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RESEARCH ARTICLE

OPTIMIZATION CONDITIONS FOR THE PRODUCTION OF MICROBIAL TRANSGLUTAMINASE FROM A NEWLY ISOLATED STRAIN OF STREPTOMYCES SP.

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Abstract

The aim of the present study is to optimize the conditions for production microbial transglutaminase (MTGase) from Streptomyces diastaticus strain isolated from soil. The fermentation medium composition and the environmental conditions used for cell growth and enzyme production may be quite different. Therefore, it is important to optimize these parameters for the isolated strain.

Three different fermentation media were tested to study the effect of the carbon sources on the enzyme production .It was found that the starch free medium is preferred than the other two media used (basal and glycerol containing media).

The results indicated that the optimum conditions for the enzyme production were: the inoculum size of 8% of fermentation medium at temperature of 30ºC, pH 7.5 .The medium volumes was 100ml in fermentation flask of 600ml at 200 rpm.

Introduction:-

Transglutaminase (EC 2.3.2.13) is an enzyme that catalyses an acyl-transfer reaction between the γ-carboxyamide group of a peptide-bound glutamine residues (acyl donors) and a variety of primary amines (acyl acceptors).

The fermentation media composition and the environmental conditions (include: pH, temperature, aeration and agitation) are important factors affecting microbial transglutaminase (MTGase) production. The optimal conditions used for cell growth and enzyme production may be quite different. Therefore, it is important to optimize these parameters to enhance the rate, activity, yield and productivity of the enzyme (Zheng et al., 2001, 2002; Yan et al., 2005).

Fermentation media of MTGase can represent almost 30% of the cost of microbial fermentation process. Starch was the main carbon source used. It ranged between 5 and 30 g/ l (Cui et al., 2007, 2008). Some media were free of starch (Ho et al., 2000; de Souza et al., 2009, 2011). These media contained other carbon sources, mainly glycerol. Zhang et al. (2008, b) used 20 gl^{-1} of dextrin instead of starch.

For batch MTGase production, it is very important to control cell growth and product formation process under appropriate conditions during MTGase fermentation.

The early studies carried out for the production of MTGase used very small volume of cultivated seeding broth compared with that of the fresh fermentation medium. The minimum inoculum size used was that of Gerber et al.

(1994) being only one ml of inoculum per 3500 ml of the fermentation broth accompanied with the longest fermentation period. All the latter studies used an inoculum size ranged from 1:10 to 1:20 ml/ml.

Batch microbial transglutaminase fermentation by Streptoverticillium mobaraense WSH- Z2 at various pH values ranging from 5.0 to 8.5 was studied by Zheng et al. (2002). They found that the MTGase formation was observed simultaneously with the beginning of cell growth continued for 6 h after the cessation of cell growth. Relative high pH at earlier fermentation stage not only made the lag phase of cell growth shorter but also was advantageous to cell growth and MTGase formation. At mid-and later-stage, properly lowering pH can strengthen cell growth and MTGase formation, thus, increase MTGase fermentation level. During the whole process, the suitable pH for cell growth and MTGase formation are different, it is favourable to use a two-stage pH control process instead of constant pH process (pH was controlled at 7.0 during 0- 13 h, and then shifted to 6.5 within 15 min till the end of fermentation period reaching 60 h). By applying this pH shift strategy in MTGase fermentation, the maximum MTGase activity and productivity had a significant improvement and reached 3.40 U/ ml and 81.4 U/ l/ h, respectively. The other authors applied the constant pH fermentation process, the operating pHs used were ranged from 6.5 to 8.5.

The MTGase producing strains are aerobic and cultivated in liquid submerged media. So, the amount of dissolved oxygen is very important parameter affecting cell growth and enzyme production. It is observed that the enzyme is produced during sporulation and there is a possible interaction between oxygenation and spore formation. Aeration and/ or agitation were the main tools used by most authors to study the effect of oxygenation on MTGase production.

Enhancement of microbial transglutaminase production by Streptoverticillium mobarance WSH-Z2 by application of a two-stage agitation speed control strategy was adapted by Yan et al. (2005) and used latter by Cui et al. (2006). They concluded that, a relatively high dissolved oxygen concentration level was favourable for cell growth in the earlier stage of fermentation, while moderate dissolved oxygen concentration was advantageous for MTGase production in the later stage of fermentation. They suggested a two-stage dissolved oxygen strategy, in which the agitation speed was controlled at 450 rpm in the first 24 h of fermentation and then switch 350 rpm.

De Souza et al. (2009) studied the effects of oxygenation in cultures of Bacillus circulans BL 32 on TGase production and cell sporulation were studied by varying the agitation speed and the volume of aeration. They adapted a two-stage aeration rate control strategy: first stage under aerobic growth condition (500 rpm; 2 v/v/min air flow) to induce biomass formation, followed by a second anoxic condition (no air supply) in which cell sporulation and TGase production was stimulated. Under these conditions the enzyme yield was 61% higher than that obtained in shake cultures and the cultivation time for the highest TGase activity was reduced from 192 just to 50 h.

There are few reports concerning optimization of the culture media for M TGase production. So, the aim of the present study is to optimize the production conditions (composition of fermentation media, inoculum size, temperature, pH, and fermentation medium in shake flask) in order to maximize the MTGase yield.

Materials and Methods:-

Materials:-

Microorganism:-

The actinomycetes strain used in the present study was isolated from soil samples collected from Alexandria Governorate, Egypt.The strain was identified as Streptomyces diastaticus as reported in the previous paper (Eshra et al., 2015)

Chemicals and reagents:-

N-carboxybenzoyl-L-glutaminyl-glycine (CBZwas purchased from Sigma-Aldrich, Co., USA. All the other chemicals were of analytical grade.

Methods:-

Preparation of tryptic casein hydrolyzate (TCH):-

Twenty grams of alkaline soluble casein were dissolved in 100 ml Tris-buffer pH 8.0, and then 0.2 g of trypsin (E-Merck) were added and incubated for 18 h at 37°C. The mixture was boiled for 5 min, cooled in ice bath and centrifuged at 3000 rpm for 20 min and preserved at - 4°C. The activity of equal volumes of pure enzyme solution

(Ajinomoto Activa WM MTGase) was measured separately by the both substrates TCH and CBZ. A correction factor was calculated to convert the enzyme units measured by TCH to the more common units measured by the synthetic substrate CBZ.The correction factor was measured for each prepared batch of the tryptic casein hydrolyzate. The average of this factor was ranged between1.42 to 1.55.
MTGase activity

The enzyme activity was measured by the colorimetric hydroxamate procedure using TCH as substrate. The reaction mixture (1.8 ml of citrate buffer ;0.2 M pH 6.0, 0.6 ml of enzyme preparation, 0.45 ml of the substrate solution, and 0.15 ml of 4 N neutral hydroxylamine and incubated at 37°C for 30 min then the reaction was stopped by adding an equal volume (0.75 ml) of each of the following solutions 1:3 HCl, 5% $FeCl_3$ in 0.1 M HCl, and 12% TCA. The absorbance was measured at 525 nm (Spectronic 20, Bausch & Lomb, USA). One unit of MTGase activity was defined as the amount of enzyme which causes the formation of one micromole of hydroxamic acid per min at 37°C. A calibration curve was prepared using γ-glutamic acid γ-monohydroxamate (Macedo et.al., 2007).

Dry cell weight:-
Aliquots of the fermentation culture were centrifuged at 3,000 g for 20 min. Cell pellets were washed twice with cold distilled water and dried at 80°C to a constant weight in vacuum oven (Zheng et al., 2002).

Residual sugars:-
Residual sugars were measured as reducing sugars according to the method of Miller (1959). Starch was completely hydrolyzed before measurement as described by (Zheng et al., 2002).

Effect of fermentation medium composition:-
Three different fermentation media were used. The first was the basal fermentation medium (gl^{-1}), 20.0 peptone, 2.0 yeast extract, 20.0 starch, 5.0 glucose, 1.0 $MgSO_4$ and 2.0 KH_2PO_4, pH 7.0 in 900 ml bottles and cultivated at 30°C for 5 days at 200 rpm using GallenKamp Orbital Incubator. All runs were made in duplicate (Macedo et al., 2007). The second was starch free medium, the same as the basal medium but without starch. While the third was glycerol containing medium which contained 10.0 gl^{-1} glycerol instead of both starch and glucose used in the basal medium.

Effect of temperature:-
The effect of temperature on the production of MTGase was carried out by adjusting the temperature of the fermentation medium from 20 to 45°C other conditions of fermentation were carried out as mentioned before (Zheng et al., 2001).

Effect of pH:-
The effect of pH on the production of MTGase was carried out by adjusting the pH of the fermentation medium between 5.5- 8.5 pH values using phosphate buffer (Zheng et al., 2002).

Influence of inoculum size:-
The influence of inoculum size on MTGase activity was tested. Five bottles of the fermentation medium were inoculated with, 2, 4, 6, 8 and 10% of the pre-inoculated seeding medium. The seeding medium was prepared as mentioned by Eshra et.al.,(2015). All the bottles were incubated in a rotatory shaker for four days under the best conditions of temperature and pH, and the MTGase activity was measured. (Macedo et al., 2008).

Effect of medium volume in shake flask:-
The selected strain was cultivated in 600 ml shake flasks containing various volumes of fermentation media (25 to 150 ml) to give different levels of air from 575 to 450 ml, while agitation speed was fixed at 200 rpm (Yan et al., 2005).

Results and Discussion:-

Effect of the fermentation medium composition:-
Three different media were tested to study the effect of the carbon sources on the enzyme production namely; basal medium, starch free medium and glycerol medium. Tryptic casein hydrolyzate was used as a substrate .The enzyme activity obtained was corrected to be CBZ units using the calculated correction factor as estimated before. The data presented in Figure (1) showed that maximum enzyme formed after the fourth day of fermentation for the three

media tested. The MTGase activity decreased by prolonging the fermentation period to 5 days. The maximum MTGase activity was obtained in the starch free medium reaching 7.02 U/100 ml of the fermentation medium after 4 days. The least activity was observed in the glycerol containing media (5.43 U/100 ml). The basal medium produced 5.73 U/100 ml. Different amounts of MTGase were stated by other authors using Streptomyces sp. strains. Téllez-luis et al. (2004) reported that 0.63 ± 0.05 U/ml was obtained after 72 h under optimized conditions. Macedo et al. (2008) stated that the highest enzyme activity was 0.2 U/ ml after 5 days of fermentation using the basal medium. From the previous results, it could be concluded that the starch free medium was the best media for the enzyme production. It was found that starch pellets were formed in the fermentation flask of the basal medium, so this medium will be discarded in the following part of the study.

The dry cell weight (DCW) and the units of enzyme produced per gram dry cell weight (U/ g DCW) were also determined through the fermentation period of the starch free and the glycerol containing media. The results given in Table (1) indicated that, for both fermentation media used (starch free and glycerol containing media), a noticeable increase in the DCW was occurred till the second day of fermentation being nearly the same for both medium (≈ 0.22 g/100 ml). The cell growth, expressed as DCW was somewhat constant from the second day till the fourth day of fermentation with slight increase for the glycerol containing medium.

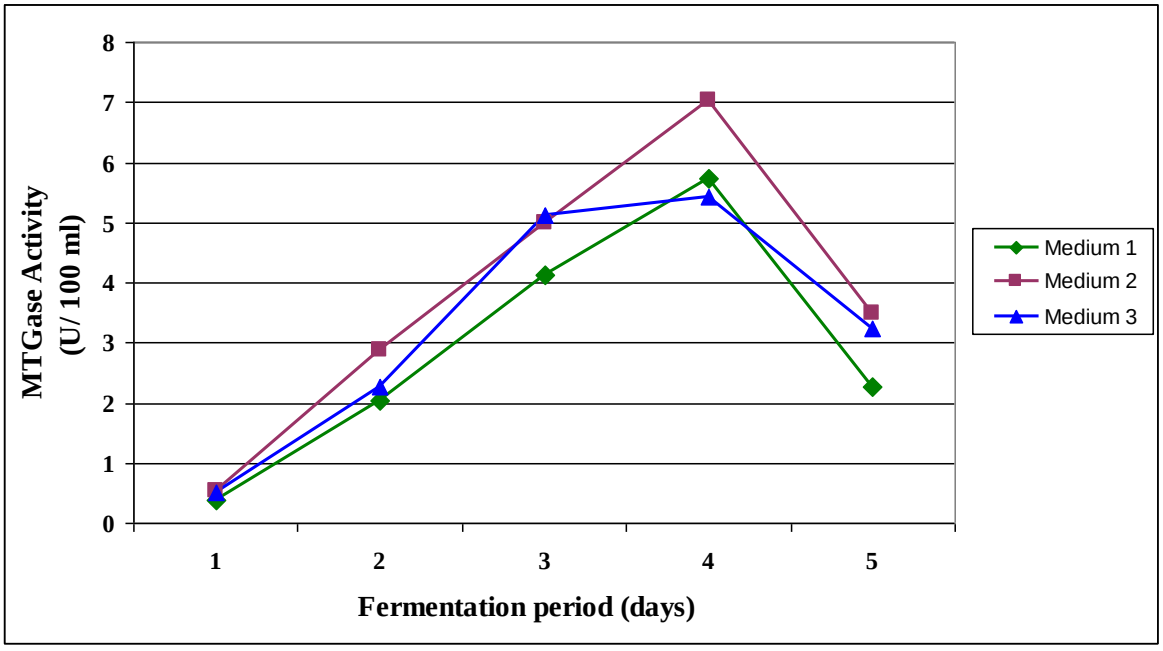


Figure1:- Effect of fermentation medium composition on the MTGase produced from Streptomyces diastaticus.

Medium (gl^{-1})	Starch	Glucose	Glycerol
1- Basal medium	20	5	0
2- Starch free medium	0	5	0
3- Glycerol containing medium	0	0	10

Table1:- Effect of fermentation medium composition on the dry cell weight (DCW) and MTGase units produced per gram of DCW.

Fermentation period (days)	Medium 1		Medium 2	
	DCW (g/ 100 ml)	Units / g DCW	DCW (g/ 100 ml)	Units / g DCW
1	0.0543	9.76	0.0685	7.59
2	0.2151	13.34	0.2251	10.04
3	0.2137	13.35	0.2221	23.05
4	0.2187	32.09	0.2349	23.12
5	0.2060	16.99	0.1887	17.12

Medium (gl^{-1})	Starch	Glucose	Glycerol
1- Starch free medium	0	5	0
2- Glycerol containing medium	0	0	10

The efficiency of enzyme production may be represented by units of enzyme produced per gram of dry cell weight U/g DCW, which is directly reflect the relationship between the cell growth and the amount of enzyme produced. The enzyme was produced from the first day of fermentation, and its production was gradually increased and reached the maximum amount at the fourth day of fermentation for the two media used. The amount of enzyme produced was 32.09 U/g DCW for the starch free medium, whereas it was 23.12 U/g DCW for the glycerol containing medium after the fourth day of fermentation. Slight to moderate decrease of enzyme production was occurred at the fifth day of fermentation. It could be concluded that during the fermentation period the cell growth and enzyme production were not parallel to each other. For the both media used maximum cell production was at the second day of fermentation, while the peak of enzyme production attained at the fourth day of production. The starch free medium is preferred than the glycerol containing medium, hence, it will be used for four days of fermentation in the following experiments.

To confirm these results, the residual sugar content (RSC) was also measured through the fermentation period using the starch free medium. The results showed that the amount of residual sugar content at the fourth day of fermentation was 0.133 gl^{-1} which represent 26.57% of the total starting sugar content (0.500 gl^{-1}). So, the amount of glucose used was adequate for the growth of the strain used even after the maximum production of MTGase. In other words the amount of glucose used is not a limiting factor for the growth of the strain or the production of the enzyme. The same trend of residual sugar content was observed through the production of MTGase be many authors (Ando et al., 1989; Zheng et al., 2002 and Yan et al., 2005).

Effect of inoculum size:-

The influence of inoculum size on the production of transglutaminase from the isolated strain Streptomyces diastaticus was tested. Five flasks of fermentation medium were inoculated with 2, 4, 6, 8 and 10% (v/v) of the seeding medium and the enzyme activity was measured at the end of the fermentation period (4 days). The results presented in Figure (2) indicated that the maximum enzyme activity was observed when 8% inoculum was used. By increasing the inoculum size to 10% the activity decreased to 88.12% of the maximum activity. So, the inoculum size of 8% was quite suitable for reaching maximum production of the enzyme.

Different inoculum sizes were used by other authors; Zheng et al. (2001 and 2002) used an inoculum size of 10%, while Yan et al. (2005) and Cui et al. (2007) used a 8% (v/v) of seed culture to inoculate the fermentation medium.

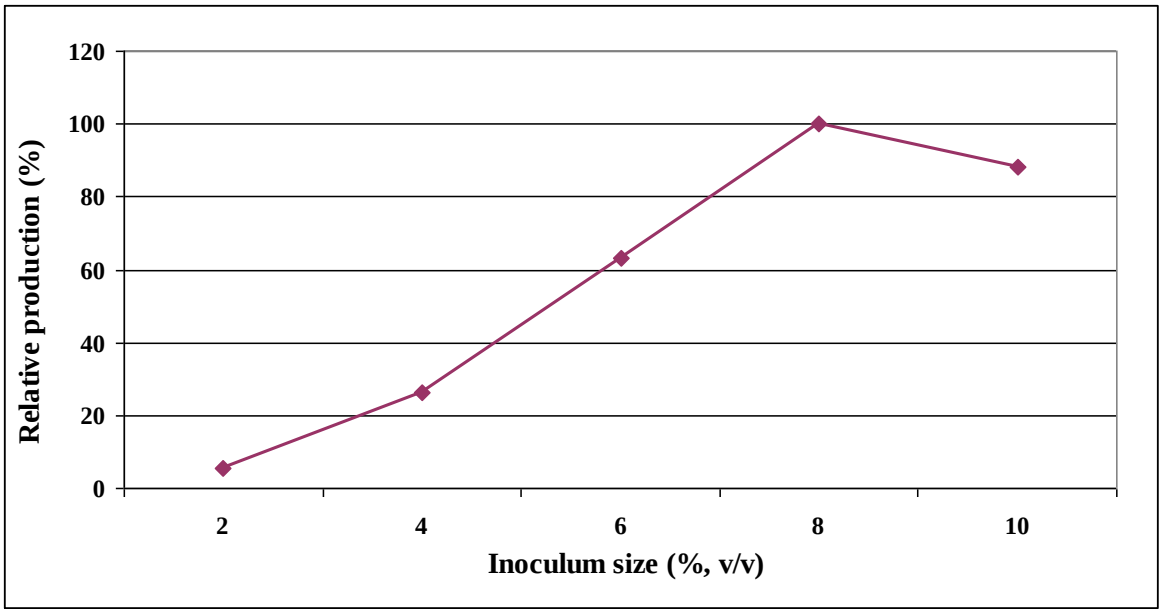


Figure 2:- Effect of inoculum size on the production of MTGase produced from Streptomyces diastaticus.

Effect of fermentation temperature:-

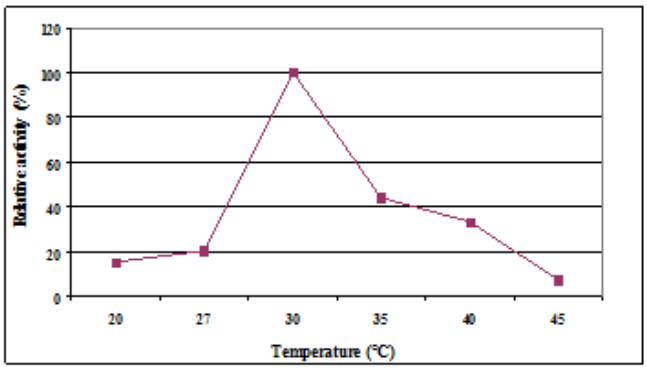
The effect of fermentation temperature ranged from 20 to 45°C on the production of MTGase from Streptomyces diastaticus was tested. The relative activity was determined at the end of the fermentation period. The data presented in Figure (3a) indicated that the maximum activity was observed at 30°C and the relative activity was highly decreased when the temperature was higher or lower than the optimum temperature. The relative activity was only 15.48 and 7.14% at 20 and 45°C, respectively.

Most of the studied on the fermentation temperature of MTGase from different actinomycetes strains indicated that 30°C was the most suitable fermentation temperature (Ando et al., 1989; Gerber et al., 1994; Zheng et al., 2002; Lu et al., 2003; Yan et al., 2005; Macedo et al., 2007, 2008, 2010 a, b; Zhang et al., 2008 a, b). Other fermentation temperatures were used by other authors, 28°C (Ho et al., 2000) and 32°C (Cui et al. 2007, 2008).

Effect of fermentation pH:-

The effect of pH of the fermentation medium on the production of MTGase was carried out using different pH values ranged from 5.5-8.5 at the optimized conditions stated before. The results presented in Figure (3b) showed that the optimum pH of the isolated strain was at pH 7.5 and the pH curve is a typical bell shape of the enzyme pH curves. The relative activity was only 18.78 and 11.68 % of the maximum activity at 5.5 and 8.5 pHs, respectively. The stated pH values for the fermentation medium of different strains of actinomycetes strains ranged from 6.5 to7.0. The pH 6.5 was used by Zheng et al. (2001) and Cui et al. (2007, 2008), while pH 7.0 was reported by many authors as optimum pH for MTGase activity; (Ho et al., 2000; Zheng et al., 2002; Lu et al., 2003; Zhang et al., 2008 a, b, Macedo et al., 2007, 2008, 2010 a, b) .

a



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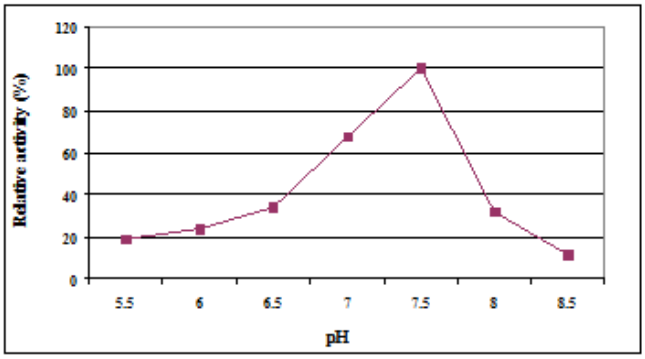

Figure3: Effect of fermentation temperature (a) and pH (b) on the MTGase activity produced from Streptomyces diastaticus.

Effect of the fermentation medium volume in shake flasks:-

Actinomycetes are aerobic microorganisms, so dissolved oxygen in the fermentation submerged medium is one of the most important environmental parameters that could affect cell growth and enzyme production. Different levels of dissolved oxygen (DO) were controlled by changing agitation speed and/ or aeration rate (Zheng et al., 2002 et al., Cui et al., 2005).

In the present study different levels of DO were carried out using different volumes of fermentation medium (25 to 150 ml) in constant volume (600 ml) fermentation flasks at fixed agitation speed (200 rpm) under the optimized conditions as reported by Yan et al.,(2005).

The results given in Table (2) indicated that using 75 and 100 ml of fermentation medium gave the highest enzyme activities. Increasing the fermentation medium than 100 ml decreased the obtained enzyme activity being 100 and 93.69% , respectively .When the medium volume reached to 125 and 150 ml the relative enzyme activity decreased to 62.15 and 49.53%, respectively. The ratio between the volume of fermentation medium (V_m) and that of fermentation flask (V_f) obtained in the present study is 1:7. Yan et al. (2005) reported that the highest MTGase activity was achieved when the medium volume was 100 ml in a shaking flask 600 ml (V_m: V_f ratio 1:6) and the enzyme activity was decreased with further increase of medium volume.

Table 2:-Effect of the fermentation medium volume in shake flasks on MTGase activity produced from Streptomyces diastaticus.

Fermentation medium volume (V_m; ml)	Fermentation medium volume: Fermentation flasks volume (V_m: V_f)	Relative activity (%)
25	1:24	18.92
50	1:12	46.37
75	1:8	100
100	1:6	93.69
125	1:4.8	62.15
150	1:4	49.53

Conclusion:-

The optimum conditions for the production of MTGase from a newly isolated strain of Streptomyces sp.were determined. It is important to measure these conditions for any new isolated strain since they may differ from one strain to another even within the same species .The CBZ is the synthetic substrate usually used for determining the MTGase activity and the enzyme unit is defined by CBZ .It is very important to search about another substrate more cheaper than CBZ to measure the MTGase activity. In the present study TCH which is a new, cheap and common MTGase substrate was prepared especially isolation and screening of MTGase producing strains as well as optimization of the enzyme production since these studies need lot of enzyme activity measurements. The enzyme units estimated by TCH was corrected to be CBZ units .

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