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Production of Microbial-Transglutaminase (MTG) from *Streptoverticillium mobaraense*

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Mineral salts in medium usually profoundly influence microorganism growth and protein synthesis. In order to produce microbial transglutaminase (MTG) with a high yield from *Streptoverticillium mobaraense*, we screened the minerals NaCl, $CaCl_2$, $CoCl_2$, $FeSO_4$, $ZnSO_4$, $MnSO_4$ and $CuSO_4$ for MTG fermentation. The results indicated that appropriate $FeSO_4$ concentrations could significantly promote cell growth and stimulate the production of MTG. With 15 mg/L of $FeSO_4$ added to medium, 58% improvements were noted in MTG productivity (2.24 U/mL). NaCl, $CaCl_2$, and $CoCl_2$ enhanced MTG productivity by less than 15%, and the optimal concentrations were determined as 1 g/L, 2 g/L, and 30 mg/L respectively. Furthermore, it was determined that 7.5 mg/L of $ZnSO_4$ in medium could augment MTG productivity by 20% and induce the stationary phase for MTG production to a period 24 hr earlier. This basic and novel discovery should result in the development of a good complement to the previously defined culture media for MTG fermentation.

Key Words : Microbial transglutaminase (MTG), mineral, *streptoverticillium mobaraense*

INTRODUCTION

Microbial transglutaminase (MTG; protein-glutamine EC 2.3.2.13) is an enzyme which is capable of catalyzing acyl transfer reactions, which introduce covalent cross-links between proteins, peptides, and cost and broad applications from human health to industrial usages(1-4).

MTG was purified from *Streptoverticillium mobaraense* for the first time in 1989, by Ando et al.(5). Up to now Until the present, the commercial microbial transglutaminase is has also mainly been produced nerated principally from derivatives of this strain. In order to obtain a high yield, studies on optimizing regarding the optimization of the fermentation conditions for producing MTG production had been have been continuously reported continuously conducted (6-12). Zhu et al. and Junqua et al.(6-9) designed media by via chemical analysis, and optimized the fermentation process using using a fed-bath culture strategy. Respectively, the The medium developed by Zhu et al.(6) had has been widely extensively used utilized for the fermentation of MTG by other researchers. GenerallyIn general, various a variety of environmental factors can (e.g. pH, oxygen and temperature) can affect the influence the cell growth, formation rate, and productivity of MTG. Therefore, it is important to optimize these parameters in MTG fermentation protocols in order to achieve attain high MTG productivity. Recently, Zheng et al. modified the fermentation conditions by strategies on concerning pH, temperature, and agitation control depending on the strains that had been mutated from Streptoverticillium mobaraense(10-12).

In addition, minerals in medium usually normally affect the process of cellular metabolic metabolism process in various a variety of ways. The appropriate composition and concentration of minerals in medium media can promote cell growth and the synthesis of certain proteins synthesis. In previous Previously, several investigations conducted by Zhu et al.(6) and Junqua et al.(7) had demonstrated the importance of Mg^{2+} and K^{+} in culture medium, to accumulate allowing for the accumulation of enough sufficient biomass, while whereas relatively little effort was has been focused on the screening of other minerals which might be prove useful for cell growth or stimulating for the stimulation of MTG production especially. A defined and efficient culture medium with efficiency will be prove meaningful for the production

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of MTG production in industry and in further researches. Thus, in the present study, We initially added varying concentrations of NaCl, $CaCl_2$, $CoCl_2$, $FeSO_4$, $ZnSO_4$, $MnSO_4$ and $CuSO_4$ on the to the base of the medium described developed by Zhu et al.. The effects on the cell growth and productivity of MTG from exerted by *Streptoverticillium mobaraense* were studied by analyzing via analyses of the dry cell weight (DCW), MTG activity, specific activity, and residual carbon sources during the process of fermentation process. For producing In order to produce MTG maximally with optimal efficiency, the suitable concentration of each mineral in the medium was demonstrated determined.

MATERIALS AND METHODS

Microorganisms

Streptoverticillium mobaraense (from the Americaln Type Culture Collection 29032) was used utilized in this study. For the formation of spores formation, cells were spread on agar plates containing malt extract (Difco, USA). After cultivation for 6-8 days of cultivation at 30℃, the spores were collected with 5.0 mL glycerol/water (1 : 1, v/v). The resulting resultant suspension was used utilized in for further experiments or stored at -20℃(13).

Culture methods

The varying concentrations of the tested minerals were modified at in suitable ranges and the media used in this study were shown are provided in Table 1. The seed medium is a modification initially described by Zhu et al. and the culture medium (A-G) was based predicated on the seed medium supplemented with the tested minerals. For seed preparation, 2.5 mL of spore suspension (1.8 x 108 CFU/50 was inoculated to into 50 seed medium and cultured for 24 hr at 30℃. Then, 75 mL of culture medium (medium A-G) was inoculated with the 5 mL of seeds and cultured for 96 hr at 30℃ in baffled flasks (250 rpm). All of the flasks were of 300 mL volumes, and the chemicals (e.g. hydroxylamine) used in employed in this study were purchased from Sigma (St. Louis, USA).

Biomass dry weight

For the measurement In order to measure of the dry cell weight (DCW), 5.0 mL of culture broth was withdrawn and centrifuged (3.000 rpm) for 10 min at room temperature. The collected cells were then washed twice with in distilled water and dried at 105℃ until a constant weight was achieved.

Enzyme assay

The supernatant of cell culture was used for determinations of the activity of MTG activity. A The colorimetric procedure previously described by Grossowicz et al.(14) was modified as follows: Mix 0.1 mL of enzyme supernatant was mixed with 0.2 mL of N-CBZ-gln-gly (0.1 M dissolved in 0.5 M Tris-HCl buffer, pH 7.0) and 0.05 mL of hydroxylamine (1.0 M), and 0.05 mL of EDTA (0.02 M).

Vortex and incubate the The samples were vortexed and incubated for 10 min at 37℃. And then add 0.1 mL of 15% TCA, 0.15 mL of 5% $FeCl_3$ (dissolved in 0.1 N HCl) and 0.25 mL of HCl (2.5 N) were then added, to produce producing a yellow-brown color. The absorbance of the samples was then measured at 525 nm using a UV spectrophotometer (Mecasys Co., Ltd., Korea).

One unit of transglutaminase was defined as the formation of 1.0 of L-glutamic acid per minute at 37℃. A calibration curve was made constructed using L-glutamic acid monohydroxamate. The specific activity of MTG was calculated as U/mg (MTG activity/Dry cell weight).

Measurement of residual sugar

The carbon source for MTG fermentation is in this study was D-glucose. Thus, the supernatant of cell culture supernatant was used utilized to for determining determine the quantity of D-glucose as a residual sugar. And the The 3,5-dinitrosalicylic acid (DNS) assay described by Miller (15)

Table 1. Media used in this study

Media compositions	Seed medium	Medium A	Medium B	Medium C	Medium D	Medium E	Medium F	Medium G
D-glucose (g/L)	20	20	20	20	20	20	20	*20*
Peptone (g/L)	20	20	20	20	20	20	20	*20*
Yeast (g/L)	2	2	2	2	2	2	2	*2*
K_2HPO_4 (g/L)	2	2	2	2	2	2	2	*2*
$MgSO_4$ (g/L)	2	2	2	2	2	2	2	*2*
NaCl (g/L)		0-5						
$CaCl_2$ (g/L)			0-5					
$CoCl_2$ (mg/L)				0-50				
$FeSO_4$ (mg/L)					0-25			
$MnSO_4$ (mg/L)						0-12.5		
$CuSO_4$ (mg/L)							0-12.5	
$ZnSO_4$ (mg/L)								0-12.5

was also employed in this study.

Dialysis assay

The procedure was modified from that described by Eckhard and Loffler(16). After 96 hr of fermentation with each tested mineral, the 25 mL of supernatant of cell culture supernatants was were dialyzed with 10 mM EDTA (dissolved in 0.05 M Tris-HCl buffer solution, pH 7.0) for 2 hr at room temperature. The enzyme supernatants was were then dialyzed in the same solution but with 1.0 mM EDTA for 12 hr at 4℃. To remove In order to remove the EDTA, the supernatant was dialyzed repeatedly dialyzed in Tris-HCl buffer without EDTA. The obtained enzyme solution obtained was then used utilized for the activity assays.

RESULTS AND DISCUSSIONS

Effect of tested minerals on MTG activity

To investigate In order to determine the effects of minerals on enzyme activity, dialysis assays was were manipulated conducted for the tested minerals after fermentation process. The results showed indicated that the activity of MTG activity was rarely affected by the process of dialysis process, which was utilized to remove removed the minerals from the enzyme solution. Although Zn^{2+} and Cu^{2+} had been were previously reported to inhibit MTG activity(5), in this study, the used concentrations of these two minerals did not obviously affect MTG activity apparently(data not shown).

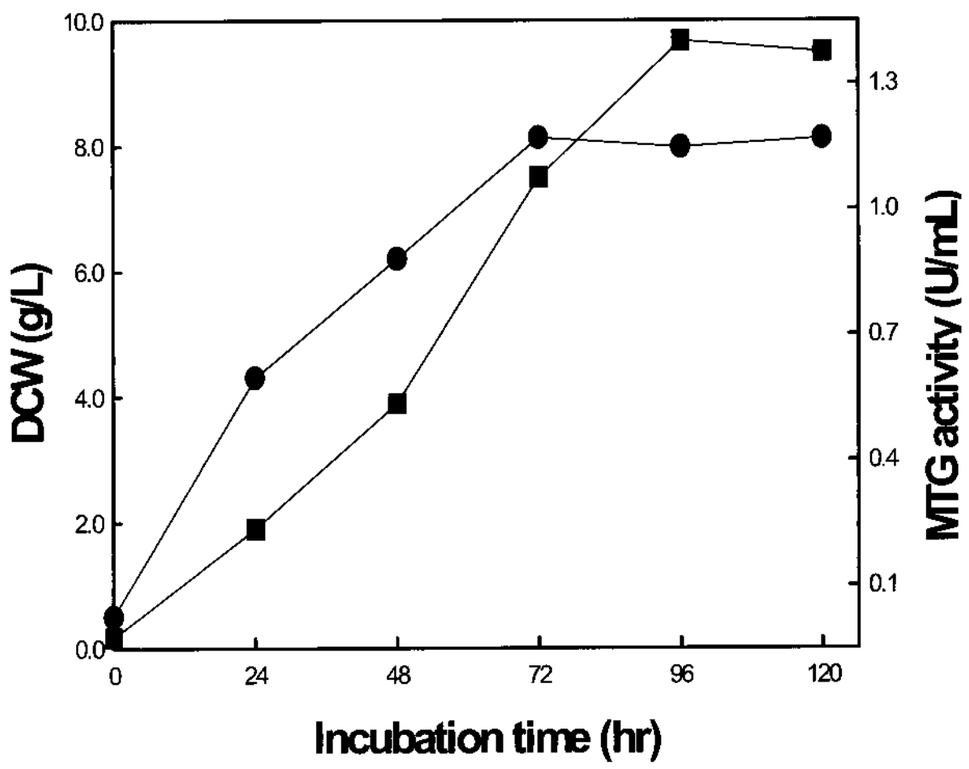

Figure 1. Time course of cell growth and MTG production by sStreptoverticillium mobaraense on the a base of seed medium base (symbols: (●) Dry cell weight (DCW), (■); MTG activity).

Effect of NaCl, $CaCl_2$, $CoCl_2$, $FeSO_4$ on cell growth and MTG productivity

Fig. 1 shows the fermentation process by seed medium, demonstrating showing that the cells grew fast for the first 48 hr and reached achieveda stationary phase at 76 hr.

Table 2. Effect of NaCl, $CaCl_2$, $CoCl_2$ and $FeSO_4$ concentration on cell growth and MTG activity

Media & mineral	mineral concentration (g/L)	DCW* (g/L)	MTG activity (U/mL)	Specific activity (U/mg)	Residual sugar (g/L)
Media A NaCl	0	7.58	1.29	0.17	2.16
	1	**8.12**	**1.46**	**0.15**	**2.05**
	2	7.82	1.26	0.16	2.11
	3	6.25	1.01	0.16	2.23
	4	5.30	0.80	0.15	2.39
	5	4.94	0.69	0.14	2.54
Media B $CaCl_2$	0	7.77	1.31	0.16	2.21
	1	8.91	1.34	0.15	2.13
	2	**9.10**	**1.45**	**0.15**	**1.57**
	3	8.78	1.31	0.15	1.64
	4	8.58	1.03	0.12	2.36
	5	7.75	1.06	0.14	2.40
Media C $CoCl_2$	0	7.69	1.30	0.17	2.23
	10 X10-3	7.89	1.36	0.16	2.08
	20 X 10-3	8.66	1.40	0.16	1.85
	30 X 10-3	9.23	1.49	0.15	**1.54**
	40 X 10-3	8.79	1.36	0.14	1.88
	50 X 10-	7.80	1.11	0.13	2.15
Media D $FeSO_4$	0	7.86	1.34	0.17	2.1
	5 X 10-3	8.18	1.68	0.20	1.7
	10 X 10-3	9.76	1.84	0.19	1.3
	15 X 10-3	11.79	2.21	0.20	**1.1**
	20 X 10-3	8.56	1.71	0.19	1.7
	25 X 10-3	7.66	1.48		1.

Each value represents the mean of three independent experiments performed in duplicate. Standard errors of mean were within limits of 5% to the average value ($p<0.05$).
DCW*: Dry cell weight

However, the maximum activity (1.36 U/mL) of MTG in culture was not achieved until 96 hr.

Medium A and medium B were used for evaluating utilized to evaluate the effects of NaCl and $CaCl_2$ on the fermentation process, respectively. Both of the two minerals tested had no effects on the time course of fermentation. Therefore, the dry cell weight, MTG activity, MTG specific activity and residual sugar in the culture broth were investigated assessed after 96 hr of fermentation(Table 2). According to In the our results, the residual sugar could also reflect might also be reflective of the cell growth, because as most the majority of the sugar consumption in the medium was used utilized for cell growth but not for MTG production, which began to increase exponentially at an exponential rate after 72 hr when cell growth had reached a achieved stationary phase(9).

The results of our experiments showed that appropriate concentrations of NaCl and $CaCl_2$ had exerted positive effects on cell growth and MTG activity in the culture broth. 8.12 g/L of dry cell weight (DCW) and 1.46 U/mL of MTG were obtained acquired in the presence of 1 g/L of NaCl in medium A. With 2 g/L of $CaCl_2$, 9.10 g/L of DCW and 1.45 U/ mL of MTG were obtained after 96 hr of fermentation. However, more than 2 g/L of these two minerals in medium would was determined to inhibit the cell growth.

In the case of $CoCl_2$ and $FeSO_4$, all of the samples were drawn after 96 hr of fermentation. Significantly improved improvements of DCW (9.23 g/L) and MTG activity (1.49 U/L) were achieved in present the presence of 30 mg/L of $CoCl_2$(Table 2). The apparent effects on cell growth might may be attributable due to the stimulation of glucose transport in the response of Co^{2+} during the fermentation process(17). With 15 mg/L of $FeSO_4$ in medium D, the highest levels of DCW (11.79 g/L) and MTG production (2.24 U/mL) were obtained observed among the tested minerals(Table 2). The much greatly enhanced MTG was mainly contributed principally attributed by the to the improved cell density. On the other hand, the synthesis of MTG synthesis could be stimulated by $FeSO_4$, since as the specific activity observed was higher than that seen in seed medium.

Effect of $MnSO_4$, $CuSO_4$ and $ZnSO_4$ on cell growth and MTG productivity

Medium E was used to test assess the effects of MnSO4 on the fermentation of MTG fermentation. With 2.5 mg/L of $MnSO_4$, the maximum levels of MTG activity (1.38 U/mL) in culture was just improved were improved only by 4%. Furthermore, even little a small amount of $MnSO_4$ (5 mg/L) significantly caused induced cell death significantly after 72 hr. According to the results of this study, no improved DCW or MTG activity was found detected with concentrations of $CuSO_4$ (between 0-12.5 mg/L) in medium F(data not shown).

$ZnSO_4$ not only had exerted an effect on the producing production of MTG, but also induced the stationary MTG production producing phase to a period 24 hrs earlier period. Fig. 2 showed shows that the MTG activity in culture reached the a peak at 72 hr and the highest levels of observed MTG activity (1.71 U/mL) was were obtained with 7.5 mg/L of $ZnSO_4$ in medium G. In the case of seed medium, a yield of 1.41 U/mL of MTG was achieved at 96 hr. Although both the DCW and MTG were enhanced by $ZnSO_4$ during the fermentation process, the DCW value began to decrease be reduced after 72 hr(Fig. 3). This indicates shows that the excess of $ZnSO_4$ in the medium inhibited cell growth after 72 hr, and the environment was no longer proper appropriate for cell growth.

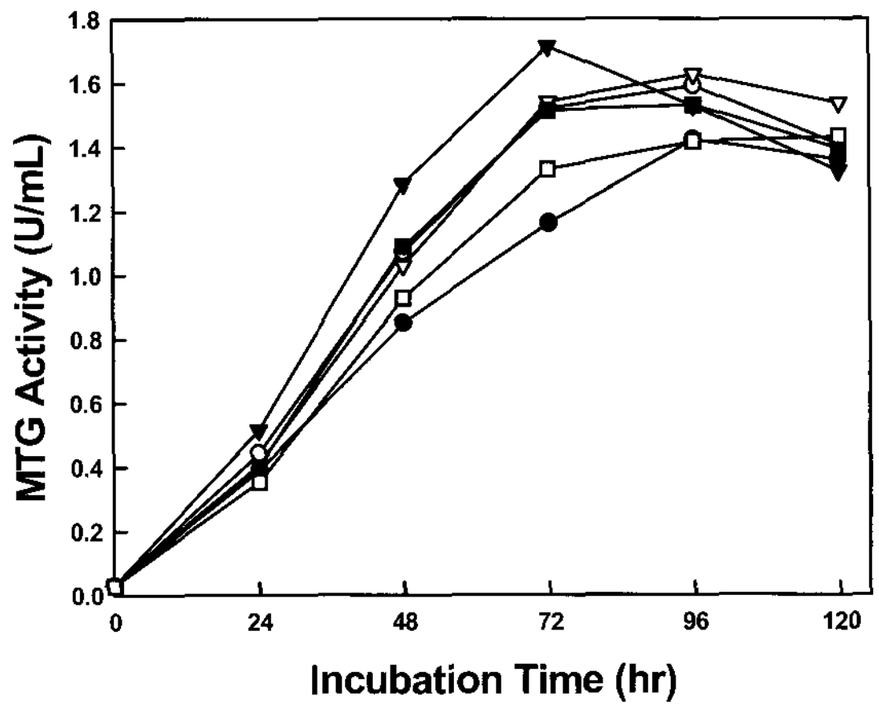

Figure 2. Effects of different initial concentrations of $ZnSO_4$ on MTG production with medium G (symbols: (●) 0 mg/L, (○) 2.5 mg/L, (▼); 5 mg/L, (△); 7.5mg/L, (■); 10mg/L, (□); 10.0 mg/L).

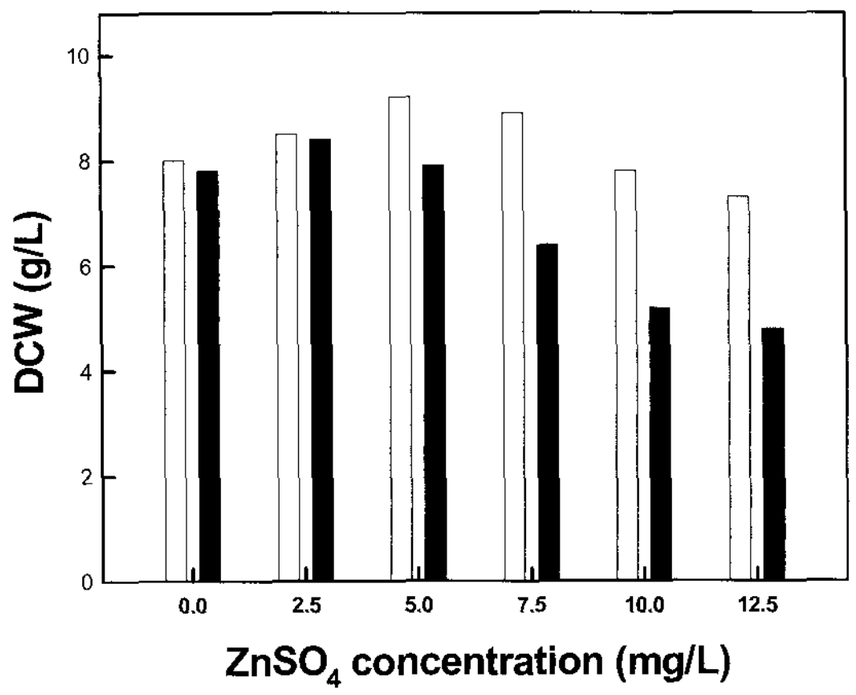

Figure 3. Dry cell weight (DCW) at 72 hr and 96 hr of the fermentation course with various initial concentrations of $ZnSO_4$ in medium (symbols: (□); the 72 hr, (■); the 96 hr).

Comparison of improved DCW and MTG productivity by the tested minerals

The optimal improved rate of dry cell weight and MTG activity by each tested mineral was is summarized in Fig. 4. It This demonstrated that $FeSO_4$ (15 mg/L) initially added in to culture medium media could effectively improve the activity of MTG, by approximately 58%. Although ZnSO4 (7.5 mg/L, 72 hr) only had yielded an MTG production rate of only 22.2% achievement of MTG production, it really could may save save time as many as much as 24 hours in the fermentation process. NaCl, $CoCl_2$ and $CaCl_2$ contributed could give a contribution of less than 15% to the process, while whereas $MnSO_4$ did not apparently improve MTG yields apparently.

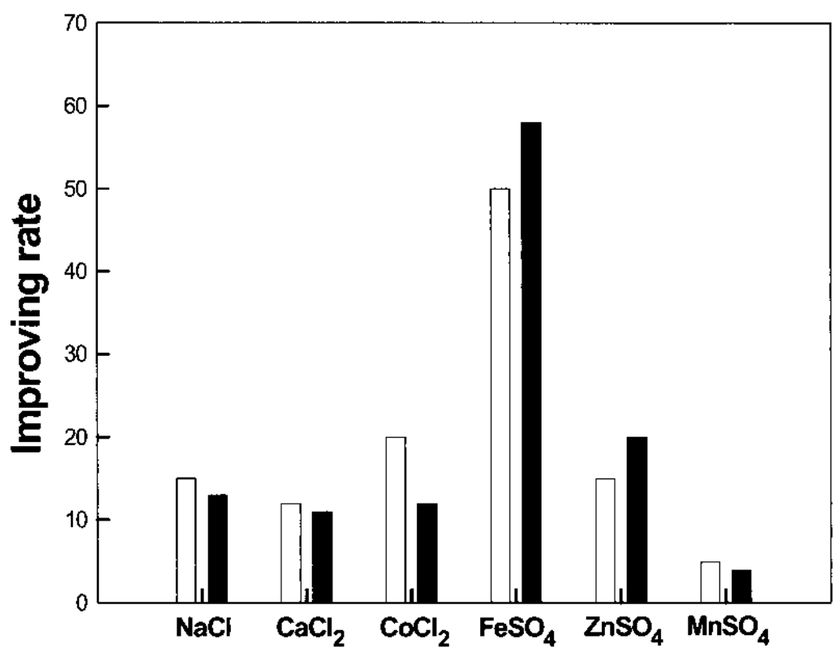

Figure 4. Comparison of optimal improving improvements rate on in dry cell weight (DCW) and MTG production by different minerals (symbols: (□); dry cell weight improving rate, (■); MTG production improving rate).

Determination of the final concentrations of minerals in medium

Except the With the exception of the added tested minerals, there are little only small amounts of relative metals ions were observed in the in seed medium, which was used as the a control medium. The content of metal ions in the seed medium was determined in triplicate by via AAS using a Varian SpectrAA 220FS instrument. Standard solutions for sodium, calcium, iron, cobalt, zinc, and manganese were prepared from analytical stock solutions (mMerck) using MilliQ water. After analysis, the quantities of Na, Ca, Fe, Zn and Mn in the seed medium were 0.36 g/L, 0.8 mg/L, 0.4 mg/L, 0.2 mg/L, and 1.1 mg/L. The little minimal quantity quantities of Co in the seed medium was were not determined assessed. Thus, the finally final optimal concentration of each mineral element in medium for MTG fermentation was calculated as follows: 0.75 g/L Na, 0.72 g/L of Ca, 13.50 mg/L of Co, 5.89 mg/L of Fe, 3.11 mg/L of Zn and 2.00 mg/L Mn.

CONCLUSION

A range of mineral salts was screened in an effort to optimize a the culture medium previously defined culture medium descried described by Zhu and his coworkers et al. (6). It was has been demonstrated that appropriate concentrations of $FeSO_4$ and $CoCl_2$ in medium could improve the MTG yield to significantly a much higher levels. This effect was mainly achieved principally by accumulating via the accumulation of more cells in culture broth, whereas the relative specific activities were not much substantially alteredchanged. Another novel discovery is that $ZnSO_4$ affected the process of MTG synthesis process and shifted the stationary phase of MTG activity in culture broth to a period 24 hrs earlier period. In industry, this effect might may prove be meaningful in that it saves a great deal of for saving time. Furthermore, the mineral salts used utilized in this study are both cheap and commercially available, which means the is indicative of low cost for highly improved MTG production. For further workIn further investigations, we will study attempt to assess the synthetic effects on producing MTG production exerted by with these minerals work in combination.

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