

Original Research Article

Influence of Ammonium Salt and Fermentation pH on Acarbose Yield from *Streptomyces* M37

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Abstract

Purpose: To investigate the effect of ammonium salts and fermentation pH on the biosynthesis of acarbose by *Streptomyces* M37.

Methods: Different ammonium salts were added to the fermentation broth of *Streptomyces* M37 to explore their effects on acarbose production. The concentration and addition time of ammonium salts, and the fermentation pH on acarbose biosynthesis were investigated. To study the effect of pH on acarbose yield, the activities of glutamate dehydrogenase (GDH) and glucose-6-phosphate dehydrogenase (G6PDH) were also studied.

Results: The optimal supplemental concentration and addition time of NH₄Cl were 0.1 M and 72 h, respectively. Regulation of pH at 8.5 until the stationary phase was reached favoured acarbose production. Furthermore, GDH and G6PDH exhibited higher activity at pH 8.5 than at other pHs. Increase in acarbose yield was 53.4 % (compared with control) by NH₄Cl, and was approximately 5460 mg/L.

Conclusion: The results suggest that the addition of NH₄Cl and maintenance of fermentation pH at 8.5 for 72 h after inoculation was an effective strategy for enhancing acarbose biosynthesis.

Keywords: Acarbose, Ammonium salts, pH, *Streptomyces*, Glutamate dehydrogenase, Glucose-6-phosphate dehydrogenase, Biosynthesis, Fermentation

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INTRODUCTION

Diabetes mellitus is a chronic disease caused by inherited and/or acquired deficiency in production of insulin by the pancreas, or by the ineffectiveness of the insulin produced. If it is not controlled, diabetes can lead to serious chronic complications in the eyes, kidneys, peripheral nerve system and arteries, and result in impaired quality of life, disability and mortality [1].

As a competitive α -glucosidase inhibitor, acarbose is widely used in the therapy of non-

insulin-dependent diabetes mellitus owing to its good therapeutic and non-toxic effects [2,3].

Acarbose, composed of an aminocyclitol and valienamine linked via a nitrogen bridge to C-4 of 6-deoxy-D-glucose, is produced by strains of the genus *Actinoplanes* [4]. Many aspects of acarbose fermentation have been well studied and reported, ranging from producer strains, media formula, fermentation conditions, and acarbose isolation and purification [5-9]. The importance of nitrogen sources in industrial microbiology is very significant since it affects the

synthesis of enzymes involved in both primary and secondary metabolism [10-12]. It is known that glutamate is the primary source of N-glycosidic bond in the biosynthesis of acarbose [13]. Wang *et al* [7] found that raising monosodium glutamate (MG) concentration up to 5 g/L promoted acarbose production with *A. utahensis* ZJB-08196. However, the effects of inorganic nitrogen source and pH on acarbose fermentation have not been reported thus far.

In this study, the effect of concentration of NH₄Cl and its addition time on the biosynthesis of acarbose by *Streptomyces* M37 were determined. The impact of fermentation pH and the activities of glutamate dehydrogenase (GDH) and glucose-6-phosphate dehydrogenase (G6PDH) on acarbose yield were also investigated.

EXPERIMENTAL

Microorganism

Streptomyces M37 (GenBank KJ888155) with high acarbose yield was isolated and mutated in our laboratory and used for acarbose production. The strain was maintained in a 20 % (v/v) sterile glycerol stock solution and stored at -80 °C. Before carrying out the study, *Streptomyces* M37 was activated on agar plates at 28 °C for about 6 days until visible pink colonies emerged. The components (in g/L) of agar medium were: sucrose, 20; K₂HPO₄•3H₂O, 1.0; FeSO₄•7H₂O, 0.02; MgSO₄, 1.0; NaNO₃, 2.0; peptone, 1.0; agar, 20. Unless otherwise stated, the initial pH value of all media was adjusted to 7.4 with 1 M NaOH prior to sterilization, and all media were sterilized by steam autoclaving at 121 °C for 20 min.

Medium and culture conditions

For inoculation, a colony of about 1 × 1 cm² from a freshly prepared agar plate was inoculated into a 500 mL Erlenmeyer flask containing 100 ml of seed medium and cultivated at 28 °C, 200 rpm for 72 h. The seed cultures for the fermentation inoculum were prepared by medium which consisted of the following (in g/L) starch, 15; glucose, 20; soybean meal, 15; peptone, 2.0; yeast extract, 1.0; K₂HPO₄•3H₂O, 1.0; MgSO₄, 1.0; CaCO₃, 3.0.

Acarbose fermentation

Batch and fed-batch fermentation was carried out in 500 mL Erlenmeyer flasks. A 10.0 mL of seed culture was used to inoculate 90 mL of

fermentation medium. Batch fermentation was carried out at 28 °C and 200 rpm for 7 days, in a medium which contained (in g/L): sucrose, 30; maltose, 25; soybean meal, 20; peptone, 2.0; yeast extract, 1.0; NaNO₃, 1.0; K₂HPO₄•3H₂O, 1.5; MgSO₄, 1.0; FeSO₄•7H₂O, 0.03; and CaCO₃, 3.0.

After 72 h of cultivation, MG or ammonium salts (NH₄Cl, (NH₄)₂SO₄ and NH₄NO₃) were added separately in the cultivation medium. According to results of our preliminary experiments, ammonium salt containing 0.1 M of NH₄⁺ was added to the fermentation medium at different cultivation times: 48, 72, 96 and 120 h.

The experiment to investigate the effect of different concentrations of NH₄Cl on acarbose production was carried out at initial pH of 7.4. After the addition of 0.1 M NH₄Cl at 72 h, the pH was adjusted to 7.0 with 2 M NaOH. The experiment on the effect of different pH values (ranging from 6.0 to 10.0) on acarbose production was carried out in 5 L fermentor with a working volume of 3.0 L. The fermentation temperature was 28 °C and the impeller speed was set at 150 rpm with the aeration rate of 1 vol/min. The pH was maintained stable at required values according to the experimental need during the fermentations with 2 M HCl or 2 M NaOH.

Analytical methods

The mycelia were harvested by centrifugation (10 min, at 9,000 × g) and suspended in 10 mL of 10 mM potassium phosphate buffer (pH 7.2) containing 2 mM ethylene diamine tetraacetic acid (EDTA) and 2 mM dithiothreitol (DTT). The mycelia were sonicated on ice for 15 min and cell debris was removed by centrifugation (9000 ×g) for 30 min at 4 °C. The supernatant was used as crude enzyme for measuring GDH activity.

Reductive GDH activity was measured spectrophotometrically at 30 °C by monitoring the oxidation of NADH or NADPH at 340 nm. The 3 mL reaction mixture contained 13 mM 2-oxoglutarate, 1.7 mM NADH or NADPH, 15 mM NH₄Cl, 15 mM NH₃•H₂O, or 7.5 mM (NH₄)₂SO₄ was added separately for the reductive GDH activity. Reactions were started by adding the enzyme. One unit of activity was defined as the amount of enzyme catalyzing the oxidation of 1 μmole of the substrate per min at 30 °C. Protein was measured by the method of Bradford using bovine serum albumin (Sigma) as a standard [14]. G6PDH was detected according to Ruijter [15].

The dry cell weight (DCW) was determined according to Wang *et al* [8]. pH was determined by using a pH meter. Glucose was estimated with a SBA-40E biosensor (Shangdong, China). Maltose was determined by ion chromatography according to Xue *et al* [16]. Acarbose titer was analyzed by HPLC according to Li *et al* [5]. NH_4^+ -N was determined according to Mulder *et al* [17].

Statistical analysis

All experiments were performed in triplicate. One-way analysis of variance (ANOVA) was performed to determine significant differences among variables. Differences with a probability value of <0.05 were considered significant and all data were reported as mean \pm SD. Statgraphics Centurion XV program (version 15.1.02) was used.

RESULTS

Effect of time on acarbose fermentation

The time course studies on acarbose yield by *Streptomyces* M37 were carried out for a period of 168 h in the fermentation media, as shown in Figure 1. After 24 h of lag phase, *Streptomyces* M37 entered the exponential growth phase and reached the maximum DCW of 21.5 g/L at 72 h. The microorganism entered stationary phases after 72 h cultivation. Acarbose production

increased significantly to the maximum of 3560 mg/L within 120 h and tended to be stable afterwards. The pH value of the fermentation broth decreased to 5.9 after 96 h of cultivation. The pH value decreased to 5.9 after 96 h during the fermentation. And the final pH was lower than 5.6. Actinomycetes prefer to grow under alkaline environment. For this reason, the acidic environment is not conducive to actinomycetes growth and synthesis of metabolites. Wang *et al.* reported the pH varied in a narrow range between 6.0 and 8.0 throughout the fed-batch fermentation during acarbose production with *Actinoplanes utahensis* ZJB-08196 [8]. Therefore, it was likely that the biosynthesis of acarbose was inhibited by the lower fermentation pH.

Effect of various ammonium salts on acarbose production

The concentrations of the various salts were optimized separately through single factor experiment in our pilot studies (data not shown). The suitable addition concentration of ammonium salts was shown in Table 1. With the addition of 0.1 M NH_4Cl , the acarbose yield reached 4875 mg/L. The result showed that NH_4Cl exhibited better promoting effect than other ammonium salts and MG.

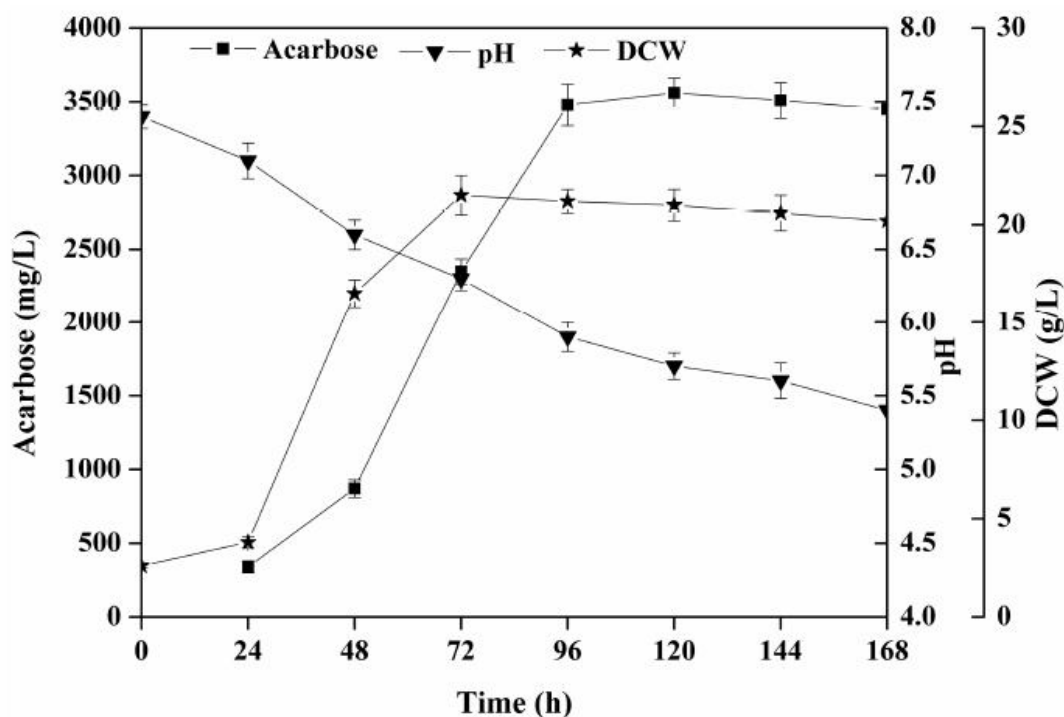


Figure 1: Effect of time on acarbose fermentation. Bars represent the standard deviation. Data are shown as mean \pm SD (n=3)

To investigate the possible effect of Cl^- on acarbose formation, 0.1 M NaCl was added to the fermentation broth, and the biomass concentration and acarbose yield were found unaffected (data not shown). Hence, the influence of Cl^- on acarbose biosynthesis was excluded.

Effect of addition time of NH_4Cl on acarbose production

The effects of the addition time of NH_4Cl on biomass and acarbose yield are shown in Table 2. NH_4Cl (0.1 M) was added to the fermentation broths in different fermentation stages: 48 h, 72 h, 96 h, and 120 h. For cell growth, when NH_4Cl was added at 48 h (log phase), the DCW increased about 10%. There was an obvious increase in acarbose production when NH_4Cl was added to the fermentation broths at all of the above times. Notably, when NH_4Cl was added at 72 h, the highest acarbose yield (4875 mg/L) was obtained. Therefore, 72 h was chosen as the optimal addition time.

Effect of concentration of NH_4Cl on acarbose yield

As shown in Table 3, acarbose yield was significantly affected by the concentrations of NH_4Cl . The highest acarbose yield was achieved when 0.1 M NH_4Cl was added to the fermentation broth. Results in Table 2 and Table 3 demonstrate that the optimal addition concentration and time were 0.1 M and 72 h. In addition, more than 30 % of the initial glucose (10.0 g/L) and maltose (8.0 g/L) remained in the culture medium at the end of all the fermentations, thus ensuring enough supply of carbon sources and precursors for cell growth and acarbose formation.

Impact of pH on acarbose production

The pH was one of the important fermentation parameters affecting cell growth and product formation [5]. Actively growing microorganisms acidified their medium through a combination of differential ion uptake,

Table 1: Effect of addition of different ammonium salts or monosodium glutamate on DCW, pH and acarbose yield at 168 h

Cultivation conditions ^a	DCW(g/L)	Final pH	Acarbose (mg/L)
Control	20.2 ± 0.5	5.50 ± 0.11	3560 ± 105
0.1 M monosodium glutamate	20.4 ± 0.8	6.41 ± 0.05	3980 ± 124
0.1 M NH_4Cl	21.3 ± 0.7	6.34 ± 0.10	4875 ± 146
0.1 M NH_4NO_3	21.0 ± 1.0	6.40 ± 0.06	4760 ± 130
0.05 M $(\text{NH}_4)_2\text{SO}_4$	20.8 ± 0.9	6.26 ± 0.10	4650 ± 112

^aMonosodium glutamate and ammonium salts were added at 72 h after inoculation with the pH adjusted to 7.0 using 2 M NaOH. Data are shown as mean ± SD (n=3)

Table 2: Effect of addition time of NH_4Cl on DCW, final pH and acarbose yield at 168 h

Addition time (h) ^a	DCW(g/L)	Final pH	Acarbose (mg/L)
Control	20.2 ± 1.1	5.50 ± 0.11	3560 ± 102
48	22.4 ± 0.6	6.14 ± 0.10	3580 ± 105
72	21.2 ± 1.0	6.38 ± 0.08	4875 ± 128
96	20.3 ± 1.2	6.42 ± 0.12	4690 ± 115
120	19.8 ± 1.2	6.46 ± 0.10	4330 ± 104

^aThe addition concentration of NH_4Cl was 0.1 M; pH was adjusted to 7.0 with 2 M NaOH after addition of NH_4Cl . Data are shown as mean ± SD (n = 3)

Table 3: Effect of addition concentrations of NH_4Cl on DCW, pH and acarbose yield at 168 h

Cultivation conditions ^a	DCW(g/L)	Final pH	Acarbose (mg/L)
Control	20.2 ± 1.1	5.50 ± 0.10	3463 ± 120
0.06 M ^a	20.4 ± 0.9	6.47 ± 0.10	3980 ± 105
0.08 M	20.7 ± 1.1	6.45 ± 0.08	4390 ± 120
0.10 M	21.2 ± 1.0	6.40 ± 0.07	4875 ± 125
0.12 M	20.7 ± 1.2	6.36 ± 0.10	4630 ± 96
0.14 M	20.5 ± 0.9	6.35 ± 0.11	4470 ± 84

^a NH_4Cl was added at 72 h after inoculation. The pH was adjusted to 7.0 with 2 M NaOH after the addition of NH_4Cl . Data are shown as mean ± SD (n = 3)

proton secretion during nutrient transport, direct secretion of organic acids, and CO₂ evolution [18]. As shown in Table 2 and Table 3, decrease of the medium pH was observed. This might be due to the transport of ammonium leading to acidify of the extracellular medium [19]. It was reported that controlling the pH of the culture medium increased the biomass and polysaccharide production [20]. Therefore, it was

of great importance to control the fermentation pH for acarbose biosynthesis.

Effect of pH on acarbose yield was investigated by controlling the medium pH between 6.0 and 10.0 in 5 L fermentor. According to the results presented in Figure 2 and Figure 3, acarbose yield continuously increased from 72 h to 168 h with decreases in NH₄⁺-N. Acarbose increased

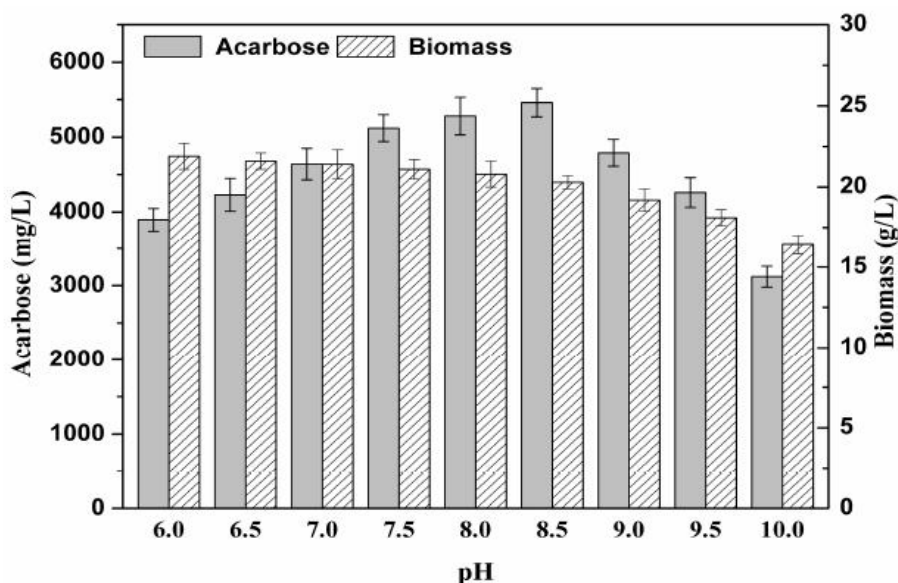


Figure 2: Effect of pH on acarbose yield during *Streptomyces* M37 fermentation. Experiments were carried out in 5 L fermentor. NH₄Cl (0.1 M) was added at 72 h after inoculation. pH was controlled with 2 M NaOH and 2 M HCl since the addition of NH₄Cl. Bars represent the standard deviation (n=3). Data are shown as mean ± SD (n = 3)

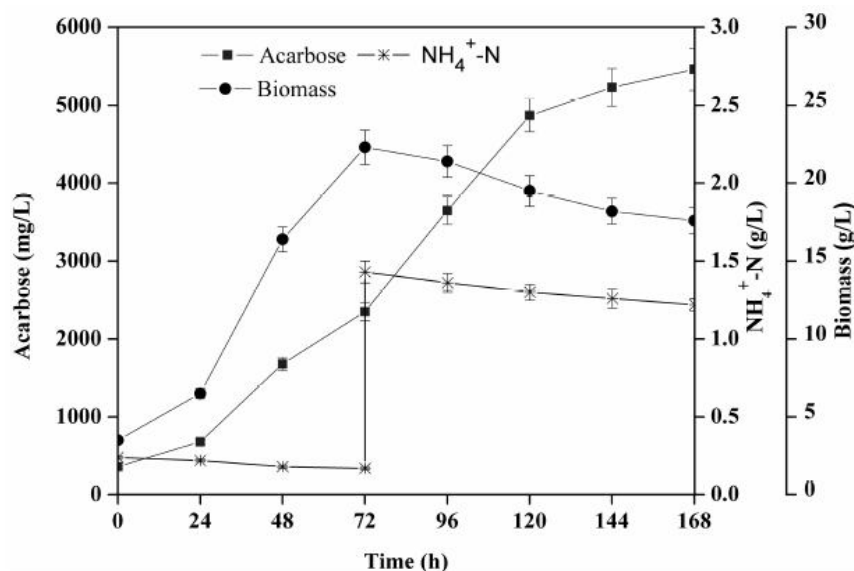


Figure 3: Time course of acarbose titer, biomass and NH₄⁺-N during fed-batch cultivation of with an optimized feeding strategy. Experiments were carried out in 5 L fermentor. NH₄Cl (0.1 M) was added at 72 h after inoculation. pH was controlled at 8.5 with 2 M NaOH and 2 M HCl since the addition of NH₄Cl. Bars represent the standard deviation. Data are shown as mean ± SD (n = 3)

obviously in the pH range of 6.0 - 9.5. A maximum acarbose yield of 5460 mg/L was obtained at pH 8.5, which was increased by 53.4 % when compared with the control. However, when the pH was higher than 9.5, the acarbose yield decreased. It was probably due to the alkaline environment inhibits the enzymes involved in acarbose biosynthesis. Therefore, it was necessary to control the fermentation pH at 8.5 since 72 h after inoculation.

Effect of fermentation pH on GDH and G6PDH activities

GDH are widely distributed enzymes that link carbon and nitrogen metabolism [21]. The physiological role of GDH might be anabolic and/or catabolic. Moreover, biosynthesis studies revealed that the cyclitol moieties of acarbose and validamycin were derived from the HMP pathway, presumably via sedoheptulose 7-phosphate or ido-heptulose 7-phosphate as intermediate [13,22]. G6PDH is the key enzyme of HMP pathway. The activities of GDH and G6PDH might affect acarbose fermentation by *Streptomyces* M37.

The impact of pH on the activity of GDH and G6PDH was shown in Figure 4. GDH and G6PDH exhibited the highest level of enzyme activities at pH 8.5. Higher enzyme activity might

stimulate *Streptomyces* M37 to produce more glutamate and sedoheptulose 7-phosphate for acarbose synthesis during the fermentation process. Using inorganic nitrogen (NH_4Cl) and controlling the pH during acarbose fermentation can significantly lower the production cost of acarbose.

DISCUSSION

There is no consensus in the fermentation literature concerning the influence of ammonium on aminoglycoside productivity. Some studies reported that ammonium impedes aminoglycoside synthesis, whereas others revealed stimulatory effects. Some reports have shown that addition of ammonium to the culture medium enhanced streptomycin and other aminoglycoside processes such as neomycin and gentamicin production [23,24]. Ammonium stimulated acarbose formation probably due to the direct requirement of *Streptomyces* M37 for exogenous glutamate was not so strict, attributing to other sources available from in vivo anabolic pathway like transamination. Similar conclusion was achieved in gentamicin and streptomycin biosynthesis. That is, glutamate, glutamine and glucosamine stimulated the formation of antibiotics containing aminoglycoside group [25].

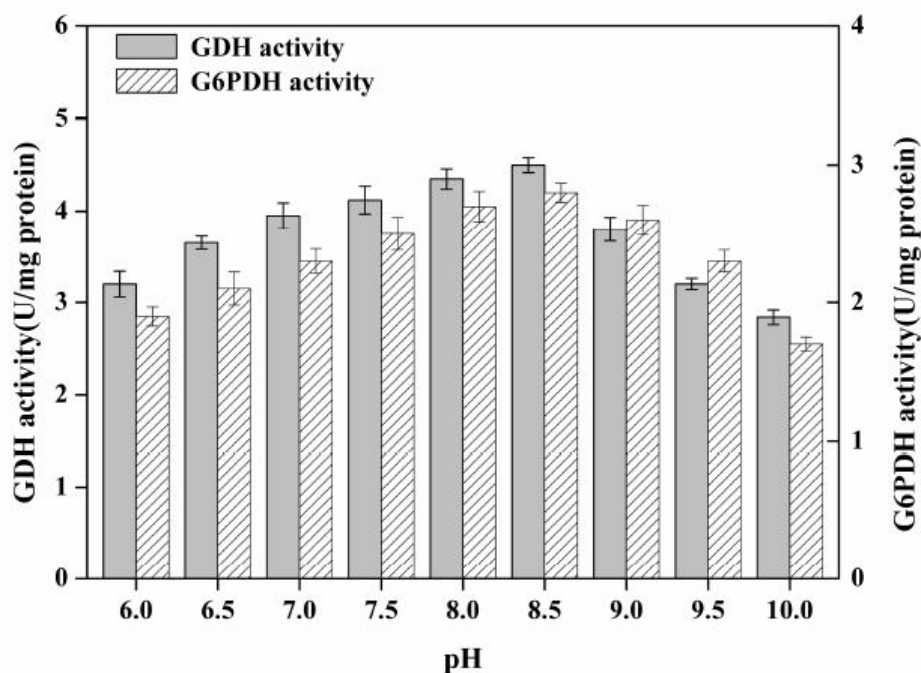


Figure 4: GDH and G6PDH activities at different pH values. Experiments were carried out in 5 L fermentor. NH_4Cl (0.1 M) was added at 72 h after inoculation. pH was controlled with 2 M NaOH and 2 M HCl since the addition of NH_4Cl . The enzyme activities were measured at 120 h. Bars represent the standard deviation. Data are shown as mean \pm SD (n = 3)

Medium pH affects enzyme synthesis, enzymatic processes, and transport across cell membranes [7]. The concentration of ammonia was primarily determined by the ammonium concentration, but under increasing pH the fraction of nitrogen in the form of ammonia increased. Ammonia is generally toxic to microbes, as it was uncharged and could pass through cell membrane [26,27]. In our study, the alkaline stress (pH 8.5) accelerated *Streptomyces* M37 consuming ammonia and producing acarbose, as a strategy to attenuate alkalization of the cytoplasm. Inhibitory action on hydrolases was one of the functions of acarbose [28]. In the present research, the results demonstrated that alkaline environment stimulated *Streptomyces* M37 to synthesis acarbose thus relieving the poison of ammonia and facilitating its survival.

CONCLUSION

The results indicate that acarbose yield is significantly increased by a supplement of 0.1 M NH₄Cl at 72 h while maintaining pH at 8.5. Furthermore, addition NH₄Cl to the medium also enhances the activities of GDH and G6PDH, probably due to stimulation of transamination in cells by ammonium to form glutamate. Glutamate can be used to synthesize acarbose from glucose and maltose, thus minimizing the harmfulness of ammonia. The strategy proposed in this work is potentially applicable to fermentation of secondary metabolites containing the aminoglycoside group.

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