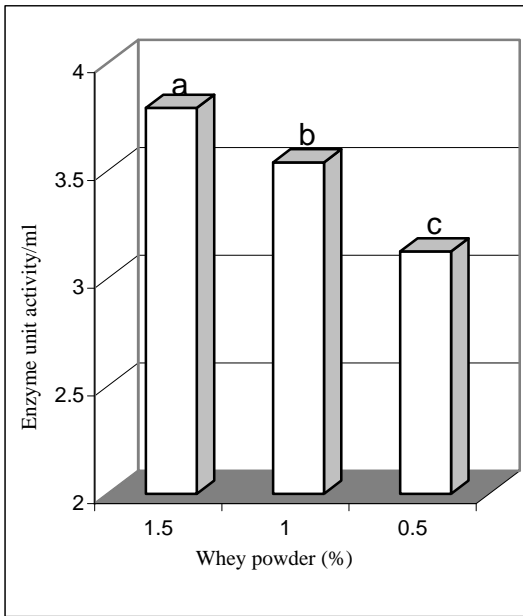
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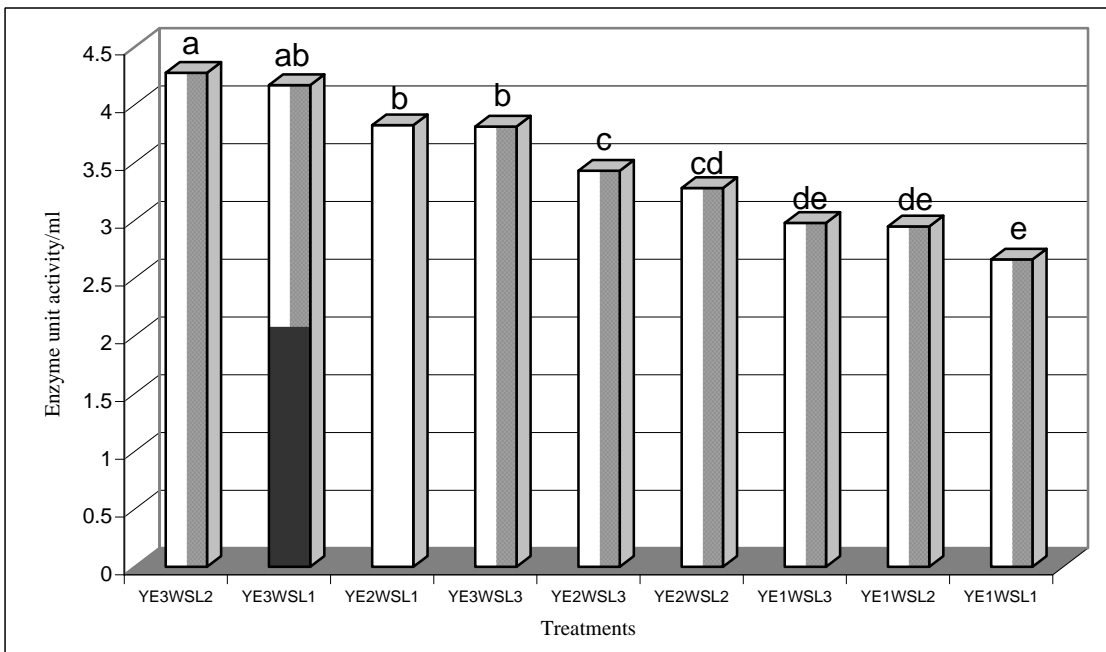
**Figure 1:** Effect of different amounts of yeast extract on producing CEE



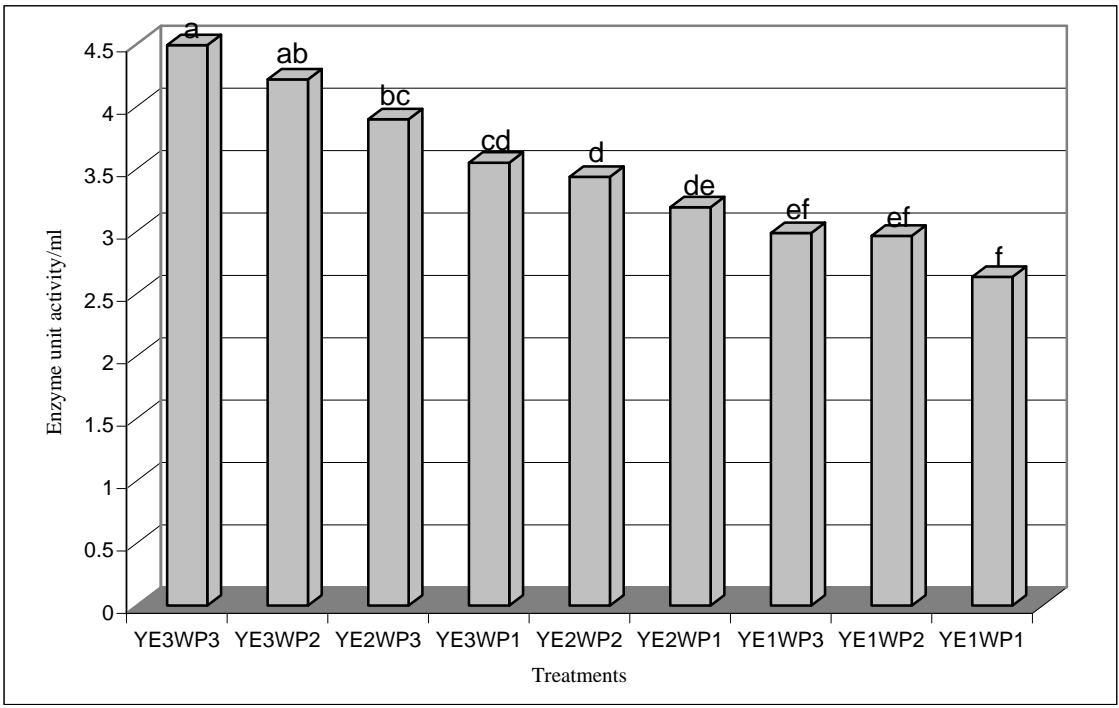
**Figure 2:** Effect of different amounts of wheat steep liquor on producing CEE



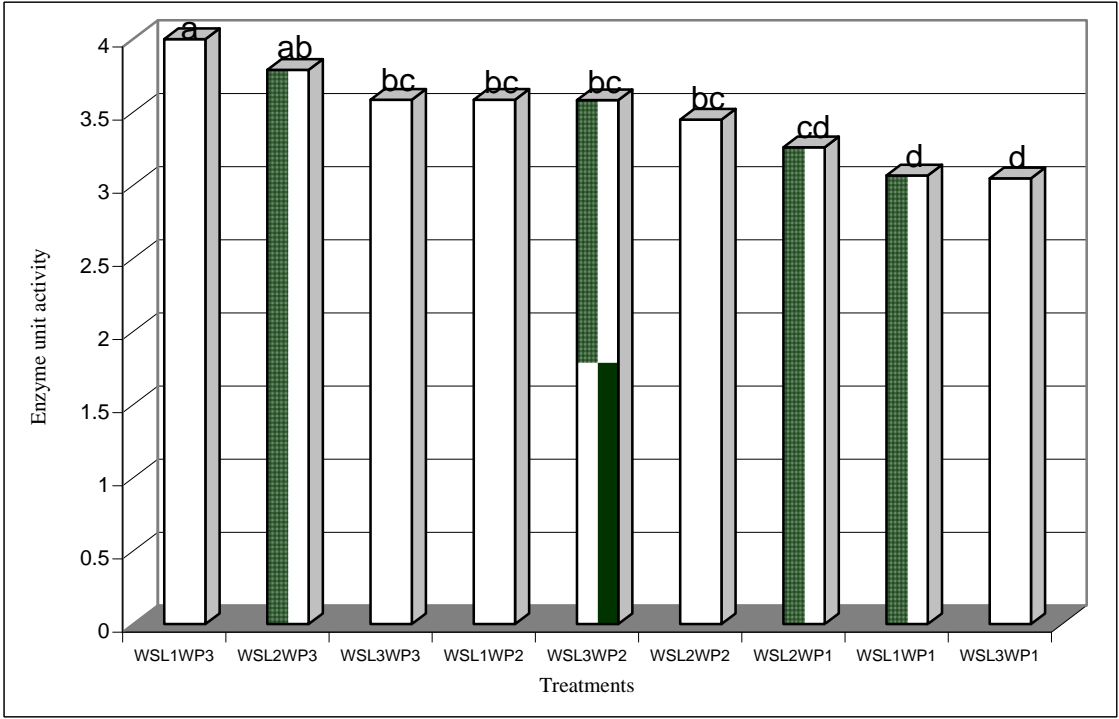
**Figure 3:** Effect of different amounts of whey powder on producing CEE



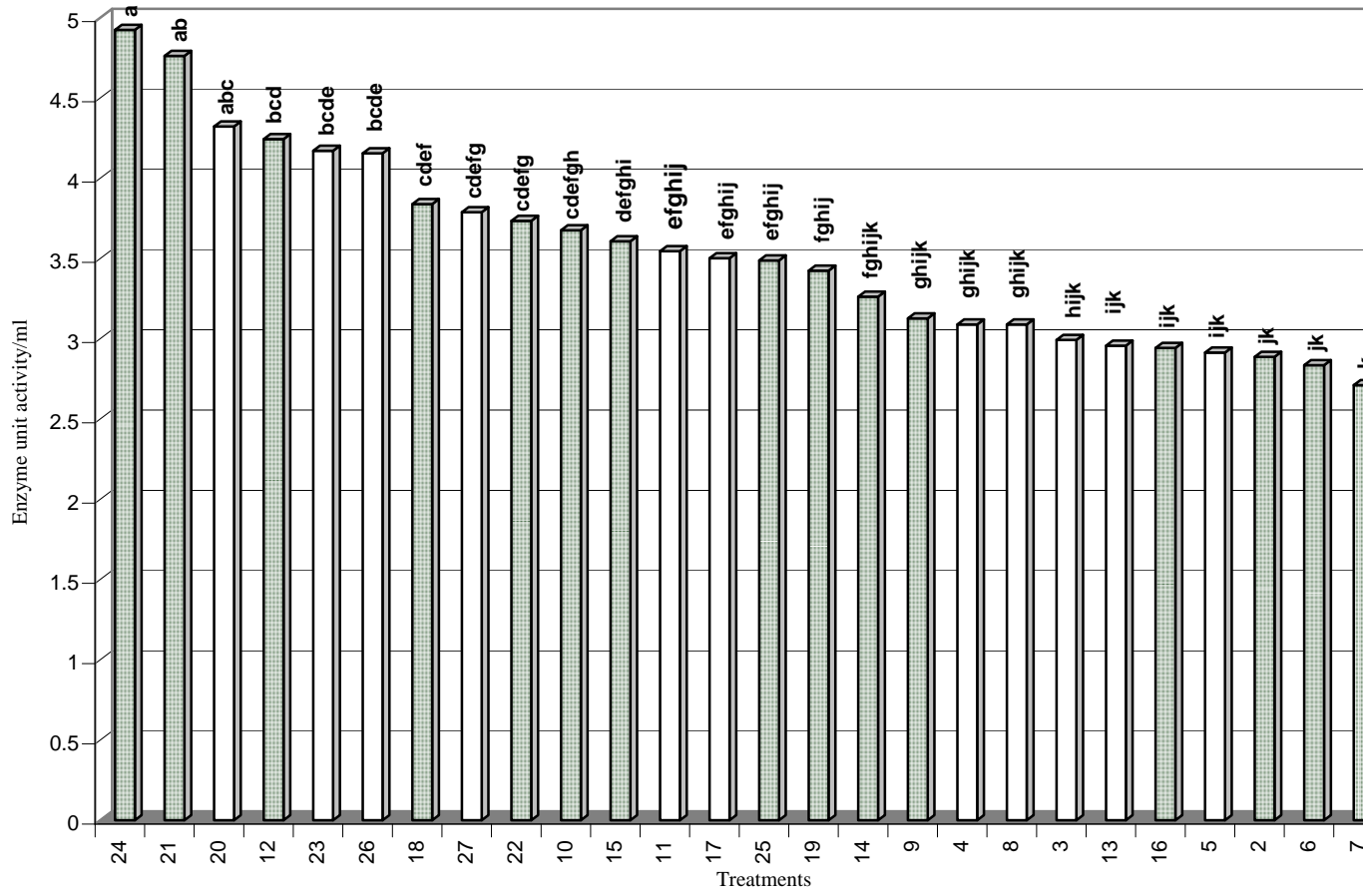
**Figure 4:** Interaction effect of yeast extract and wheat steep liquor on producing CEE



**Figure 5:** Interaction effect of yeast extract and whey powder on producing CEE



**Figure 6:** Interaction effect of wheat steep liquor and whey powder on producing CEE



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