Comprehensive evaluation and selection of the potential complex medium for industrial glutamate decarboxylase (GAD) production by *Escherichia coli*

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Abstract: To address the need for producing large quantities of an enzyme, glutamate decarboxylase (GAD), this study evaluated the performance of the currently used complex and defined culture media in terms of biomass production, GAD yield, and byproducts accumulation, and selected the candidate(s) that could maximize the production of GAD by *Escherichia coli* (AS 1.505, the China General Microbiological Culture Collection Center, Beijing, China). The highest yield of the enzyme per biomass in the final broth (4431 U/g) corresponded to the Luria Bertani medium (LBM), which was 1.16 times the second highest from the Terrific Broth medium (TBM), while the highest biomass production (4.5 g/L) was achieved in the cultivation with TBM. For the byproducts, the profiles of all the media except the TK medium displayed a distinct exponential phase after six hour cultivation with a rapid byproducts generation, which could be attributed to the mixed acid fermentation caused by oxygen limitation. In addition, the subsequent re-assimilation of acetate could reduce the loss of GAD accumulated by stabilizing the media pH. The comprehensive analysis showed that although the highest biomass production was achieved by TBM, the fastest intracellular accumulation of GAD occurred in the cultivation with LBM (184.64 U/(g·h)), which also was the cheapest medium (0.56$/$10^3$ U). Therefore, LBM was considered the potentially competitive candidate medium for large-scale production of GAD by *E. coli*.

Keywords: culture media, biomass yield, GAD accumulation, byproducts, economic analysis, *Escherichia coli*
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1 Introduction

Glutamate decarboxylase (GAD, EC 4.1.1.15) is a unique enzyme that catalyzes the conversion of L-glutamic acid (Glu) to γ-aminobutyric acid (GABA), a chemical with several important physiological functions. It is known that GABA functions in animals as a major inhibitory neurotransmitter[1,2] and is involved in the regulation of cardiovascular functions, such as blood pressure and heart rate, and plays a role in the sensations of pain and anxiety[2]. Treatments for sleeplessness, depression, autonomic disorders[3,4], chronic alcohol-related symptoms and stimulation of immune cells[5] have also been related to the administration of GABA. Recently, GABA has been found to be a strong secretagogue of insulin from the pancreas that may prevent diabetic conditions[6].

Owing to these physiological benefits, GABA has been used extensively in pharmaceuticals such as aminalone, gammalone, pycamilone, and pantogam for the treatment of disturbances of brain activity[7]. Also

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several functional foods containing GABA are manufactured such as GABA-enriched green tea, GABA-enriched fermented beverages[8], dairy products[9], and red-mold rice[4,10,11] that have been reported to lower the elevation of systolic blood pressure in spontaneously hypertensive rats.

The growing effort in studying the physiological functions of GABA in recent years has increased the market demand for GAD, leading to numerous research attempts made to obtain sufficient quantities of purified, enzymatically active GAD for extensive studies on its structure, function, regulation, and therapeutic use[12-18]. In addition, results from animal studies indicate that GAD itself may be used in the treatment and/or prevention of beta-pancreatic islets in insulin-dependent diabetes mellitus (IDDM)[19-21]. Clearly, to enjoy all these benefits, production of sufficient quantities of purified GAD becomes essential.

GAD is widely present among eukaryotes and prokaryotes, such as mammalian brain[21,22] plants[23,24], *E. coli*[25,26], *Aspergillus oryzae*[27], and lactic acid bacteria[28]. Among all these sources, *E. coli* is recognized as a preferential vehicle for producing large quantities of GAD due to its fast growth and ability to reach high cell density culture, which enables high productivity of GAD due to the increased total biomass as well as the increased GAD yield per cell. To that end, finding the most efficient culture medium to enhance *E. coli* growth is one of the important strategies deserving more investigations.

A number of culture media are currently in use by researchers in *E. coli* cultivation. Luria Bertani (LB), the popular complex medium, allows the growth of *E. coli* up to a cell density of 1 g/L dry cell weight (DCW). Macaloney et al.[29] replaced the glucose in LB with glycerol to grow *E. coli* to a higher cell density. Shiloach and Bauer[30] designed a semi-defined medium consisting of organic salts, glucose, yeast extract, and a solution of trace elements which achieved a cell density of 54 g/L DCW. After adding citric acid, ethylenediaminetetraacetic acid (EDTA), and thiamine-HCl to the defined medium (containing only simple inorganic salts and a defined carbon source), Riesenberg et al.[31] fed the solution with 80% glucose, 2% $MgSO_4$ and trace elements and achieved 110 g/L DCW. Matsui[32] modified this medium to ensure a high cell density of *E. coli* JM109, followed by optimizing the culture conditions for the strain. However, most of these reports were related to the success of harvesting more *E. coli* cells, with only a limited number of reports contributing to the investigation of a suitable medium targeting accumulation and productivity of GAD in cells, a critical step towards large-scale production. Meanwhile, little has been done in evaluating the available media in terms of their performance for *E. coli* cultivation for GAD production.

In this study, the performance of the currently reported complex and defined media for *E. coli* cultivation was comprehensively evaluated with respect to biomass yield, GAD accumulation per biomass, and byproducts generation. Economic analysis was also conducted to provide useful information for selecting a cost-effective medium for potential large-scale production of GAD and for planning future research aimed at employing feeding strategies.

2 Materials and methods

2.1 Strain and cultivations

The *E. coli* (AS 1.505) utilized in this study was obtained from the Northeast Agricultural University in Harbin, China. It was a mutant derived from A 101, which has been reported to contain a GAD gene[33] and was originally obtained from the China General Microbiological Culture Collection Center (CGMCC), Beijing, P. R. China. The pre-culture was conducted in a 250 mL flask containing 50 mL LB medium for 6 h. Flask culture experiments were performed in 250 mL flasks each containing 50 mL culture medium after inoculated with 2% (v/v) of the seed culture. The strain was cultivated at 37℃, 200 r/min (rotation per minute) for 24 hours prior to use.

2.2 Culture media

The media selected for study were listed below. M1 referred to the medium for *E. coli* to accumulate GAD. Media of Terrific Broth (TB), TK-10 and LB were the frequently cited media for *E. coli* culture in the literature.

Medium 4 (M4) and Medium 5 (M5) were the specific media for GAD production by *E. coli*[16,34]. The chemical compositions of each medium were presented below.

1) Medium 1 (M1): 18 g/L casein hydrolysate, 5 g/L yeast extract, 1 g/L NH_4Cl, 100 mL phosphate buffer pH 7.0 ($Na_2HPO_4.12H_2O$ 118.7 g, KH_2PO_4 45.4 g) and 100 mL of glucose solution (glucose 30 g, $MgSO_4.7H_2O$ 5 g), pH 7.0.

2) TB medium (TBM): tryptone 12 g/L, yeast extract 24 g/L, 100 mL 170 mmol/L KH_2PO_4/0.72 mol/L K_2HPO_4.

3) TK-10 medium (TKM): 40 g/L glucose, 10 g/L $(NH_4)_2HPO_4$, 2 g/L K_2SO_4, 0.3 g/L NaCl, 2 g/L $MgSO_4{\cdot}7H_2O$, 300 mg/L $FeSO_4{\cdot}7H_2O$, 68 mg/L $ZnSO_4{\cdot}7H_2O$, 30 mg/L $CuSO_4{\cdot}5H_2O$, 15.2 mg/L $MnSO_4{\cdot}5H_2O$, 60 mg/L $CaCl_2{\cdot}2H_2O$, 6.8 mg/L $Na_2B_4O_7{\cdot}10H_2O$, 3 mg/L $(NH_4)_6Mo_7O_{24}$, 20 mg/L thiamine-HCl, and 100 mg/L ampicillin, pH 7.0.

4) M4: 10 g/L glucose, 30 g/L peptone, 3 g/L NaCl, 1 g/L K_2HPO_4, 0.2 g/L $MgSO_4$, 0.1 g/L glutamic acid, 15 g/L corn steep liquor, 30 μg/L biotin, 40 g/L bran, pH 6.5.

5) M5: 30 g/L soybean paste hydrolysates, 10 g/L peptone, 10 g/L yeast extract, 5 g/L corn steep liquor, 2 g/L K_2HPO_4, pH 7.0

6) LB medium (LBM): 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, pH 7.0

2.3 Analytical methods

2.3.1 Biomass

Broth samples were collected from flasks after cultivation at 37℃ for 24 hours and centrifuged at 2 340 g for 20 min. The precipitated biomass was washed twice with deionized water (DI) and the biomass concentration was determined by weighing the mass after drying at 100℃ overnight.[35]

2.3.2 GAD

The reaction mixture (pH 5.2) consisted of 200 μL of 50 mM sodium phosphate, 100 mM L-glutamate, 0.05 mM pyridoxal 5-phosphate monohydrate (PLP), and 100 μL of GAD containing liquid and was incubated at 40℃ for 60 min with periodic shaking. The reacted solution (1 mL, pH 8.7) was mixed with 1 mL of dabsyl chloride (1 mg/mL, in acetone) and reacted at 65℃ for 10 min, the pH of which was kept at 9 by adding 1 M $NaHCO_3$ solution. After that, the reaction was stopped by placing the sample tube in an ice bath, and then the dabsyl sample was filtered through a 0.45 μm nylon filter membrane[36,37]. The filtrate was analyzed for its GABA content by HPLC with a spectrophotometric detector at 440 nm. A reversed phase column (Hypersil ODS, 250 mm × 4.6 mm) coupled with a C18 cartridge was used. The column temperature was maintained at 40℃ and the flow rate was 1 mL/min. The composition of the optimized mobile phase was kept at 40% of acetonitrile and 60% of 0.006 M CH_3COONa (pH 4). One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of GABA in 1 h.

2.3.3 Organic acids

The content of organic acids in the culture broth was analyzed using an HPLC (Agilent Technologies, Inc., Santa Clara, CA) with a Bio-Rad Aminex HPX 87H column (Bio-Rad Laboratories, Hercules, CA) and a UV detector at 210 nm. The column temperature used was 40℃. The mobile phase, 6 mM H_2SO_4, was used at a flow rate of 0.6 mL/min[38].

All the experimental results presented were the mean values of at least three measurements (only data with variation ≤5% about the mean were used) on a minimum of three replicates (each an independent experiment) for every partition data point. One-way ANOVA was employed for statistical evaluation of the data at $p \leq 0.05$.

2.4 Economic analysis of culture media

Medium cost is calculated by summing up the costs of all the components in the medium for obtaining 10^3 units of GAD, which were determined with reference to the price supplied by the Fisher Scientific Inc. (Massachusetts, USA).

3 Results and discussion

3.1 Performances of various media on biomass production

The time courses of biomass accumulation by *E. coli* fermentation in six different culture media studied are shown in Figure 1. The highest production of biomass (4.5 g/L) was achieved in the cultivation with the TB

medium, which was not as high as that reported by Macaloney et al.[29] where *E. coli* was cultivated to a higher biomass concentration using the same medium (5.8 g/L). The lower biomass production in this study may be explained by the different *E. coli* strains used as well as the uncontrolled feeding strategy employed in this investigation.

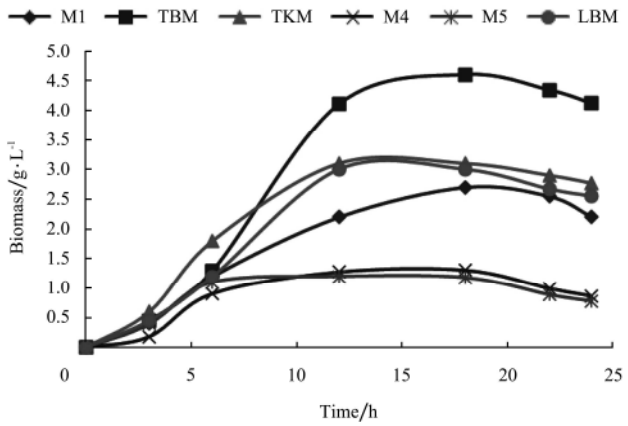


Figure 1 Performances of various media on the biomass accumulation

In the cultivations of M4 and M5, although the stationary phase was reached 6 hours after inoculation, which was faster than the rest, the maximum biomass obtained were lower than all other cultivations, ending with the amounts of 1.37 g/L and 1.3 g/L, respectively. The rapid cell growth in the early phase comparable to the others could be attributed to the presence of more than three nitrogenous nutrients in M4 and M5. Nevertheless, the low yield of biomass indicated the low efficiencies (substrate consumed/initial substrate) of these media, which could be caused by the unbalanced proportions of nutrients in the media, as reported by Vazquez et al.[39]. Another potential problem with these two media is the use of corn steep liquor as the nitrogenous materials. Although it is an inexpensive source of essential microbial nutrients for a variety of applications, the tiny particles of corn steep liquor could result in turbidity of culture broth and negatively affect the product purity, thus requiring additional downstream separation that could drive up the production cost[40,41].

Stationary cell growth in TKM was observed after 12-hour into cultivation and the sample yielded a higher dry cell weight of 3 g/L than those of M4 and M5. TKM subject to feeding strategies was reported to be a suitable medium for *E. coli* production[42]. In our case, the cease of the cellular growth in TKM could be the result of growth inhibition by the accumulated acid, which was supported by the low pH observed (4.69) in the cultivation (Table 1) under uncontrolled feeding in this study. For LBM and M1, the stable phases were reached after 18-hour cultivation, with the biomass content achieving 3.0 g/L and 2.75 g/L, respectively Figure 1.

The results suggest that the rapid cell growth in the initial stage of cultivation did not necessarily guarantee the final high biomass production possibly because of the fast accumulation of byproducts and/or the unbalanced nutrients supply, leading to a rapid inhibition to cell growth. This implies that it may be necessary to consider the inclusion of buffer solution in the culture media for *E. coli* or the employment of feeding strategies to improve cell growth. In addition, the production of biomass exhibited a declining trend after the exponential phase, which could result from cell lysis[43,44]. Take M1 and LBM, the biomass concentration was 12.5% and 20.4%, respectively, lower than at their peaks at around 18-hour cultivation. Thus, control of the cultivation time in the batch mode is important to avoid product losses.

Table 1 Summary of product yield and economic study results obtained for the various media investigated in shake flasks

Item	Medium					
	M1	TBM	TKM	M4	M5	LBM
Biomass/g · L^{-1}	2.73	4.5	3	1.35	1.3	2.5
Biomass productivity/g · $(L \cdot h)^{-1}$	0.09	0.17	0.12	0.04	0.03	0.11
GAD yield in final broth/ U · g^{-1} cell	2 353	3 811	1 805	2 299	2 423	4 431
GAD productivity/U · $(g \cdot h)^{-1}$	98.04	158.8	75.2	95.8	100.9	184.6
Total Units/U	5 200	15 700	5 000	2 001	1 890	11 300
Acetic acid/g · L^{-1}	1.85	0.7	1.73	2.78	6.24	0.5
Lactic acid/g · L^{-1}	0.11	2.3	0.26	0	1.9	1.01
Formic acid/g · L^{-1}	0.003	0.016	0	0.31	0.273	0.01
Initial pH	7	7	6.54	5.73	7.3	7.22
Adjusted pH			7	6.95	7	7
Final pH	6.81	6.59	4.69	4.83	4.37	8.19
*Medium cost $/$10^3$ U GAD	1.56	1.33	1.03	5.37	7.09	0.56

Note: *The costs of media are estimated based on the purchasing prices of media ingredients listed in the catalogs of suppliers (2010 edition) such as Thermo-fisher Scientific, Inc., Sigma-Aldrich Company, etc.

3.2 Performances of various media on GAD accumulation in *E. coli* cell

Figure 2 shows the time course of GAD obtained per gram biomass in different culture media. Although the highest yield of biomass was harvested from TBM, the highest yield of the enzyme per biomass (5 333 U/g) went with LBM, which was 1.15 times higher than TBM, and 1.34 times higher than M4. When TKM media were utilized for *E. coli* cultivations, only 3 296 U GAD/g biomass was detected in 12-h cultivations, which might suggest that TKM, a basic-salt defined medium, probably did not provide the balanced nutrients required by *E. coli* to synthesize GAD.

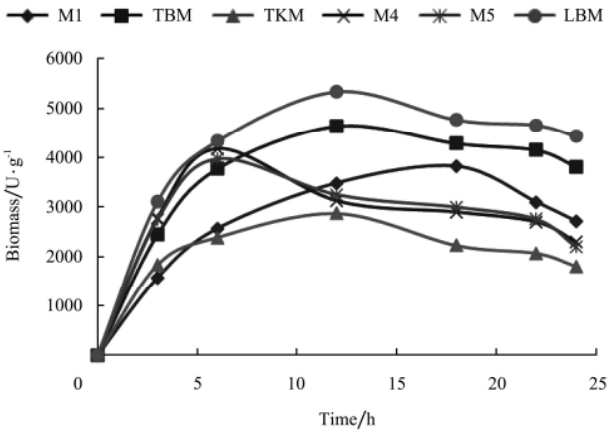


Figure 2 Performances of various media on the yield of GAD/g biomass

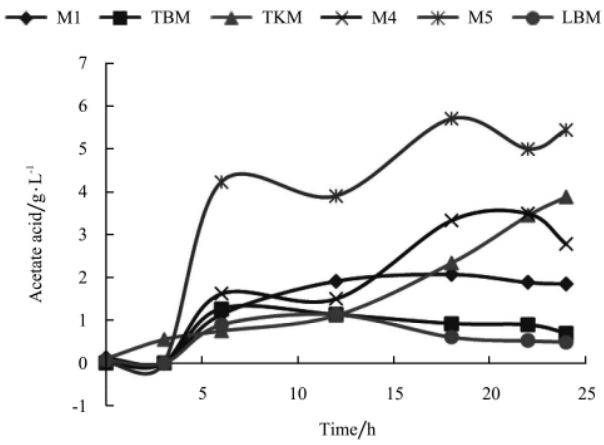
Data in Figure 2 also indicate the relationships between biomass production and enzyme accumulation. For all cultivations, the yields of GAD reached the peak when the cell growth reached stationary phase (Figures 1 and 2). Thus it can be concluded that the maximum yield of GAD correlated with the cell physiology and growth, which is consistent with the past findings that the rapid cell growth facilitated the accumulation of enzyme in cells[16,45]. However, the enzyme yield in all cases declined after the peak. The harvested GAD in LBM had the least loss of 16.91%, followed by TBM with 17.77% loss. One reason might be due to the unfavorable change in pH in the cultivation that poses a detrimental effect on the production of proteins by the cells.[46] Another reason could be due to cell lysis, which is common in *E. coli* cultivation[47,48], because the protease excreted during cell lysis[49,50] may cause the loss of GAD activity[16].

3.3 Effects of various media on the by-products generation

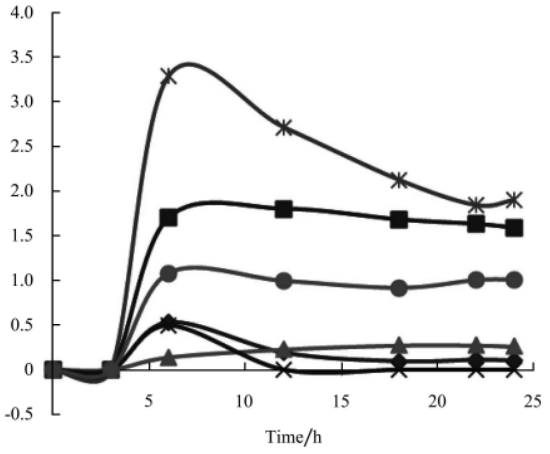
The aim of this part of the study was to evaluate the effects of various culture media on the byproducts formation such as acetic, formic, and lactic acid because byproducts accumulation in culture media could cause significant inhibition to the productivity of GAD by *E. coli*. The byproduct profiles for M1 displayed a distinct exponential phase (Figures 3a, 3b, 3c) after 6-hour cultivation with a rapid byproducts generation and the acetate accumulation was observed following the exponential phase. Similar trends were observed for TBM, M4, M5, and LBM. The observation of acetic acid accumulation after exponential phase can be explained by the over-flow metabolism of which acetate is the main by-product[51]. The initial byproducts accumulation indicated that the cultivation was dominated by acid fermentation due to shortage of oxygen as a result of the microbial cells inoculated into the cultivation that consumed oxygen to satisfy their rapid growth[52]. In addition, the complex nitrogen sources utilized in the culture broth could cause the increased viscosity that hampered oxygen dissolution[53,54]. When *E. coli* cultures are exposed to a degree of oxygen limitation, mixed acid fermentation products (acetic acid, formic acid, lactic acid, succinic acid, ethanol) will appear[55,56].

The exponential phase of M1 was followed by a phase showing a gradual decline of formic acid and lactic acid with the final contents being 0.18 g/L and 0.003 g/L, respectively. An explanation for this phenomenon could be found from a study reported by Enfors et al.[57] where re-assimilation of formic acid and lactic acid was observed when the cells returned to the substrate limited or oxygen sufficient compartment of the cultivation. In the case of TBM, the profiles of three acids showed a gradually decline after 6-hour cultivation (Figure 3) and the lactic acid concentration decreased to almost zero at the end of cultivation (0.002 g/L). This implies that the re-assimilation rate of lactic acid was higher than those of acetic and formic acid, which was in accordance with the results found in literature[58,59]. Additionally, since

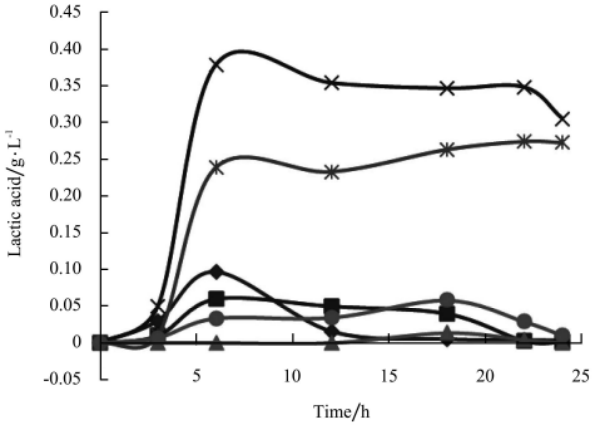
29.9% of acetic acid was re-assimilated, the byproduct inhibition to cell physiology was reduced, as reflected by the continuous cell growth in the latter period of cultivation. Coupled with pH control, it was observed that the loss of GAD accumulated (17.8%) was reduced. A similar trend was obtained for LBM with 56.2% of acetic acid re-assimilated, leading to the least loss of GAD accumulated (16.9%).



a. Acetate



b. Formic acid



c. Lactic acid

Figure 3 Performances of various media on the byproducts generation

When using M4 and M5, a fluctuation was observed for acetic acid accumulation, which could in part be caused by the repeated production and re-assimilation of acetic acid from the overflow metabolism and mixed acid fermentation products. The reduced biomass yield can be attributed to the fluctuation of acetic acid.[57]. The cultivation of M5 yielded the highest acetic acid concentration of 3.2 g/L at 6-hour incubation, while M4 corresponded to the highest lactic acid concentration of 0.35 g/L at the same time. An interesting and unexpected result was that the accumulated formic acid was consumed while the accumulated lactic acid was maintained after the peak. The reason that caused the fast consumption of formic acid was unclear. For TKM, although no obvious exponential phase was observed for acetic acid generation, its concentration continued to increase with no re-assimilation. On the other hand, the cultivation of TKM caused the least accumulation of lactic and formic acid.

3.4 Preliminary economic analyses for scaling - up production of GAD

Medium selection is a fundamental and challenging undertaking as it requires a balance between the cost of nutrients, product yield, and quality. A criterion used in this study for media evaluation for cost-effective GAD production is based on the GAD yield per cell as well as the total cell biomass. Another equally important part of an economic study is the productivity per batch, which helps determine the amount of capital and labor costs. Table 1 summarizes the batch results for the six media studied. The highest biomass (4.5 g/L) corresponded to TBM while the fastest accumulation of GAD in cell occurred in the cultivation with LBM with the productivity being 184.64 U/g-h. The comparison indicates that although LBM achieved lower biomass accumulation than TBM, it greatly increased the GAD productivity of each cell by 16.3% as compared to TBM, which is phenomenal. One thing that could not be determined according to the experiments in this study is whether the high biomass production by TBM was a result of the medium pH control. Buffer solution was contained in the formula of TBM, which showed the ability to maintain pH with the final value around 6.59.

On the contrary, the pH of LBM was not controlled, which reached 8.19 at the end of study and might be caused by the hydrolysis of the nitrogenous material into amino acids. This could be a reason for lower biomass production by LBM because as discussed early, the byproducts accumulation due to pH change would interfere with cell physiology, leading to less biomass formed. Cost wise, the significantly lower medium cost of LBM than TBM (0.56$ vs. 1.33$/$10^3$U, see Table 1) makes LBM even more appealing from the economics perspective. Therefore, based on the results from this study, it may be concluded that LBM can be a promising candidate that deserves further research in large-scale applications of GAD fermentation by *E. coli.* Having said that, it is understood that evaluation of the medium cost for large scale production of GAD involves the recognition and inclusion of all costs related to the medium production, such as the costs of material handling and storage, labor, and analytical requirements [60, 61]. It is our suggestion that all these factors should be taken into account in the next-step research using LBM in scale-up applications.

4 Conclusions

The study performed a comprehensive evaluation of the various reported media for GAD production. Cultivations in M4 and M5 exhibited the earliest exponential growth phase. However, the rapid growth in the initial cultivation did not lead to the final high biomass yield unless the byproduct accumulation could be controlled. The highest yield of biomass in the final broth (4.5 g/L) was achieved in the cultivation with TBM, but the highest yield of enzyme per biomass (4 431 U/g) corresponded to LBM, which was 1.16 times higher than the second highest from TBM. Similarly, the two highest performers in GAD productivity were LBM (184.6 U/g-h) and TBM (158.8 U/g-h).

For the byproducts, all media except TKM displayed a distinct exponential phase of a rapid byproduct generation after three hour cultivation, which could be attributed to the mixed acid fermentation caused by oxygen limitation. The subsequent re-assimilation of acetate could reduce the loss of GAD accumulated. LBM achieved the least loss of GAD (16.9%) with 56.2% of acetic acid re-assimilated, followed by TBM that achieved the second lowest loss of GAD (24.7%) with 29.9% of acetic acid re-assimilated. The economic analysis showed that the cheapest GAD production medium was LBM (0.56$/$10^3$ U). Based on the evaluation conducted in this study, it can be concluded that LBM can be a promising candidate medium for large-scale production of GAD.

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[References]

[1] Krogsgaard P, Gaba receptors, New York: Marcel Dekker Inc, 1989.

[2] Mody I, Dekoninck Y, Otis T S, Soltesz I. Bringing the cleft at gaba synapses in the brain. Trends Neurosci, 1994; 17: 517–525.

[3] Oh S H, Soh J R, Cha Y S. Germinated brown rice extract shows a nutraceutical effect in the recovery of chronic alcohol-related symptoms. J.Med.Food, 2003; 6: 115–121.

[4] Okada T, Sugishita T, Murakami T, Murai H, Saikusa T, Hotorino T, et al. Effect of the defatted rice germ enriched with gaba for sleeplessness, depression, autonomic disorder by oral administration. Nippon Shokuhin Kagaku Kaishi, 2000; 47.

[5] Oh S H, Oh C H. Brown rice extracts with enhanced levels of gaba stimulate immune cells. Food Science and Biotechnology, 2003; 12: 248–252.

[6] Hagiwara H, Seki T, Ariga T. The effect of pre-germinated brown rice intake on blood glucose and pai-1 levels in streptozotocin-induced diabetic rats. Bioscience Biotechnology and Biochemistry, 2004; 68: 444–447.

[7] Adeghate E, Ponery A S. Gaba in the endocrine pancreas: Cellular localization and function in normal and diabetic rats. Tissue & Cell, 2002; 34: 1–6.

[8] Huang J, Le-He M, Wu H, Lin D Q. Biosynthesis of gamma-aminobutyric acid (gaba) using immobilized whole cells of lactobacillus brevis. World Journal of Microbiology & Biotechnology, 2007; 23: 865–871.

[9] Aoki H, Furuya Y, Endo Y, Fujimoto K. Effect of gamma-aminobutyric acid-enriched tempeh-like fermented soybean (gaba-tempeh) on the blood pressure of spontaneously hypertensive rats. Bioscience Biotechnology

and Biochemistry, 2003; 67: 1806–1808.

[10] Hayakawa K, Kimura M, Kasaha K, Matsumoto K, Sansawa H, Yamori Y. Effect of a gamma-aminobutyric acid-enriched dairy product on the blood pressure of spontaneously hypertensive and normotensive wistar-kyoto rats. British Journal of Nutrition, 2004; 92: 411–417.

[11] Inoue K, Shirai T, Ochiai H, Kasao M, Hayakawa K, Kimura M, et al. Blood-pressure-lowering effect of a novel fermented milk containing gamma-aminobutyric acid (gaba) in mild hypertensives. European Journal of Clinical Nutrition, 2003; 57: 490–495.

[12] Wu J Y, Matsuda T, Roberts E. Purification and characterization of glutamate decarboxylase from mouse brain. Journal of Biological Chemistry, 1973; 248: 3029–3034.

[13] Yagasaki M, Azuma M, Ishino S, Ozaki A. Enzymatic production of d-glutamate from l-glutamate by a glutamate racemase. Journal of Fermentation and Bioengineering, 1995; 79: 70–72.

[14] Plokhov A Y, Gusyatiner M M, Yampolskaya T A, Kaluzhsky V E, Sukhareva B S, Schulga A A. Preparation of gamma-aminobutyric acid using e-coli cells with high activity of glutamate decarboxylase. Applied Biochemistry and Biotechnology, 2000; 88: 257–265.

[15] Ueno H. Enzymatic and structural aspects on glutamate decarboxylase. Journal of Molecular Catalysis B-Enzymatic, 2000; 10: 67–79.

[16] Yang S Y, Yu B, Lu Z X, Bie X M, Lin Q, Sun L J. Optimization of the culture conditions for production of glutamate decarboxylase by streptococcus salivarius ssp thermophilus. Journal of Chemical Technology and Biotechnology, 2008; 83: 389–392.

[17] Xu H, Liao P, Xiao J B, Zhang Q F, Dong Y J, Kai G Y. Molecular cloning and characterization of glutamate decarboxylase cdna from the giant-embryo oryza sativa. Archives of Biological Sciences, 2010; 62: 873–879.

[18] Wang L, Liu M, Lv Y G, Zhang H. Purification of calmodulin from rice bran and activation of glutamate decarboxylase by ca2+/calmodulin. Journal of the Science of Food and Agriculture, 2010; 90: 669–675.

[19] Baekkeskov S, Aanstoot H J, Christgau S, Reetz A, Solimena M, Cascalho M, et al. Identification of the 64k autoantigen in insulin-dependent diabetes as the gaba-synthesizing enzyme glutamic-acid decarboxylase. Nature, 1990; 347: 151–156.

[20] Yoon J W, Yoon C S, Lim H W, Huang Q Q, Kang Y, Pyun K H, et al. Control of autoimmune diabetes in nod mice by cad expression or suppression in beta cells. Science, 1999; 284: 1183–1187.

[21] Kaufman D L, Claresalzler M, Tian J D, Forsthuber T, Ting G S P, Robinson P, et al. Spontaneous loss of t-cell tolerance to glutamic-acid decarboxylase in murine insulin-dependent diabetes. Nature, 1993; 366: 69–72.

[22] Conrad B, Weidmann E, Trucco G, Rudert W A, Behboo R, Ricordi C, et al. Evidence for superantigen involvement in insulin-dependent diabetes-mellitus etiology. Nature, 1994; 371: 351–355.

[23] Denner L A, Wu J Y. Two forms of rat brain glutamic acid decarboxylase differ in their dependence on free pyridoxal phosphate. J.Neurochem., 1985; 44: 957–965.

[24] Nathan B, Hsu C C, Bao J, Wu R, Wu J Y. Purification and characterization of a novel form of brain l-glutamate decarboxylase a ca2+-dependent peripheral membrane-protein. Journal of Biological Chemistry, 1994; 269: 7249–7254.

[25] Johnson B S, Singh N K, Cherry J H, Locy R D. Purification and characterization of glutamate decarboxylase from cowpea. Phytochemistry, 1997; 46: 39–44.

[26] Oh S H, Choi W G, Lee I T, Yun S J. Cloning and characterization of a rice cdna encoding glutamate decarboxylase. Journal of Biochemistry and Molecular Biology, 2005; 38: 595–601.

[27] Rice E W, Johnson C H, Dunnigan M E, Reasoner D J. Rapid glutamate-decarboxylase assay for detection of escherichia-coli (vol 59, pg 4347, 1994). Applied and Environmental Microbiology, 1995; 61: 847–847.

[28] Kato Y, Kato Y, Furukawa K, Hara S. Cloning and nucleotide sequence of the glutamate decarboxylase-encoding gene gada from aspergillus oryzae. Bioscience Biotechnology and Biochemistry, 2002; 66: 2600–2605.

[29] Macaloney G, Draper I, Preston J, Anderson K B, Rollins M J, Thompson B G, et al. At-line control and fault analysis in an industrial high cell density escherichia coli fermentation, using nir spectroscopy. Food and Bioproducts Processing, 1996; 74: 212–220.

[30] Shiloach J, Bauer S. High-yield growth of escherichia-coli at different temperatures in a bench scale fermentor. Biotechnology and Bioengineering, 1975; 17: 227–239.

[31] Riesenberg D, Schulz V, Knorre W A, Pohl H D, Korz D, Sanders E A, et al. High cell-density cultivation of escherichia-coli at controlled specific growth-rate. Journal of Biotechnology, 1991; 20: 17–28.

[32] Matsui T, Yokota H, Sato S, Mukataka S, Takahashi J. Pressurized culture of escherichia-coli for a high-concentration. Agricultural and biological chemistry, 1989; 53: 2115–2120.

[33] Yang F, Li J H, Xu Y, Fang J. Optimization of fermentation condition for high-production glutamic acid decarboxylase (gad). Journal of Food Science and Biotechnology, 2008; 27: 107–111.

[34] Hu S Q, Zhou K X, Zhang E H. Cultivation conditions for l-glutamate decarboxylase. Jounrnal of ChongQing Teachers College, 1992; 9: 56–60.

[35] Thongchul N. Lactic acid production by immobilized

rhizopus oryzae in a rotating fibrous bed bioreactor. Ohio State University, 2005.

[36] Romero R, Bagur M G, Sanchez-Vinas M, Gazquez D. Optimization of experimental variables in the dabsyl chloride derivatization of biogenic amines for their determination by rp-hplc. Chromatographia, 2000; 51: 404–410.

[37] Syu K Y, Lin C L, Huang H C, Lin J K. Determination of theanine, gaba, and other amino acids in green, oolong, black, and pu-erh teas with dabsylation and high-performance liquid chromatography. Journal of Agricultural and Food Chemistry, 2008; 56: 7637–7643.

[38] Yao W Y, Wu X, Zhu J, Sun B, Miller C. Utilization of protein extract from dairy manure as a nitrogen source by rhizopus oryzae nrrl-395 for l-lactic acid production. Bioresource Technology, 2010; 101: 4132–4138.

[39] Vazquez J A, Gonzalez M P, Murado M A. Peptones from autohydrolysed fish viscera for nisin and pediocin production. Journal of Biotechnology, 2004; 112: 299–311.

[40] Rivas B, Moldes A B, Dominguez J M, Parajo J C. Development of culture media containing spent yeast cells of debaryomyces hansenii and corn steep liquor for lactic acid production with lactobacillus rhamnosus. International Journal of Food Microbiology, 2004; 97: 93–98.

[41] Demirci A, Pometto A L, Lee B, Hinz P N. Media evaluation of lactic acid repeated-batch fermentation with lactobacillus plantarum and lactobacillus casei subsp. Rhamnosus. Journal of Agricultural and Food Chemistry, 1998; 46: 4771–4774.

[42] Matsui T, Sato H, Yamamurob H, Misawa S, Shinzato N, Matsuda H, et al. High cell density cultivation of recombinant escherichia coli for hirudin variant 1 production. Journal of Biotechnology, 2008; 134: 88–92.

[43] Gardner J G, Keating D H. Requirement of the type ii secretion system for utilization of cellulosic substrates by cellvibrio japonicus. Applied and Environmental Microbiology, 2010; 76(15): 5079–5087.

[44] Castan A, Nasman A, Enfors S O. Oxygen enriched air supply in escherichia coli processes: Production of biomass and recombinant human growth hormone. Enzyme and Microbial Technology, 2002; 30: 847–854.

[45] Roychoudhury S, Parulekar S J, Weigand W A. Cell-growth and alpha-amylase production characteristics of bacillus-amyloliquefaciens. Biotechnology and Bioengineering, 1989; 33: 197–206.

[46] Krishna C, Chandrasekaran M. Banana waste as substrate for alpha-amylase production by bacillus subtilis (cbtk 106) under solid state fermentation. Applied Microbiology and Biotechnology, 1996; 46: 106–111.

[47] Shiloach J, Fass R. Growing e-coli to high cell density - a historical perspective on method development. Biotechnology Advances, 2005; 23: 345–357.

[48] Rinas U, Hoffmann F. Selective leakage of host-cell proteins during high-cell-density cultivation of recombinant and non-recombinant escherichia coli. Biotechnology Progress, 2004; 20: 679–687.

[49] Wang W, Hollmann R, Deckwer W D. Comparative proteomic analysis of high cell density cultivations with two recombinant bacillus megaterium strains for the production of a heterologous dextransucrase. Proteome Science, 2006; 4.

[50] Park S J, Georgiou G, Lee S Y. Secretory production of recombinant protein by a high cell density culture of a protease negative mutant escherichia coli strain. Biotechnology Progress, 1999; 15: 164–167.

[51] Xu B, Jahic M, Blomsten G, Enfors S O. Glucose overflow metabolism and mixed-acid fermentation in aerobic large-scale fed-batch processes with escherichia coli. Applied Microbiology and Biotechnology, 1999; 51: 564–571.

[52] Bajpai R K, Reuss M. A mechanistic model for penicillin production. Journal of Chemical Technology and Biotechnology, 1980; 30: 332–344.

[53] Nigam V K, Verma R, Kumar A, Kundu S, Ghosh P. Influence of medium constituents on the biosynthesis of cephalosporin-c. Electronic Journal of Biotechnology, 2007; 10: 230–239.

[54] Agarwal L, Isar J, Meghwanshi G K, Saxena R K. A cost effective fermentative production of succinic acid from cane molasses and corn steep liquor by escherichia coli. Journal of Applied Microbiology, 2006; 100: 1348–1354.

[55] Castan A, Enfors S O. Formate accumulation due to DNA release in aerobic cultivations of escherichia coli. Biotechnology and Bioengineering, 2002; 77: 324–328.

[56] Varma A, Boesch B W, Palsson B O. Stoichiometric interpretation of escherichia-coli glucose catabolism under various oxygenation rates. Applied and Environmental Microbiology, 1993; 59: 2465–2473.

[57] Enfors S O, Jahic M, Rozkov A, Xu B, Hecker M, Jurgen B, et al. Physiological responses to mixing in large scale bioreactors. Journal of Biotechnology, 2001; 85: 175–185.

[58] Soini J, Ukkonen K, Neubauer P. High cell density media for escherichia coli are generally designed for aerobic cultivations - consequences for large-scale bioprocesses and shake flask cultures. Microbial Cell Factories, 2008; 7.

[59] Bylund F, Castan A, Mikkola R, Veide A, Larsson G. Influence of scale-up on the quality of recombinant human growth hormone. Biotechnology and Bioengineering, 2000; 69: 119–128.

[60] Danquah M K, Forde G M. Growth medium selection and its economic impact on plasmid DNA production. Journal of Bioscience and Bioengineering, 2007; 104: 490–497.

[61] Castilho L R, Polato C M S, Baruque E A, Sant' Anna G L, Freire D M G. Economic analysis of lipase production by penicillium restrictum in solid-state and submerged fermentations. Biochemical Engineering Journal, 2000; 4: 239–247.