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Fermentative production of ribavirin by overexpressing purine nucleoside phosphorylase in *Bacillus*

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ABSTRACT

Ribavirin, a well-known broad spectrum antiviral agent, has been produced successfully by chemical or enzymatic method. However, the complex synthetic route and expansive material cost limit its large-scale production. In this work, we report a novel fermentative method for ribavirin production by engineered nucleoside producing strain. Two kinds of purine nucleoside phosphorylase (PNPase), i.e. high-molecular-mass PNPase (deoD) and low-molecular-mass PNPase (pupG) were intracellularly or extracellularly overexpressed in different nucleoside (adenosine, inosine, and guanosine) producing strains to accumulate ribavirin. The highest titer of 13.8 ± 0.9 g/L ribavirin was obtained by extracellularly overexpressed PNPase in the guanosine producing strain B. amyloliquefaciens TA208-LS. To further increase the ribavirin yield, the adding time of fermentative precursor 2H-1,2,4-triazole-3-carboxamide (TCA) and fermentation temperature were investigated. Under the optimal condition, a titer of 19.1 g/L ribavirin was obtained after 60 h fermentation in 7.5 L fermenter. This fermentation strategy provides a novel approach for industrial ribavirin production.

Keywords: *Bacillus amyloliquefaciens*; purine nucleoside phosphorylase; purine nucleoside fermentation; ribavirin

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INTRODUCTION

Ribavirin is an analog of purine nucleoside which plays important roles in the treatment of various kinds of virus infection [1-6]. It can be produced by synthetic method, enzymatic method, or fermentative method. In industry, chemical synthesis is currently the most frequently used method. However, because of the cumbersome manipulation and the environmental unfriendly process, synthetic method is not ideal [7, 8]. Since ribavirin was firstly synthesized via chemical method in 1970 s [9], the investigation of producing ribavirin by biological methods was also prosperous [10-12]. Among which the enzymatic method was developed where purine nucleoside phosphorylase (PNPase, EC 2.4.2.1) was employed to catalyze the synthesis of ribavirin from the precursors, purine nucleoside and 2H-1,2,4-triazole-3-carboxamide (TCA) (Fig. 1). However, the cost of ribavirin production was high due to the expensive enzyme source and the high price of precursors, since both nucleoside and TCA were indispensably added in the enzymatic reaction system. In 1976, Ochiai demonstrated a fermentative method where TCA was added to the culture of nucleoside producing strains of *Bacillus* or *Brevibacterium*, and ribavirin was synthesized from endogenous nucleoside catalyzed by the natural PNPase of the strain [13]. Nevertheless, because of the low activity of natural PNPase from the origin strain, the yield of ribavirin was only 0.28 g/L.

In order to generate fermentative method for ribavirin production that is industrially competitive to the chemical method, our laboratory has conducted a series of studies for about 10 years, and a lot of knowledge and techniques were achieved so far. In our previous work, a guanosine-overproducing strain *B. subtilis* TM903 accumulated 5 g/L ribavirin in 60 h under the optimal fermentation condition [14-16]. However, it was difficult to further enhance the ribavirin yield because a nucleoside-overproducing strain could hardly have a high PNPase activity simultaneously,

since the genes related to nucleoside catabolism (inevitably including PNPase encoding genes) were attenuated or inactivated [17-19]. Thus, the top priority was to improve the PNPase activity in nucleoside-overproducing strain. We then conducted some basic studies to investigate the properties of different molecularly expressed PNPase, namely their substrate specificity, activity, and kinetic parameters related to ribavirin production [20, 21]. PNPase is a class of enzyme that has special affinity to purine nucleoside, and the affinity differ according to different enzyme sources and/or different purine nucleosides [22-25]. Inosine, guanosine, and adenosine are the typical purine nucleosides, which have been produced by *Bacillus sp*. in industry [26-29].

On the basis of the previous results, this study respectively overexpressed two kinds of PNPase, low-molecular-mass PNPase (L-PNPase) encoded by *pupG* and high-molecular-mass PNPase (H-PNPase) encoded by *deoD*, in several nucleoside-overproducing *Bacillus* strains to redefine their purine synthesis pathway. Furthermore, considering the nucleoside was largely accumulated in medium rather than in cytoplasm and TCA was exogenously added during fermentation process, all PNPase were also secretly expressed with signal peptides to verify the notion that extracellular expression of PNPase could enhance the contact probability between PNPase and substrates, and thus improve ribavirin production. Finally, shaking flask cultivations and batch fermentation in 7.5 L fermenter were conducted to produce ribavirin using the optimal strain constructed, and to the best knowledge of the authors the highest ribavirin yield (19.1 g/L) via fermentative method was achieved.

EXPERIMENTAL SECTION

Strains and growth conditions

E. coli DH5α was used as host strain for the construction of plasmids and was grown in Luria-Bertani (LB) broth medium at 37°C, ampicillin (100 mg/L) was added to the medium when necessary. For the maintenance of strain on agar plate, 2% (w/v) agar was supplemented. *B. subtilis* WB600 [30], *B. subtilis* XGL (adenosine-producing strain), *B. subtilis* Q60 (inosine-producing strain), *B. amyloliquefaciens* TA208 (guanosine-producing strain), and their derivatives were used for the study of ribavirin fermentation. Seed and production media were constituted as below. Seed medium (per liter): glucose 20 g, yeast extract 10 g, NaCl 2 g, $MgSO_4$ 1 g, KH_2PO_4 1 g, and corn steep liquor 30 ml; the final medium was adjusted to pH 7.0 with 4 M NaOH and added with 10 μg/ml kanamycin. Production medium (per liter): glucose 80 g (separated sterilization), yeast extract 20 g, $(NH_4)_2SO_4$ 15 g, $MgSO_4$ 5 g, KH_2PO_4 5 g, $FeSO_4$ 10 mg, $MnSO_4$ 4 mg, and corn steep liquor 20 ml, the final medium was adjusted to pH 6.7 with 4 M NaOH.

Construction of plasmids

The primers used in this study were listed in Table 1. The vectors pBE43 and pBSA43 were respectively used for intracellular and extracellular expression.

Table 1 Primers used in this study

Primers	Sequence (5'-3')	Relevant Characteristics
H-F_{In}	AT**GGTACC**AAAGGAGACATCAACGATGAGTGTACATATAGGTGCTG	*Kpn* I; RBS; ATG
H-F_{Ex}	CCGG**CTCGAG**CAGTGTACATATAGGTGC	*Xho* I
H-R	AT**CTGCAG**CCAGCACGCCTCTTGATAT	*Pst* I
L-F_{In}	AT**GGTACC**AAAGGAGACATCAACGATGAAGGACAGAATTGAACG	*Kpn* I; RBS; ATG
L-F_{Ex}	AGGC**CTCGAG**CAAGGACAGAATTGAACGC	*Xho* I
L-R	AT**CTGCAG**GCTGTCTTTTGGTCCTG	*Pst* I

Restriction enzyme sites are in bold, ribosome binding sites are bordered, and start codons are given shadow effect.

For the construction of pBE43, the fragment of P43 promoter was cut from pWB980 by *Eco*R I and *Kpn* I and cloned into the *Eco*R I/*Kpn* I sites of pBE2 vector. To construct pBSA43, the fragment from P43 promoter to *SacB* signal peptide sequence was cut from pWB980 by *Eco*R I and *Pst* I and cloned into the *Eco*R I/*Pst* I sites of pBE2 vector.

To construct the *deoD* intracellular expression plasmid pBE43-*deoD*, *deoD* gene was amplified from the genomic DNA of *B. subtilis* 168 by PCR using the primers of H-F_{In} and H-R. The PCR product was digested with *Kpn* I and *Pst* I and cloned into the *Kpn* I/*Pst* I sites of pBE43 vector. The construction of *pupG* intracellular expression plasmid pBE43-*pupG* was similar to that of pBE43-*deoD* but using the primers of L-F_{In} and L-R.

To construct the *deoD* extracellular expression plasmid pBSA43-*deoD*, *deoD* gene was amplified from the genomic DNA of *B. subtilis* 168 using primers H-F_{Ex} and H-R. The PCR product was digested with *Xho* I and *Pst* I and cloned into the *Xho* I/*Pst* I sites of pBSA43 vector. The construction of *pupG* extracellular expression plasmid pBSA43-*pupG* was similar but using the primers of L-F_{Ex} and L-R.

Transformations of bacteria

The transformations of both *B. subtilis* and *B. amyloliquefaciens* were performed as described previously using an electroporation method [31]. To effectively transform TA208, plasmids extracted from DH5α were firstly transformed into WB600. All the competent cells were stored at -80˚C less than one month.

Fermentation

The bacteria from agar slants were inoculated into flasks containing seed medium and cultivated at 36˚C in a rotary shaker at 200 r/min for 10 h, which were then inoculated (10% v/v) into 30 ml production medium. The production cultures were shaken at 32-36˚C in a rotary shaker at 200 r/min for 96 h. TCA (10 g/L) was added into medium at 24 h. Glucose was supplied to the final concentration of 40 g/L when the residual glucose was lower than 20 g/L.

Analysis

All the experiments were performed in triplicate. For each experiment, two technical replicates were performed. Samples were diluted 1:20 with deionized water to determine the absorbance at 600 nm on MJ33 (Mettler Toledo, Switzerland), and one unit OD_{600nm} was equal to 0.3 g/L dry cell weight (DCW). Samples were diluted 1:10 with 1 M NaOH first and then diluted 1:100 with deionized water to determine the content of nucleoside. Samples were centrifuged (10,000×g, 4°C, 5 min), and the supernatants were analyzed for glucose and ribavirin. Glucose was determined using a biosensor analyzer (SBA-40B, Institute of Biology, Shandong Academy of Sciences, China). Ribavirin and purine nucleosides were measured by HPLC (Series 1200, Agilent Technologies, Santa Clara, CA, USA) as described previously, but detection was performed at 254 nm for guanosine and guanine and 207 nm for ribavirin [32].

RESULTS AND DISCUSSION

Effect of overexpression of two PNPase on ribavirin production in different purine nucleoside-producing strains

The main difference between fermentative and enzymatic method for ribavirin production is that whether nucleoside needs to be added into the culture. Overexpressing purine nucleoside phosphorylase (PNPase) in purine nucleoside-producing strain enabled the engineered strain to use endogenous purine nucleoside as substrate to synthesize ribavirin, when 2H-1,2,4-triazole-3- carboxamide (TCA) was added into the culture.

Table 2 Effect of overexpression of two PNPase on ribavirin production in different purine nucleoside-producing strains

Strains	Plasmids	Product titer (g/L)	
		Ribavirin	Nucleoside
intracellular expression			
WB600	pBE43	0.4 ± 0.1	0.1 ± 0.1
(negative control)	pBE43-*pupG*	0.6 ± 0.1	0.1 ± 0.1
	pBE43-*deoD*	0.6 ± 0.1	0.1 ± 0.1
Q60	pBE43	1.7 ± 0.2	8.3 ± 0.4
(inosine-producing)	pBE43-*pupG*	6.2 ± 0.6	3.5 ± 0.3
	pBE43-*deoD*	4.9 ± 0.3	4.8 ± 0.6
TA208	pBE43	1.5 ± 0.2	8.1 ± 0.4
(guanosine-producing)	pBE43-*pupG*	8.3 ± 0.4	1.4 ± 0.8
	pBE43-*deoD*	6.6 ± 0.7	3.8 ± 0.7
XGL	pBE43	1.8 ± 0.1	7.5 ± 0.4
(adenosine-producing)	pBE43-*pupG*	2.9 ± 0.6	4.3 ± 0.3
	pBE43-*deoD*	2.1 ± 0.4	4.9 ± 0.5

Engineered strains were cultivated in 500 ml flask at 36˚C for 60 h. In each culture, 0.3 g TCA (2H-1,2,4-triazole-3-carboxamide) was added into the culture to a final concentration of 10 g/L at 24 h. XGL, Q60, TA208, and WB600 are adenosine-, inosine-, and guanosine-producing and non-nucleoside-producing strain, respectively. The plasmid pBE43 is an E. coli-Bacillus shuttle vector containing P43 promoter. Gene pupG encodes low-molecular-mass purine nucleoside phosphorylase and deoD encodes high-molecular-mass purine nucleoside phosphorylase. Data are represented as Mean ± SD (n = 3).

In this study, *B. subtilis* WB600 was regarded as negative control, whose PNPase activity was probably superfluous relative to its poor purine nucleoside yield. In other words, the purine nucleoside amount was the limitation for ribavirin production in WB600. As shown in Table 2, little ribavirin was detected in WB600 and its derivates, however, those purine nucleoside-producing strains and their derivates, which provided purine nucleoside abundantly by themselves, both exhibited higher yield of ribavirin. In adenosine-producing strain group, no obvious difference of ribavirin yield was detected among PNPase expressed derivates and the original strain. The reason might be that the current PNPase showed weak activity in catalyzing adenosine, thus leading most of the adenosine remained in the

culture. This was similar to the results of Shirae [12] that ribavirin was hardly produced from adenosine and TCA. While in inosine- and guanosine-producing strain groups, both L-PNPase and H-PNPase overexpressing derivates showed higher ribavirin yield and lower residual nucleoside than original strains. Although inosine had the highest affinity to PNPase among all nucleosides, the product of phosphorolysis of inosine, hypoxanthine, was a strong competitive inhibitor to TCA for PNPase as in ribavirin production [12]. This probably led the equilibrium of the reversible reaction that was less inclined to ribavirin synthesis. The highest ribavirin yield was produced by guanosine-producing strain derivate, indicating guanosine was the optimal substrate for ribavirin synthesis via the PNPase overexpressed in this study.

On the other hand, within the same incubation time frame, overexpressing L-PNPase achieved higher yield of ribavirin than H-PNPase in both inosine- and guanosine-producing strains. It was implied that the L-PNPase had higher catalytic activity for ribavirin synthesis than H-PNPase [21].

Effect of secreted overexpression of two PNPase on ribavirin production in different purine nucleoside-producing strains

B. amyloliquefaciens and *B. subtilis* have excellent property in protein secretion, especially for homologous proteins [33]. There were abundant study verified that enzymes could be successfully secreted into culture of these two species [34, 35]. For the fact that in nucleoside fermentation process nucleoside largely accumulated in medium rather than in cytoplasm, we extracellularly overexpressed PNPase by fusing signal peptide (Fig. 1) in order to increase ribavirin yield. Because enhancing the contact probability between PNPase and substrates by secreted expression was expected to increase the utilization efficiency of substrates.

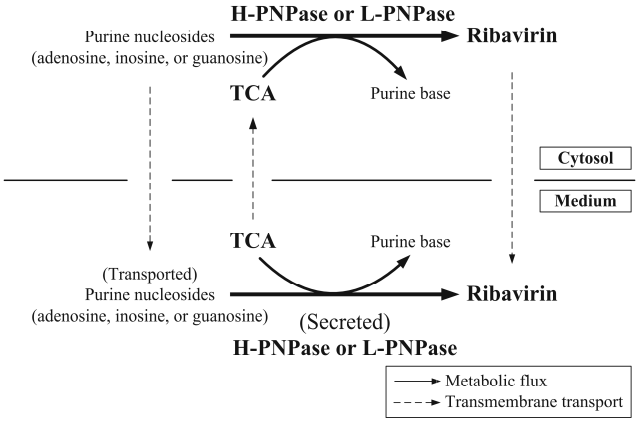

Figure 1 The sketch of PNPase secretion, product transform, and ribavirin synthetic reaction catalyzed by PNPase. H-PNPase: high-molecular-mass purine nucleoside phosphorylase; L-PNPase: low-molecular-mass purine nucleoside phosphorylase; TCA: 2H-1,2,4-triazole-3-carboxamide

Table 3 Effect of secreted overexpression of two PNPase on ribavirin production in different purine nucleoside-producing strains

Strains	Plasmids	Product titer (g/L)	
		Ribavirin	Nucleoside
extracellular expression			
WB600	pBSA43-*pupG*	0.6 ± 0.2	0.1 ± 0.1
(negative control)	pBSA43-*deoD*	0.6 ± 0.3	0.1 ± 0.1
Q60	pBSA43-*pupG*	6.9 ± 0.4	2.3 ± 0.4
(inosine-producing)	pBSA43-*deoD*	5.5 ± 0.5	3.3 ± 0.5
TA208	pBSA43-*pupG*	9.5 ± 0.8	0.5 ± 0.4
(guanosine-producing)	pBSA43-*deoD*	7.6 ± 0.5	2.4 ± 0.8
XGL	pBSA43-*pupG*	2.8 ± 0.6	4.9 ± 0.7
(adenosine-producing)	pBSA43-*deoD*	2.4 ± 0.5	4.8 ± 0.1

Engineered strains were cultivated in 500 ml flask at 36˚C for 60 h. In each culture, 0.3 g TCA (2H-1,2,4-triazole-3-carboxamide) was added into the culture to a final concentration of 10 g/L at 24 h. XGL, Q60, TA208, and WB600 were adenosine-, inosine-, and guanosine-producing and non-nucleoside-producing strain, respectively. The plasmid pBSA43 was an E. coli-Bacillus shuttle vector containing P43 promoter and SacB signal peptide which enabled target protein to be secreted into medium. Gene pupG encodes low-molecular-mass purine nucleoside phosphorylase and deoD encodes high-molecular-mass purine nucleoside phosphorylase. Data are represented as Mean ±SD (n = 3).

As shown in Table 3, secreted expression of L-/H-PNPase achieved a 0.14-/0.15-fold increase in ribavirin yield and

64%/63% decrease in residual guanosine compared with intracellular expression. Likewise, in inosine-producing strain derivates, 0.11-/0.12-fold increase in ribavirin yield, and 65%/69% decrease in residual inosine were observed. Secreted expression of PNPase extended the scope of reaction space, meanwhile, when PNPase was secreted into medium, the function of substrate transmembrane transportation was less important. The decrease of the concentration of inosine or guanosine confirmed that extracellular expression of PNPase was indeed beneficial for increasing the utilization efficiency of substrates. But in adenosine-producing strain derivates, secretion of PNPase did not promote the synthesis of ribavirin due to the weak catalytic activity of PNPase to adenosine.

Therefore, with the secreted expression of L-PNPase, guanosine-producing strain derivate *B. amyloliquefaciens* TA208 (pBSA43-*pupG*) was screened as the optimal ribavirin-producing strain. We thus designated this strain as *B. amyloliquefaciens* TA208-LS, which was used for further optimization of the fermentation condition.

Effect of TCA adding time on ribavirin production in *B. amyloliquefaciens* TA208-LS

As the essential precursor, TCA was added into the culture of all original and PNPase overexpressed strains at 24 h (Table 2). In order to investigate the effect of TCA adding time on ribavirin production, we added TCA into the culture of the optimal ribavirin-producing strain *B. amyloliquefaciens* TA208-LS at 0, 12, 24, and 36 h, respectively.

As shown in Fig. 2a, in the culture of PNPase overexpressed guanosine-producing strain, the previous product, guanosine, no longer largely accumulated. The highest concentration of guanosine had not exceeded 2 g/L in the entire fermentation process. This phenomenon proved that the activity of L-PNP overexpressed in guanosine-producing strain was sufficient for guanosine utilization. Even in the group without TCA supplement, the concentration of guanosine was low, despite slightly higher than those in other groups. Meanwhile, TCA adding terminated the otherwise increase of guanosine concentration (Fig. 2a).

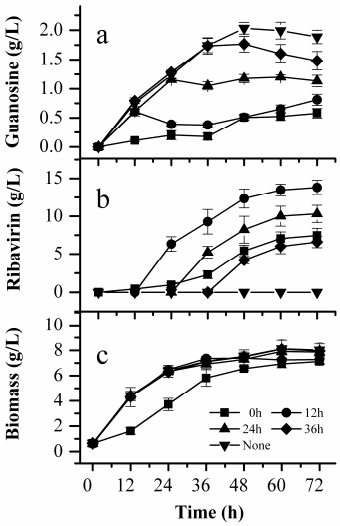

Figure 2 Fermentation profiles of *B. amyloliquefaciens* TA208-LS with different TCA adding time. The optimal ribavirin-producing strain *B. amyloliquefaciens* TA208-LS was cultivated in 500 ml flask at 36˚C for 60 h. In each culture, 0.3 g TCA , H-1,2,4-triazole-3-carboxamide) was added into the culture to a final concentration of 10 g/L respectively at the following time: (-■-): 0 h, (-●-): 12 h, (-▲-): 24 h, and (-♦-): 36 h. (a): Guanosine production (g/L); (b) Ribavirin production (g/L); (c) Biomass yield (g/L, DCW). Data are represented as Mean ± SD (n = 3).

Beyond our expectations, one of the phosphorolysis products of purine nucleoside, purine base, was not detected during the whole fermentation course whether TCA was added. Another phosphorolysis product of purine nucleoside was ribose-1-phosphate (R-1-P), which could be bonded with TCA by PNPase to form ribavirin (Fig. 1). But R-1-P normally could not accumulate in the culture due to its fast transport and utilization [36, 37]. Hence, TCA should be added early enough to capture R-1-P for ribavirin production, in other words, the earlier the addition of TCA, the higher the yield of ribavirin. As shown in Fig. 2b, adding TCA at 12 h achieved the highest ribavirin yield 13.8 ± 0.9 g/L. With the delay of TCA adding, 10.3 ± 1.1 g/L and 6.6 ± 0.8 g/L ribavirin were respectively gained as TCA added at 24 h and 36 h. When TCA added at 0 h, however, a ribavirin production “lag phase” was observed, and the yield of ribavirin was only 7.5 g/L. That probably because TCA had inhibiting effect to cell growth in the early phase of fermentation; the biomass of “0 h” group, as shown in Fig. 2c, was lower than other groups until 36 h. While in the groups where TCA was added after 12 h, there was no obvious difference in cell growth. For the reasons above, TCA should be added neither too early nor too late in the fermentation course. It had important impact on the growth of cell and yield of ribavirin.

Since the phosphorolysis product of purine nucleoside, purine base, was not detected during the whole fermentation process, we proposed a novel pathway for ribavirin biosynthesis as in Fig. 3. Specifically, the intermediate product in the pentose phosphate pathway (HMP), ribose-5-phosphate (R-5-P), is converted to R-1-P catalyzed by the phosphopentomutase (PPMase) [38], which can be further utilized directly by PNPase to synthesize ribavirin as TCA supplemented. Thus, enhancing the enzyme system in HMP together with PNPase overexpression might generate an efficient pathway for ribavirin production. We will make great efforts to investigate this possibly novel pathway in detail.

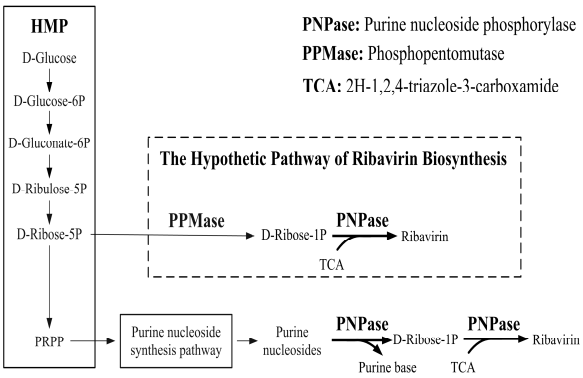

Figure 3 The hypothetic pathway of ribavirin biosynthesis. In ribavirin biosynthesis pathway, R-1-P can be supplied either by pentose phosphate pathway or purine nucleoside synthesis pathway. PRPP: 5-Phosphoribosyl diphosphate; TCA: H-1,2,4-triazole-3-carboxamide; PNPase: purine nucleoside phosphorylase; PPMase: phosphopentomutase

Effect of fermentation temperature on ribavirin production in *B. amyloliquefaciens* TA208-LS

Fermentation temperature could affect the ribavirin yield via cell growth rate, PNPase activity, and guanosine synthesis rate. Ribavirin fermentation is based on nucleoside fermentation and enzymatic catalysis process, thus it is crucial to keep the appropriate balance between nucleoside production and PNPase activity. Any imbalance would decrease ribavirin yield. In this study, the low concentration of guanosine in the culture implied that the limiting factor of ribavirin yield was the production of guanosine. The most suitable fermentation temperature for guanosine production was 32-36˚C [39], under which PNPase retained 60% relative activity but was more stable [21].

The biomass, ribavirin yield, and glucose consumption at 36˚C were respectively 8.9 ± 0.3 g/L, 13.8 ± 0.9 g/L, and 89.6 ± 6.2 g/L (Fig. 4). While incubated at 34˚C, the biomass, ribavirin yield, and glucose consumption were respectively 8.8 ± 0.4 g/L, 13.1 ± 1.2 g/L, and 82.6 ± 6.4 g/L. It can be seen that there were no significant difference on cell growth (biomass) between these two temperatures. Moreover, the yield of ribavirin and consumption of glucose were very close.

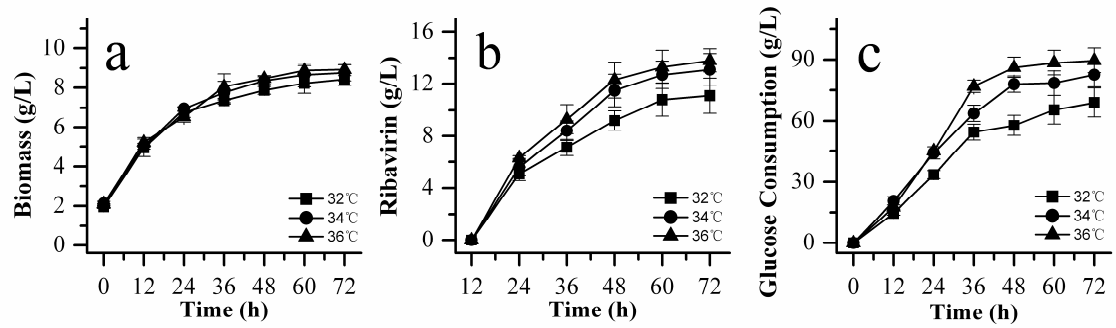

Figure 4 Fermentation profiles of *B. amyloliquefaciens* TA208-LS at different temperature. The optimal ribavirin-producing strain *B. amyloliquefaciens* TA208-LS was cultivated in 500 ml flask at different temperatures for 60 h. In each culture, 0.3 g TCA , 2H-1,2,4-triazole-3-carboxamide) was added into the culture to a final concentration of 10 g/L at 12 h. The fermentation temperature was as follows: (-■-): 32˚C; (-●-): 34˚C; and (-▲-): 36˚C. (a): Biomass yield (g/L, DCW); (b) Ribavirin production (g/L); (c) Glucose consumption (g/L). Data are represented as Mean ± SD (n = 3).

Whereas, at 32˚C, the biomass, ribavirin yield, and glucose consumption were respectively 8.4 ± 0.3 g/L, 11.1 ± 1.1 g/L, and 68.9 ± 7.3 g/L, which were lower than those at 34 and 36˚C. These variations were probably caused by the effects of temperature on the activity of some key enzymes related to glucose catabolism, such as glucose-6-phosphate dehydrogenase and glucose-6-phosphatase [40-42]. As the enzyme activity altered, the allocation proportion of carbon

skeleton into HMP was changed [42], leading to the change of the synthesis of phosphoribosyl pyrophosphate (PRPP), a precursor for purine pathway [43]. Although the yield of ribavirin decreased at lower temperatures according to the mechanisms mentioned above, the glucose-ribavirin conversion rate increased. As shown in Fig. 4c, the glucose-ribavirin conversion rate at 32, 34, and 36˚C was respectively 16.1%, 15.9%, and 15.4%.

Batch fermentation with strain *B. amyloliquefaciens* TA208-LS

The fermentation process was performed in 7.5 L fermenter based on the optimized condition of shaking flask cultivation. The main results are shown in Fig. 5. The optimal dissolved oxygen control strategy for guanosine fermentation was to keep a relative high dissolved oxygen level in the early phase for cell growth and a relative low dissolved oxygen level in the middle and late phase to benefit guanosine synthesis. The same dissolved oxygen control strategy should be employed for ribavirin fermentation. Before 24 h, we improved dissolved oxygen level along with cell growth by increasing agitation rate; after 24 h, agitation rate was discontinuously reduced to keep the dissolved oxygen at a relative low level (Fig. 5).

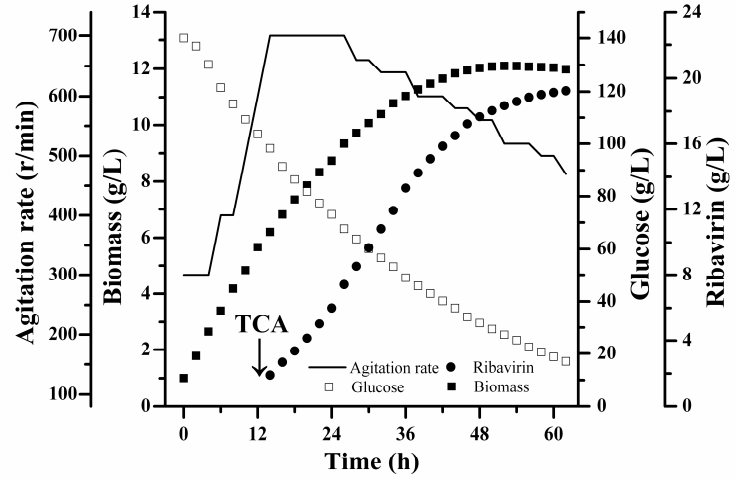

Figure 5 Ribavirin batch fermentation with *B. amyloliquefaciens* TA208-LS. The optimal ribavirin-producing strain *B. amyloliquefaciens* TA208-LS was cultivated in 7.5 L fermenter at 36˚C for 60 h. The pH was automatically maintained at 6.7-7.0 using 25% (v/v) ammonia. TCA (2H-1,2,4-triazole-3-carboxamide) was added into the culture to a final concentration of 15 g/L at 12 h. (■): Biomass (g/L, DCW); (●): Ribavirin yield (g/L); (□): Glucose (g/L); (-): Agitation rate (r/min)

Compared with the flask fermentation, an increase of about 0.5-fold (about 12 g/L) of biomass was achieved. A total of 123 g/L glucose was consumed, which was 0.37-fold more than that in flask. In addition, the ribavirin yield reached to 19.1 g/L. The glucose-ribavirin conversion rate was 15.4% that was equal to that in flask fermentation under the same condition.

CONCLUSION

In the present study, we successfully combined traditional fermentation process and enzymatic synthesis process together to establish a novel ribavirin biosynthesis pathway. Via overexpressing PNPase, the previous end product purine nucleoside was successfully converted to ribavirin. Among the typical purine nucleoside-producing strains, guanosine-producing strain derivate was screened out as the optimal ribavirin-producing strain. In addition, L-PNPase exhibited higher catalytic activity than H-PNPase on synthesis of ribavirin. Furthermore, secreted expression of PNPase gained about 10% increase on ribavirin yield. Via optimizing the adding time of TCA, we found that when TCA was added at 12 h, the highest ribavirin yield of 13.8 ± 0.9 g/L was achieved. Low fermentation temperature (32˚C) facilitated the conversion from glucose to ribavirin, while relative high temperature (36˚C) was beneficial to higher ribavirin yield. Finally, the batch fermentation in 7.5 L fermenter under the optimal condition achieved the highest ribavirin yield reported to date that was 19.1 g/L.

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REFERENCES

[1] CM Hadi, A Goba, SH Khan, et al. *Emerg. Infect. Dis.*, **2010**, 16(12), 2009-2011.
[2] DG Bausch, CM Hadi, SH Khan, et al. *Clin. Infect. Dis.*, **2010**, 51(12), 1435-1441.

[3] O Ergonul. *Curr. Opin. Virol.*, **2012**, 2(2), 215-220.
[4] AM Bisceglie, M Shindo, TL Fong, et al. *Hepatology*, **1992**, 16(3), 649-654.
[5] S Brillanti, J Garson, M Foli, et al. *Gastroenterology*, **1994**, 107(3), 812-817.
[6] A Pelaez, GM Lyon, SD Force, et al. *J. Heart Lung Transplant.*, **2009**, 28(1), 67-71.
[7] Y Saito, V Escuret, D Durantel, et al. *Bioorg. Med. Chem.*, **2003**, 11(17), 3633-3639.
[8] T Utagawa. *Chem. Inform.*, **1998**, 29(42), 121-130.
[9] Y Togo. *Antimicrob. Agents Ch.*, **1973**, 4(6), 641-642.
[10] H Shirae, K Yokozeki, K Kubota. *Agric. biol. chem.*, **1988**, 52(6), 1499-1504.
[11] H Shirae, K Yokozeki, K Kubota. *Agric. biol. chem.*, **1988**, 52(5), 1233-1237.
[12] H Shirae, K Yokozeki, M Uchiyama, et al. *Agric. biol. chem.*, **1988**, 52(7), 1777-1783.
[13] K Ochiai, A Sato, A Furuya. *Journal of the Agric. Chem. Soc. Japan*, **1976**, 50, 423-430.
[14] GH Wu, XJ Zhao, QY Xu, et al. *J. Chem. Ind. Eng.*, **2007**, 58(6), 1535-1540.
[15] GH Wu, XJ Zhao, QY Xu, et al. *Food Ferment. Ind.*, **2007**, 33(12), 17-19.
[16] GH Wu, XJ Zhao, QY Xu, et al. *Chinese J. Pharm.*, **2007**, 38(10), 701-704.
[17] NP Zakataeva, DV Romanenkov, VS Skripnikova, et al. *Appl. Microbiol. Biotechnol.*, **2012**, 93(5), 2023-2033.
[18] T Asahara, Y Mori, NP Zakataeva, et al. *Appl. Microbiol. Biotechnol.*, **2010**, 87(6), 2195-2207.
[19] SB Shi, T Chen, ZG Zhang, et al. *Metab. Eng.*, **2009**, 11(4), 243-252.
[20] JG Xia, KF He, XX Xie, et al. *China Biotechnol.*, **2010**, 30(12),53-59.
[21] XX Xie, JG Xia, KF He, et al. *Biotechnol. Lett.*, **2011**, 33(6), 1107-1112.
[22] A Bzowska, M Luić, W Schröder, et al. *FEBS Lett.*, **1995**, 367(3), 214-218.
[23] EM Bennett, C Li, PW Allan, et al. *J. Biol. Chem.*, **2003**, 278(47), 47110-47118.
[24] KF Jensen. *Eur. J. Biochem.*, **1976**, 61(2), 377-386.
[25] KF Jensen. *Biochimica et Biophysica Acta (BBA)-Enzymology*, **1978**, 525(2), 346-356.
[26] M Schallmey, A Singh, OP Ward. *Can. J. Microbiol.*, **2004**, 50(1), 1-17.
[27] E Spoldi, D Ghisotti, S Cali, et al. *Nucleosides, Nucleotides Nucleic Acids*, **2001**, 20(4-7), 977-979.
[28] K Miyagawa, H Kimura, K Nakahama, et al. *Nat. Biotechnol.*, **1986**, 4(3), 225-228.
[29] R Aoki, H Momose, Y Kondo, et al. *J. Gen. Appl. Microbiol.*, **1963**, 9, 387-396.
[30] XC Wu, W Lee, L Tran, et al. *J. Bacteriol*, **1991**, 173(16), 4952-4958.
[31] G Cao, X Zhang, L Zhong, et al. *Biotechnol. Lett.*, **2011**, 33(5), 1047-1051.
[32] XX Xie, GL Wang, JG Xia, et al. *World J. Microbiol. Biotechnol.*, **2011**, 27(5), 1175-1181.
[33] WF Li, XX Zhou, P Lu. *Res. Microbiol.*, **2004**, 155(8), 605-610.
[34] SL Wong, R Ye, S Nathoo. *Appl. Environ. Microbiol.*, **1994**, 60(2), 517-523.
[35] A Heydari, MM Moghaddam, H Aghamollaei, et al. *New Cell. Mol. Biotechnol. J.*, **2013**, 3(9), 67-73.
[36] JE Lopilato, JL Garwin, SD Emr, et al. *J. Bacteriol*, **1984**, 158(2), 665-673.
[37] Y Park, YJ Cho, T Ahn, et al. *EMBO J.*, **1999**, 18(15), 4149-4156.
[38] TM Iverson, TD Panosian, WR Birmingham, et al. *Biochemistry*, **2012**, 51(9), 1964-1975.
[39] GH Wu, J Wang, B Zhang, et al. *Chinese J. Pharm.*, **2005**, 36(2), 79-80.
[40] GMM Bredemeijer, G Esselink. *J. Plant Physiol.*, **1995**, 145(4), 565-569.
[41] GB Jagdale, R Gordon. *Comp Biochem Physiol Part A: Physiol.*, **1997**, 118(4), 1151-1156.
[42] ME Salvucci, DL Hendrix, GR Wolfe. *J. Insect Physiol.*, **1999**, 45(1), 21-27.
[43] B Hove-Jensen. *J. Bacteriol*, **1988**, 170(3), 1148-1152.