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ORIGINAL PAPER

Fishmeal Wastewater as A Low-Cost Nitrogen Source for γ-Polyglutamic Acid Production Using *Bacillus subtilis*

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Abstract Fishmeal wastewater, a fishmeal processing waste, was used as culture medium to study the effect of *Bacillus subtilis* A3 on the production of γ-polyglutamic acid (γ-PGA). The results showed that the optimum concentration of chemical oxygen demand (COD_{Cr}) for fishmeal wastewater was 15 g/L. Moreover, addition of 30 g/L glucose and 25 g/L glutamic acid in the medium was beneficial to cell growth and production of γ-PGA. The study also showed that the high salinity of wastewater had little effect on cell growth and production of γ-PGA after dilution. Thus, the optimal medium consisted of COD 15, 30 g/L glucose, 25 g/L glutamic acid, in which the average yield of γ-PGA (25.07 ± 0.34 g/L) was obtained. The study suggested that fishmeal wastewater can be a replacement for nitrogen source for γ-PGA production, and hence it can be the cost-effective alternative in γ-PGA production. Meanwhile, the process can offset the disposal costs of the wastes.

Keywords *Bacillus subtilis* · Fishmeal wastewater · Nitrogen source · γ-Polyglutamic acid

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Introduction

A natural polymer, the γ-polyglutamic acid (γ-PGA) is composed of D- and L-glutamic acid moieties interconnected via γ-amide linkages [1–4]. This unusual anionic polymer has many advantages. For example, it is biodegradable, water soluble, and edible. And so it is non-toxic to both human and environment [5–11]. These unique properties make γ-PGA a promising product with potential applications in food, pharmaceutical and cosmetic industries. γ-PGA, in fact, has already found applications in a wide range of other industries, from textiles to fertilizer [12–18].However, the high cost of γ-PGA production is a major barrier to its commercialization, which is partially associated with the high cost of media composition. Therefore, using a low cost media composition is crucialin reducing the cost of γ-PGA production. Much research has consequently focused on finding cheaper supplements for the γ-PGA production. And waste material is one of the important options. However, only a few studies have been reported regarding the γ-PGA production using waste materials [19, 20, 23]. For example, Zhang et al. [23] reported that the low-cost canemolasses can be used for the environmental-friendly and economicalproduction of γ-PGA by *B. subtilis* NX-2.

Nitrogen source is usually the most expensive component of a medium [21–23]. Most studies have been focused on finding cheaper carbon sources, of which only a few have addressed the use of cheaper nitrogen source. For example, Hoppensack et al. [19] and Xiong chen et al. [20] reported that *Bacillus licheniformis* and *B. subtilis* could be used with swine manure for the γ-PGA production in the presence of sodium gluconate or citrate and glycerol [19, 20]. However, there has been concern with the safety of γ-PGA produced in culture media containing swine manure. Fishmeal wastewater is a waste byproduct of the fishmeal processing plants

and its disposal can cause serious environmental problems. On the other hand, it contains a lot of soluble proteins. So it can be a candidate for nitrogen source for γ-PGA production. In addition, the process can offset the disposal costs of the wastes. And this is the first attempt to use fishmeal wastewater as nitrogen source for γ-PGA production.

Bacillus species have long been established as industrial workhorses for the production of products ranging from hydrolytic enzymes, such as proteases and alpha-amylases, tospecialty chemicals, such as amino acids and vitamins [24–27]. Currently, γ-PGA is mainly generated by *B. subtilis. B. subtilis* is capable of secreting copious amounts of γ-PGA under certain conditions, indicative of their highly developed biosynthetic capacity [12–15]. In addition, *B. subtilis* can produce γ-PGA with lower nutrition requirements, and so it is very economical to be applied in industrial fermentors [16–18]. We therefore chose *B. subtilis* A3 for the production of γ-PGA in this study.

In this work, we tested the feasibility of using fishmeal wastewater as nitrogen source for the economical production of γ-PGA by *B. subtilis* A3, and meanwhile we tried to make the most of this abundant waste to develop a new way for γ-PGA production.

Materials and Methods

Strain

Bacillus subtilis A3, purchased from China Center of Industrial Culture Collection (CICC) as CICC 20646, was used as the working strain.

Fishmeal Wastewater

The fishmeal wastewater used in this study was effluent coming from Tongda Fishmeal Co Ltd, Weihai, China.The characteristic parameters of the fishmeal wastewater were shown in Table 1.

Table 1 Composition and characteristics of fishmeal wastewater

Parameter	Value
pH	6.6–7.0
TSS (g/L)	58–61
VSS (g/L)	57–60
BOD_5 (g/L)	28–30
COD_{Cr} (g/L)	66–68
NH_3-N (g/L)	1.9-2.0
TN (g/L)	5.0–5.3
Salinity (g/L)	9.6–10.0
Total sugar (g/L)	1.03–1.09
Reduced sugar (g/L)	0.17–0.19
Lipid content (g/L)	3.90–3.95

TSS Total suspended solid, *VSS* volatile suspended solid, COD_{Cr} chemical oxygen demand, *TN* total nitrogen, BOD_5 biochemical oxygen demand

Media

Media used in this work were prepared with the following recipes:

Slant medium (SM), in g/L: glucose, 20; yeast extract, 10; L-glutamate, 20; NaCl, 5; agar 18. The pH was adjusted to 7.0 by HCl or NaOH.

Seed medium (SM), in g/L:glucose, 20; yeast extract, 10; L-glutamate, 20; $K_2HPO_4{\cdot}3H_2O$, 2; $MgSO_4$, 0.1; $MnSO_4$, 0.03.

The optimal fermentation medium without wastewater for *B. subtilis* A3, in g/L: glucose, 36; tryptone, 9; L-glutamate, 28; $K_2HPO_4{\cdot}3H_2O$, 2; $MgSO_4$, 0.25 [28].

Fermentation medium (FM), in g/L: glucose, 30; COD_{Cr} of fishmeal wastewater, 15; L-glutamate, 25. The pH was adjusted to 7.0 by HCl or NaOH.

These media were autoclaved at 121 °C for 20 min.

Shake Flask Experiment

A loopful of cells from the slant were transferred into 250 ml flask containing 30 ml seed medium and cultivated on a rotary shaker operating at 37 ℃ and 180 rpm for 24 h. The flask culture was used as seed culture.

The γ-PGA production by *B. subtilis* A3 was conducted in a 250 ml flask containing 50 ml fermentation medium inoculated with 5 ml of the seed culture. The flask was cultivated on a rotary shaker operating at 37 °C and 180 rpm for 48 h [28]. Three replicates were performed for each experiment.

Fermentation

Fermentation experiments were conducted in 5-L bioreactor (Baoxing Corp., Shanghai, China) with an agitation of 200 rpm, in which 3-L FM media were initially used for fermentation. The temperature and pH of the fermentative broth were maintained at 37 °C and 7.0. The fermentation process lasted for 60 h. Samples were taken at predetermined intervals for γ-PGA production and biomass accumulation analysis.

Analytical Methods

Total suspended solid (TSS), volatile suspended solid (VSS), chemical oxygen demand (COD_{Cr}), Biochemical oxygen demand (BOD_5), NH_3–N, salinity and total nitrogen (TN) in the fishmeal wastewater were measured according

to the procedure described in Standard Methods for the Examination of Water and Wastewater [29]. Lipid contents in the fishmeal wastewater were estimated by the chloroform–methanol method [29]. Total carbohydrate (TC) and glucose content of fishmeal wastewater were estimated by the dinitrosalicylic acid method [30].

The culture broth was centrifuged and the resulting supernatant was used to measure COD and glucose. COD was measured according to the procedure described in Standard Methods for the Examination of Water and Wastewater [29]. The glucose contents were estimated by the dinitrosalicylic acid method [30].

The volumetric yield of γ-PGA was measured by gel permeation chromatography (GPC) system following the method reported previously [31]. Dry cell weight was determined after the cells were precipitated from 30 ml fermentation broth, washed once with distilled water, and dried at 105 °C overnight.

Results and Discussion

The Feasibility of Using Fishmeal Wastewater in γ-PGA Production

γ-PGA -producing strains are divided into two types: L-glutamate-dependent and L-glutamate-independent strains. For the former, γ-PGA production depends on the existence of glutamic acid in the medium, but the latter can produce considerable amount of γ-PGA without glutamic acid. As an L-glutamate-dependent bacterium, *B. subtilis* A3 cannot produce the metabolite γ-PGA without glutamate in medium. And from the optimal fermentation medium for *B. subtilis* A3 (36 g/L glucose, 9 g/L tryptone, 28 g/L L-glutamate, 2 g/L $K_2HPO_4{\cdot}3H_2O$, 0.25 g/L $MgSO_4$), we concluded that exogenous sugars and glutamate were necessary for γ-PGA production and cell growth. Therefore, γ-PGA fermentation was investigated using fishmeal wastewater under the following conditions: Fishmeal wastewater only; Fishmeal wastewater + Glucose (10 g/L); Fishmeal wastewater + Glutamate (10 g/L); Fishmeal wastewater + Glucose (10 g/L) + Glutamate (10 g/L). The results are shown in Table 2.

Table 2 The feasibility of using fishmeal wastewater

Strategies	γ-PGA (g/L)	Biomass (g/L)
Only fishmeal wastewater	1.89 ± 0.10	2.73 ± 0.13
Fishmeal wastewater + glucose	4.11 ± 0.10	3.13 ± 0.13
Fishmeal wastewater + glutamate	3.89 ± 0.10	2.98 ± 0.11
Fishmeal wastewater + glucose + glutamate	5.81 ± 0.12	3.92 ± 0.15

In the first experiment, when the fermentation medium contained only fishmeal wastewater, a small amount of metabolite was observed in this broth. This suggests that *B. subtilis* A3 can produce γ-PGA in this medium, for fishmeal wastewater may contain glutamate or some components that function as glutamate. In addition, the fishmeal wastewater contained some organic and inorganic components that can be efficiently utilized in the γ-PGA production as carbon source, and the high salt content in the effluent could not completely inhibit cell growth. Therefore, it is feasible for fishmeal wastewater to be used for γ-PGA production by *B. subtilis* A3. In the second and third experiments, the results revealed that the exogenous glucose or glutamate could improve γ-PGA production. This shows that the fishmeal wastewater medium lacked exogenous carbon source and glutamate. In the fourth experiment, the results revealed that the exogenous glucose and glutamate contributed a lot to γ-PGA production and cell growth. However, the yield of γ-PGA was too low. From the above results, we learned that the optimization of the conditions in the medium, such as the concentration of fishmeal wastewater, the concentration of carbon source and L-glutamate, can help increase the yield. The details will be discussed in the following sections.

Effect of Concentration of Fishmeal Wastewater on γ-PGA Production

The effect of the initial fishmeal wastewater concentration in the fermentation medium on γ-PGA production was studied with shake flasks and the results are shown in Fig. 1. Fishmeal wastewater with COD ranging between 5 and 20 g/L had a comparatively higher yield of γ-PGA and biomass. The maximum production of γ-PGA and biomass were about 13.62 ± 0.19 and 4.34 ± 0.11 g/L at COD 15 g/L, respectively. Otherwise stated, fishmeal

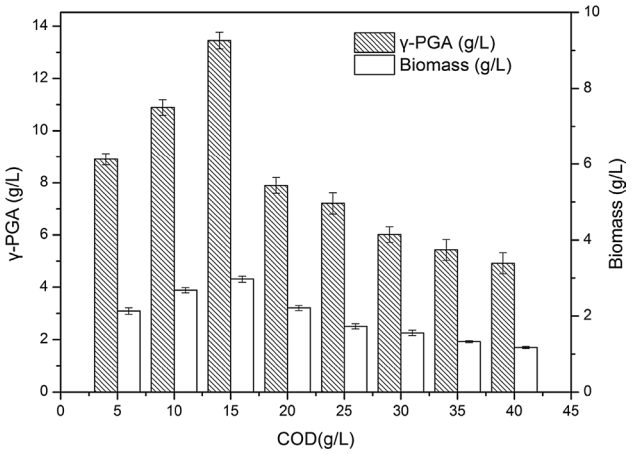

Fig. 1 Effect of concentration of fishmeal wastewater on biomass and γ-PGA production

wastewater with COD 15 g/L was chosen in the following experiments.

Effect of Different Carbon Sources on Biomass and γ-PGA Production

In order to identify the effect of different carbon sources on biomass and γ-PGA production, *B. subtilis* A3 was cultivated in the fishmeal wastewater medium with different carbon sources (glucose, lactose, maltose, citric acid and glycerol). The results are shown in Table 3. Lactose and maltose were conducive to *B. subtilis* A3 cell growth, but had no effect on γ-PGA production. In the case of glucose, this *B. subtilis* A3 had a higher production of γ-PGA.

Effect of Glucose Concentration on Biomass and γ-PGA Production

The effect of the exogenous glucose concentration in the fishmeal wastewater medium on γ-PGA production was studied with shake flasks and the results are shown in Fig. 2. The yields of γ-PGA and biomass were enhanced with the increase of glucose concentration at an optimum level of 30 g/L. Any further increase in glucose concentration resulted in a decrease of γ-PGA production and biomass. This phenomenon can be partly attributed to the repressive effect of glucose on *B. subtilis* A3 growth. In addition, from the optimal fermentation medium without wastewater for *B. subtilis* A3 (36 g/L glucose, 9 g/L tryptone, 28 g/L L-glutamate, 2 g/L $K_2HPO_4{\cdot}3H_2O$, 0.25 g/L $MgSO_4$) and Table 1 (total sugar and lipid content), sugar, lipid or other components in fishmeal wastewater, which can be used as carbon source in γ-PGA production can greatly reduce the amount of exogenous glucose.

Table 3 Effect of different carbon sources on the production of γ-PGA and biomass by *B. subtilis* A3 in the fishmeal wastewater

Carbon	Level (g/L)	γ-PGA (g/L)	Biomass (g/L)
Glucose	5	8.67 ± 0.12	4.16 ± 0.13
	10	13.62 ± 0.17	4.36 ± 0.21
Citric acid	5	4.87 ± 0.12	2.96 ± 0.08
	10	7.92 ± 0.11	3.01 ± 0.11
Glycerol	5	5.80 ± 0.12	3.06 ± 0.11
	10	8.65 ± 0.14	3.17 ± 0.12
Maltose	5	0	4.78 ± 0.15
	10	0	5.14 ± 0.11
Lactose	5	0	5.07 ± 0.16
	10	0	6.54 ± 0.12

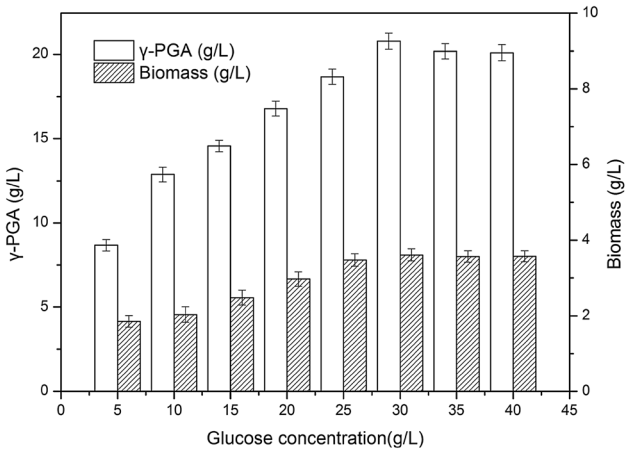

Fig. 2 Effect of different amounts of glucose on the yields of γ-PGA and biomass

Effect of L-Glutamate Concentration on Biomass and γ-PGA Production

As an L-glutamate-dependent bacterium, *B. subtilis* A3 could not produce γ-PGA without glutamate in medium. Therefore, the exogenous glutamate concentration would greatly affect γ-PGA production and biomass. The effect of L-glutamic acid on the production of γ-PGA by *B. subtilis* A3 was investigated with concentrations in the range of 5–40 g/L, and with 30 g/L exogenous glucose as carbon source (Fig. 3). When glutamate concentration increased from 5 to 25 g/L, despite the repression of biomass by high concentration of glutamate, the yield of γ-PGA increased from 6.67 ± 0.34 to 24.67 ± 0.45 g/L. In addition, from the optimal fermentation medium without wastewater for *B. subtilis* A3 (36 g/L glucose, 9 g/L tryptone, 28 g/L L-glutamate, 2 g/L $K_2HPO_4{\cdot}3H_2O$, 0.25 g/L $MgSO_4$) and

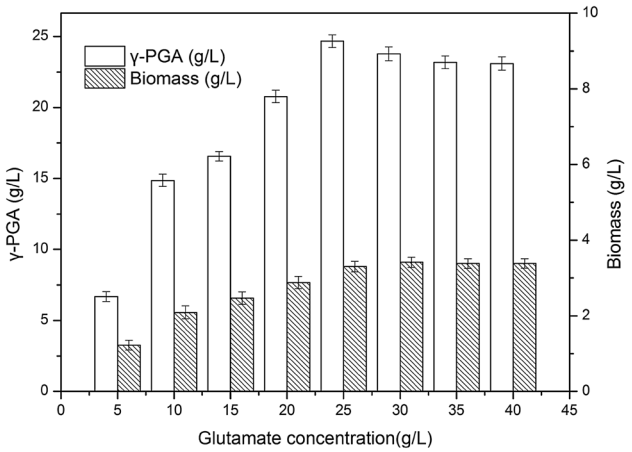

Fig. 3 Effect of different amounts of glutamate on the yields of γ-PGA and biomass

Table 1(NH_3–N), the amino acids or some components in fishmeal wastewater that function as glutamate in γ-PGA production can reduce the amount of exogenous glutamate.

Effect of NaCl Concentration on Biomass and γ-PGA Production

The effluent contained a large amount of salt and it was necessary to investigate the effect of NaCl concentration on growth and γ-PGA production of *B. subtilis* A3. This investigation was carried out in COD 15 g/L fishmeal wastewater medium with addition of 30 g/L glucose. The relationship between NaCl concentration, biomass and production of γ-PGA was shown in Fig. 4. The production of γ-PGA and biomass were greatly affected by NaCl concentrations. *B. subtilis* A3 could be easily metabolized with NaCl concentration below 4.0 g/L, while the growth was completely stopped when NaCl concentration was above 12 g/L. However, from the results in Fig. 1 and Table 1, the salinity of diluted wastewater was below 2.3 g/L, which had little effect on cell growth and production of γ-PGA.

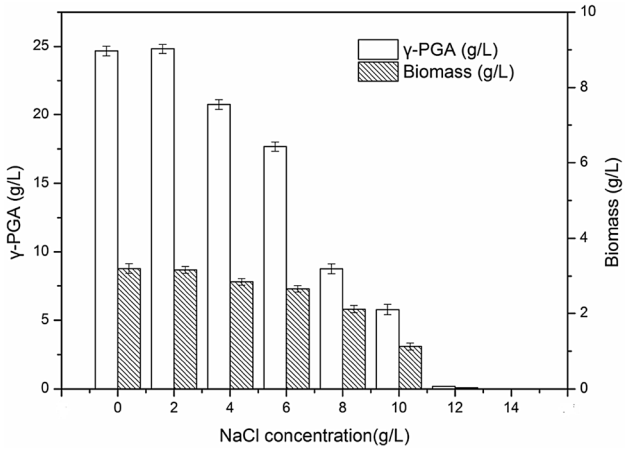

Fig. 4 Effect of NaCl concentration on the yields of γ-PGA and biomass

Comparison of Different Nitrogen Sources on Biomass, γ-PGA Production and Prize

To evaluate the potential use of wastewater as nitrogen source for the economical production of γ-PGA, *B. subtilis* A3 was cultivated in the same medium with 9 g/L different nitrogen sources (peptone, tryptone, yeast extract, ammonium sulfate, soy bean, maize flour, fish protein and fishmeal wastewater), *B. subtilis* A3 cultivated in the optimal fermentation medium without wastewater was used as the control group.

The results in Table 4 show that tryptone group and control group (t-test, data not presented) had the same beneficial effect on γ-PGA production, which suggests that fishmeal wastewater could be a replacement for nitrogen source for γ-PGA production. It also can be seen that tryptone (29.01 ± 0.30 g/L γ-PGA) and fish protein (27.05 ± 0.12 g/L γ-PGA) had more beneficial effect on γ-PGA production than the fishmeal wastewater (25.01 ± 0.32 g/L γ-PGA). However, the prices of tryptone and fish protein were dozens of times those of fishmeal wastewater, and the γ-PGA production with tryptone medium was just improved by 16% compared to that with fishmeal wastewater. Additionally, the results could explain why fishmeal wastewater can be a replacement for nitrogen source for γ-PGA production, which is the good performance of fish protein.

Time Course of γ-PGA Production

Based on the above results obtained in flask, the effects of initial concentrations of glucose, fishmeal wastewater and glutamate were studied to optimize the culture conditions for γ-PGA production. Therefore, the batch fermentation of fishmeal wastewater was carried out in 5-L bioreactor, and γ-PGA production during the growth of *B. subtilis*

Table 4 Comparison of different nitrogen sources

Nitrogen source	48 h		54 h		Prize (kg/dollar)
	γ-PGA (g/L)	Biomass (g/L)	γ-PGA (g/L)	Biomass (g/L)	
Peptone	22.85 ± 0.12	8.55 ± 0.28	22.34 ± 0.11	8.443 ± 0.22	2.5–3.0
Tryptone	29.01 ± 0.30	9.90 ± 0.33	29.32 ± 0.24	9.78 ± 0.25	5.5–6.0
Yeast extract	22.47 ± 0.31	8.45 ± 0.29	21.86 ± 0.28	8.47 ± 0.28	3.0–3.5
Ammonium sulfate	0	1.21 ± 0.12	0	1.23 ± 0.15	0.2–0.3
Soy bean	18.32 ± 0.25	8.01 ± 0.29	17.78 ± 0.20	8.05 ± 0.26	0.7–0.8
Maize flour	19.37 ± 0.18	7.86 ± 0.19	19.12 ± 0.14	7.90 ± 0.21	0.4–0.5
Fish protein	27.05 ± 0.12	8.99 ± 0.24	26.92 ± 0.10	9.01 ± 0.21	1.6–1.8
Fishmeal wastewater	25.01 ± 0.32	8.81 ± 0.22	24.67 ± 0.30	8.68 ± 0.20	0-0.1
Control group	29.39 ± 0.21	9.79 ± 0.21	29.14 ± 0.19	9.70 ± 0.13	5.5-6.0

Control group: *B. subtilis* A3 is cultivated in the optimal fermentation medium without wastewater

A3 is shown in Fig. 5. After 4 h of cultivation, the cells started to grow, when the concentration of glucose began to decrease. Meanwhile, γ-PGA was secreted to the broth, the concentration of γ-PGA rapidly increased during exponential phase and reached a plateau in the stationary phase. γ-PGA production was accompanied by the simultaneous consumption of glucose. No further γ-PGA was produced after the depletion of glucose. The highest γ-PGA yield was 25.07 ± 0.34 g/L at a fermentation time 48 h. Furthermore, to test the effects of changes in the composition content on the fermentation, we repeated the experiment ten times and assayed the γ-PGA production. The results did not show any significant difference (t-test, data not presented). Thus we can conclude that small variations in the content of nutrient composition will not affect the final output.

Conclusions

In this study, γ-PGA production was investigated with fishmeal wastewater treatment integrated by *B. subtilis* A3. The results showed that the optimum concentration of chemical oxygen demand for fishmeal wastewater was 15 g/L. Moreover, addition of 30 g/L glucose and 25 g/L glutamic acid in the medium was beneficial to cell growth and production of γ-PGA. The study also showed that the high salinity of wastewater had little effect on cell growth and production of γ-PGA after dilution. Thus, the optimal medium consisted of COD 15, 30 g/L glucose, 25 g/L glutamic acid, in which the average yield of γ-PGA (25.07 ± 0.34 g/L) was obtained. In addition, the results demonstrated that fishmeal wastewater can be the cost-effective alternative for γ-PGA production, the amino acids or some components in fishmeal wastewater that function as glutamate in γ-PGA production can reduce the amount of exogenous glutamate, and the sugar and lipid in wastewater also can reduce the amount of exogenous glucose. Therefore, fermentation of fishmeal wastewater can be a green and economical option for γ-PGA production, and the process can offset the disposal costs of the wastes.

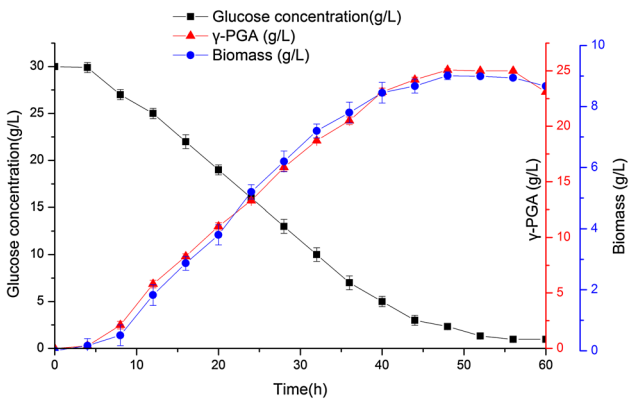

Fig. 5 Kinetics of glucose, γ-PGA and biomass

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