Metabolic Engineering of *Bacillus Amyloliquefaciens* as A Novel Cell Factory to Produce Spermidine

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Abstract

Background: Spermidine is a biologically active polyamine with extensive application potential in foods and pharmaceuticals. However, previously reported spermidine titers by biosynthesis methods are relatively low, which hinders the industrial fermentation of spermidine. To improve the spermidine titer, key genes affecting the spermidine production were mined to engineer the *Bacillus amyloliquefaciens*.

Results: Genes of S-adenosylmethionine decarboxylase (*speD*) and spermidine synthase (*speE*) from different microorganisms were expressed and compared in *B. amyloliquefaciens*. Therein, the *speD* from *Escherichia coli* and *speE* from *Saccharomyces cerevisiae* were confirmed to be optimal for spermidine synthesis, respectively. Then, these two genes were co-expressed to generate an engineering strain *B. amyloliquefaciens* HSAM2(PDspeD-SspeE) with a spermidine titer of 91.31 mg/L, improving by 10.90-fold compared with the control (HSAM2). Through further optimization of fermentation medium, the spermidine titer was increased to 227.35 mg/L, which was the highest titer among present reports. Moreover, the consumption of the substrate S-adenosylmethionine was consistent with the accumulation of spermidine, which contributed to understanding the synthetic pattern of spermidine.

Conclusions: Two critical genes for spermidine synthesis were obtained, and an *B. amyloliquefaciens* cell factory was constructed for enhanced spermidine production, which laid the foundation for further industrial production of spermidine.

Background

Spermidine is a multifunctional polyamine with the positive charge and low molecular weight [1, 2]. Generally, spermidine can electrostatically bind with negatively charged biomolecules such as DNA and RNA, which can stabilize the biomolecules and promote the cell growth [3, 4]. Moreover, spermidine has been reported to induce cell autophagy to extend life-span and reduce cardiovascular diseases-related morbidity [5–8]. In addition, spermidine also shows versatile functions such as reducing neurodegeneration, delaying senile dementia, preventing heart aging, and treating type 2 diabetes [9–12]. Therefore, spermidine has promising application potential in the fields of foods and pharmaceuticals, and development of spermidine-related products was extremely valuable.

At present, spermidine is mainly produced by chemical synthesis, while it has several disadvantages such as high energy consumption, mass toxic by-product, and heavy environmental pollution. In comparison, the microbial fermentation method is safe, efficient and environmentally friendly [13]. Currently, many microorganisms have been reported to synthesize trace spermidine [14–16]. The core metabolic pathway for spermidine synthesis is shown in Fig. 1. Putrescine and S-adenosylmethionine (SAM) are the precursors for spermidine formation. Firstly, the SAM is catalyzed to decarboxylated SAM (dcSAM) by SAM decarboxylase encoded by *speD* gene. Then, the aminopropyl group of the dcSAM is transfered to putrescine to produce the spermidine, which is mediated by the spermidine synthase encoded by *speE* gene [17–19]. In *Saccharomyces cerevisiae*, the spermidine production was increased by over-expressing

genes of ornithine decarboxylase, SAM decarboxylase and spermidine synthase and deleting genes of anti-ornithine decarboxylase and polyamine transporter [20, 21]. In *Synechocystis sp*, overexpression of arginine decarboxylase genes *Adc1* and *Adc2* could enhance the intracellular spermidine content [22]. However, previously reported fermentation titers of spermidine are relatively low, and more work is needed to improve the spermidine production, such as explaining the metabolic mechanism, mining key genes and engineering the strain.

B. amyloliquefaciens has been an efficient platform workhorse for production of various bioproducts [23–25]. The complete spermidine synthesis pathway exists in *B. amyloliquefaciens*. Moreover, the precursors of spermidine including putrescine and SAM have been highly produced in *B. amyloliquefaciens* after metabolic engineering [26, 27]. Therefore, the *B. amyloliquefaciens* has the potential to be developed as an efficient spermidine cell factory. However, the genes responsible for spermidine synthesis from putrescine and SAM have not been investigated in *B. amyloliquefaciens*. Herein, the downstream genes of spermidine synthesis including SAM decarboxylase gene (*speD*) and spermidine synthase gene (*speE*) were selected from different microorganisms, and their effects on spermidine production were evaluated in *B. amyloliquefaciens*. Two efficient genes affecting the spermidine production were obtained, and the spermidine production was enhanced dramatically by combined expression and fermentation optimization.

Results And Discussion

Effects of different SAM decarboxylase genes on spermidine production

SAM can be catalyzed to decarboxylated SAM (dcSAM) by SAM decarboxylase, which is a key step for spermidine synthesis. Therefore, efficient SAM decarboxylase genes are believed to enhance the spermidine production. Previously, we constructed a high SAM-producing strain *B. amyloliquefaciens* HSAM2 [26], which might provide abundant SAM substrate for spermidine synthesis. Therefore, the HSAM2 was used as the host strain to express SAM decarboxylase genes (*speD*) from different microorganisms. The *speD* genes from *B. amyloliquefaciens* HZ-12, *S. cerevisiae* CICC 31001 and *E. coli* DH5α were selected to construct recombinant expression strains, named HSAM2(PHspeD), HSAM2(PSspeD) and HSAM2(PDspeD) respectively. After expressing these genes, the spermidine titers were improved significantly in all recombinant strains (Fig.2a). Among them, the maximum spermidine titer of 91.91 mg/L was obtained in HSAM2(PDspeD), increasing by 8.49-fold compared with the control strain HSAM2(pHY300PLK). These results indicated that the *speD* gene from *E. coli* DH5α was the optimal gene for enhanced spermidine production.

Subsequently, the *speD* gene of *E. coli* was integrated into the genome of HASM2 by homologous recombination, resulting in the integrated expression strain HSAM2□□*DspeD*. After fermentation for 60 h, the spermidine titer reached 33.87 mg/L, which was 2.40-fold higher than that of the control strain HSAM2 (Fig.2b). In comparison, the spermidine titer of the integrated expression strain HSAM2□□*DspeD* was much lower than that of plasmid-based expression strain HSAM2(PDspeD). This phenomenon was

probably due to the low gene copy number during integration expression, while the pHY300PLK plasmid had the high copy number [28]. Therefore, recombinant plasmid expression was more suitable for the *speD* gene.

Effects of different spermidine synthase genes on spermidine production

Spermidine synthase catalyzes the transfer of the aminopropyl group from dcSAM to putrescine to form the spermidine [29], which is considered to be a rate-limiting step in biosynthesis of spermidine [30]. It is particularly essential to exploit efficient spermidine synthase genes (*speE*). Therefore, different *speE* genes were evaluated. According to the KEGG database, three spermidine synthase genes from *E. coli* DH5α, *C. glutamicum* ATCC13032 and *S. cerevisiae* CICC31001 were selected and expressed in *B. amyloliquefaciens* HZMD, resulting in recombinant strains HZMD(PDspeE), HZMD(PGspeE), and HZMD(PSspeE), respectively.

As shown in Fig.3, expressing *speE* genes from *E. coli* and *S. cerevisiae* significantly improved the spermidine production, while the gene from *C. glutamicum* showed no significant difference. Therein, the maximum spermidine titer reached 49.58 mg/L in HZMD(PSspeE), with a 23% increase than that of the control strain HZMD(pHY300PLK). Previously, overexpression of the native *speE* gene was confirmed to be efficient for spermidine synthesis in *S. cerevisiae*[31]. Herein, our results demonstrated that the *speE* gene from *S. cerevisiae* also enhanced the spermidine production in *B. amyloliquefaciens*. It can be inferred that overexpression of this *speE* gene presumably increased the enzymatic activity of spermidine synthase to promote the spermidine synthesis.

Effect of co-expressing *speD* and *speE* genes on spermidine production

Above results showed that genes of *speD* from *E. coli* DH5α and *speE* from *S. cerevisiae* CICC31001 were efficient to enhance the spermidine production. Therefore, these two genes were ligated into one pHY300PLK plasmid, co-expressed in HSAM2 to generate a recombinant strain HSAM2(PDspeD-SspeE). Then, the control strain HSAM2(pHY300PLK), single gene expression strain HSAM2(PDspeD), and co-expression strain HSAM2(PDspeD-SspeE) were compared after fermentation for 60 h. As shown in Figure 4, the spermidine titer of HSAM2(PDspeD-SspeE) reached 105.24 mg/L at 60 h, further improving by 15% compared with the HSAM2(PDspeD). It indicated that co-expression of *speD* and *speE* was effective to increase the spermidine titer, which was probably due to that more upstream substrates of SAM and putrescine were converted to form spermidine.

Optimize fermentation medium

To further improve the spermidine titer of the engineered HSAM2(PDspeD-SspeE), the key components of fermentation medium were optimized, including carbon sources, nitrogen sources and antibiotics. Carbon sources were important for cell growth and metabolites synthesis [32, 33]. Firstly, effects of carbon sources types on spermidine production were investigated. As shown in Fig. 5a, the maximum titer of spermidine was obtained using xylose as the carbon source. Furthermore, the concentration of xylose

was optimized (Fig.5b). The maximum titer of spermidine reached 151.79 mg/L when the xylose concentration was 40 g/L, and no significant increase was observed at 60 g/L of xylose.

Corn pulp was a nutrition-rich nitrogen source, and effects of different corn pulp concentrations on spermidine production were investigated. As was indicated in Fig. 5c, the corn pulp concentration significantly affected the spermidine titer, and the maximum spermidine titer was obtained at 20 g/L. Several previous studies investigated the impact of antibiotics on the fermentation process [34-36]. Herein, different concentrations of tetracycline were added into medium. The increased tetracycline concentration resulted in the improved spermidine titer, and no further increase was noted when the tetracycline concentration reached 4 mg/mL (Fig.5d). These results indicated that adding tetracycline could improve spermidine production, which was probably due to that more plasmids could be maintained under the tetracycline stress.

The spermidine production process under the optimized fermentation midium

Under the optimized fermentation medium (40 g/L xylose, 10 g/L peptone, 20 g/L corn pulp, 2 g/L urea, 6.3 g/L $(NH4)_2SO_4$, 2.5 g/L NaCl, 3 g/L KH_2PO_4, and 4.2 g/L $MgSO_4{\cdot}7H_2O$), the spermidine production process was investigated (Fig. 6). The spermidine was synthesized as the cell grew at the early stage of fermentation. The cell growth entered into the stationary phase at about 48 h, while the spermidine was further synthesized until 84 h, indicating that the spermidine synthesis was a partly growth-coupled process. At 84 h, the maximum spermidine titer reached 227.35 mg/L, which was currently the highest titer reported by microbial fermentation. SAM was the key precursor for spermidine synthesis [5, 37], and the SAM concentration was also measured to further understand the fermentation process. In the initial 24 h stage, the SAM was accumulated rapidly to reach the highest point, while the spermidine concentration did not have obvious improvement. When the fermentation time exceeded 24 h, the spermidine production showed a rapid increase accompanied by a sharp drop in the SAM concentration, indicating that the SAM was probably consumed to synthesize spermidine.

Conclusions

This study explored the possibility of synthesizing spermidine via engineering the *B. amyloliquefaciens*. Effects of different *speD* and *speE* genes on spermidine production were investigated in *B. amyloliquefaciens*. Two efficient genes of *speD* and *speE* were mined to enhance the spermidine production. Subsequently, these two genes were co-expressed to construct an engineering strain HSAM2(PDspeD-SspeE) with high spermidine production. After further optimization of the key medium components, the maximum spermidine titer of 227.35 mg/L was obtained, which was the highest titer among the microbial fermentation methods. Moreover, the detected SAM consumption also explained the accumulation of spermidine. This study excavated the key genes for spermidine synthesis and successfully acquired the highest spermidine titer, which provided the reference for breeding the industrial strain for spermidine production in the future.

Materials And Methods

Strains and plasmids

Table 1 listed all the strains and plasmids constructed in present study. All engineering *B. amyloliquefaciens* strains were modified from the wild-type strain *B. amyloliquefaciens* HZ-12 (M 2015234). *Escherichia coli* DH5α was used as the platform strain to construct expression and integration vectors based on plasmids of pHY300PLK and T2(2)-ori [38]. All designed primers in this study were showed in supplementary material (Table S1).

Table 1
Strains and plasmids used in this study.

Strains or plasmids	Characteristics	Source
B. amyloliquefaciens		
HSAM2	HZ-12 integrated with *SAM2*, *metA* and *metB*, and deficient in *mccA* and *sucC*	Stored in lab
HSAM2(pHY300PLK)	HSAM2 harboring the plasmid pHY300PLK	This study
HSAM2(PHspeD)	HSAM2 harboring the plasmid PHspeD	This study
HSAM2(PSspeD)	HSAM2 harboring the plasmid PSspeD	This study
HSAM2(PDspeD)	HSAM2 harboring the plasmid PDspeD	This study
HSAM2□□*DspeD*	HSAM2 integrated with *speD* from *E. coli* DH5α	This study
HZMD	HZ-12 integrated with *SAM2* and *DspeD*	Stored in lab
HZMD(pHY300PLK)	HZMD harboring the plasmid pHY300PLK	This study
HZMD (PHspeE1)	HZMD harboring the plasmid PHspeE	This study
HZMD (PSspeE2)	HZMD harboring the plasmid PSspeE	This study
HZMD (PDspeE3)	HZMD harboring the plasmid PDspeE	This study
HSAM2(PDspeD-SspeE)	HSAM2 harboring the plasmid PDspeD-SspeE	This study
E.coli DH5α	F^- Φ80d/*lac*ZΔM15, Δ(*lacZYA-argF*) U169, *recA*1, *endA*1, *hsdR*17 (r_K^-, m_K^+), *phoA*, *supE*44, λ^-, *thi*-1, *gyrA*96, *relA*1	Stored in lab
S. cerevisiae CICC 31001	Wild type	Stored in lab
B. subtilis 168	Wild type	Stored in lab
B. licheniformis WX-02	Wild type	Stored in lab
plasmids		

Strains or plasmids	Characteristics	Source
pHY300PLK	*E. coli-Bacillus* shuttle vector for gene expression, Ap^r, Tet^r	Stored in lab
PHspeD	pHY300PLK + P43 + TamyL + *speD* from HZ-12	This study
PSspeD	pHY300PLK + P43 + TamyL + *speD* from *S. cerevisiae*	This study
PDspeD	pHY300PLK + P43 + TamyL + *speD* from *E. coli* DH5α	This study
PDspeE	pHY300PLK + P43 + TamyL + *speE* from *E. coli* DH5α	This study
PGspeE	pHY300PLK + P43 + TamyL + *speE* from *C. glutamicum*	This study
PSspeE	pHY300PLK + P43 + TamyL + *speE* from *S. cerevisiae*	This study
PDspeD-SspeE	pHY300PLK + P43 + TamyL + *speD* from *E. coli* DH5α + P43 + TamyL + *speE* from *S. cerevisiae*	This study
T2(2)-ori	*E. coli-Bacillus* shuttle vector for gene knockout or integration; Kan^r	Stored in lab
T2-PDspeD	T2 (2) + A + B + P43 + TamyL + *speD* from *E. coli* DH5α	This study

Culture conditions

Cells were picked up from LB solid plates (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl and 15 g/L agar), and transferred into LB liquid medium to culture for 12 h at 37°C and 180 rpm to obtain seed cultures. Then, the inoculum was pipetted into the fermented medium. The initial fermentation medium contained 40 g/L sucrose, 3 g/L aspartate, 10 g/L peptone, 5 g/L corn pulp, 2 g/L urea, 6.3 g/L $(NH_4)_2SO_4$, 2.5 g/L NaCl, 3 g/L KH_2PO_4, 4.2 g/L $MgSO_4 \cdot 7H_2O$, and pH 6.5. The inoculum size was 3% (v/v) and the tetracycline concentration was 8 μg/mL. The optimized fermentation medium was consisted of 40 g/L xylose, 10 g/L peptone, 20 g/L corn pulp, 2 g/L urea, 6.3 g/L $(NH4)_2SO_4$, 2.5 g/L NaCl, 3 g/L KH_2PO_4, and 4.2 g/L $MgSO_4 \cdot 7H_2O$, 4 μg/mL tetracycline, and pH 6.5. Fermentation was carried out for 60 h at 37°C with shaking at 180 rpm.

Recombinant plasmid expression

Recombinant plasmid expression was carried out based on the procedures reported previously [39, 40]. The *speD* gene of *B. amyloliquefaciens* HZ-12 (named as *BspeD*) was amplified using primers *BspeD*-F and *BspeD*-R. By Splicing with Overlapping Extension PCR (SOE-PCR), the *BspeD* fragment was ligated with the P43 promoter from *Bacillus subtilis* 168 and the TamyL terminator from *Bacillus licheniformis* WX-02 to obtain gene expression module. Then, this module was inserted into the *Bacillus-E. coli* shuttle

plasmid (pHY300PLK) at restriction sites of *BamH*I/*Xba*I to obtain the expression vector PBspeD1, which was subsequently electro-transformed into *B. amyloliquefaciens* HSAM2 competent cells, resulting in the recombination strain named as HSAM2(PBspeD). Other genes were expressed in pHY300PLK plasmids following the same method.

Homologous recombination

T2(2)-ori mediated homologous recombination was employed in gene integration expression [39, 40]. The upstream and downstream homology arms were amplified using primers A-F/A-R and B-F/B-R, respectively, which were further fused with the expression module of *EspeD* gene by SOE-PCR. Then, the fused fragment was ligated into the T2(2)-ori plasmid at *BamH*I/*Xba*I, resulting in the integrated vector T2(2)-EspeD. Subsequently, this plasmid was electro-transformed into *B. amyloliquefaciens* HSAM2 competent cells, which were cultured in LB plates containing 20 μg/mL of kanamycin. After verification by PCR, positive clones were inoculated into kanamycin-containing LB liquid medium (20 μg/mL), and cultured at 45°C for 8 h at 180 rpm. Single-crossover strains were selected by kanamycin resistance screening and PCR identification, subcultured in LB liquid medium for several times at 37°C (8h). The final cultures were diluted and incubated on LB plates to obtain individual colonies, which were transferred into LB and kanamycin-containing LB plates to screen kanamycin-sensitive colonies. The double-crossover strain was obtained by PCR verification.

Determination of spermidine

A volume of 1.5 mL $HClO_4$ aqueous solution (0.4 M) was added into 0.5 mL fermentative sample, extracted for 1 h. By centrifugation for 10 min at 12,000×g, 250 μL supernatant was collected, and mixed with 25 μL of 2 M NaOH, 75 μL of saturated $NaHCO_3$, and 500 μL of 5 mg/mL dansylchloride. Then, the mixture was reacted at 50°C for 45 min, added with 25 μL of 25% NH_4OH to remove the residual dansyl chloride by incubating for 15 min at 50°C. Finally, the mixture volume was added to 1.5 mL with acetonitrile. After centrifugation for 5 min at 2500×g, the supernatant was collected and filtered through a 0.22 μm membrane for HPLC (high-performance liquid chromatography) analysis, which was carried out using an Agilent 1260 HPLC system with an Agilent column Zorbax Eclipse XDB-C18 (4.6 mm×250 mm, 5 μm) at 30°C. The separation was achieved using a linear gradient of mobile phase A (acetonitrile) and B (H_2O) at a flow rate of 1 mL/min. The solvent gradient was as follows: 50% A (0–3 min), 50–90% A (3–20 min), 90% A (20–29 min), 90–50% A (29–32 min), and 50% A (32–35 min). The detection was carried out at 254 nm [41].

Determination of SAM

The SAM was detected by previously reported HPLC method [26] . For sample pretreatment, 1.5 mL of 0.4 M $HClO_4$ was added into 500 μL fermentation broth to extract the total SAM for 1 h, vortexing for 10 s every 15 min. After centrifugation for 10 min at 12,000×g, 800 μL supernatant was collected and mixed with 95 μL 2 M NaOH and 15 μL saturated $NaHCO_3$. Then, the mixture was centrifuged for 5 min at

2500×g to obtain the supernatant, which was further filtered through a 0.22 μm membrane for HPLC analysis. The sample was analyzed on an Agilent 1260 HPLC system (Agilent, USA) with a Zorbax Eclipse XDB-C18 column (4.6 mm×250 mm, 5 μm). The ratio of mobile phases A (methanol) and B (40 mM $NH_4H_2PO_2$ solution containing 2 mM sodium heptane sulfonate) was set as 18:82, and the flow speed was controlled at 0.8 mL/min. The column temperature was set at 30°C, and the UV detection wavelength was controlled at 254 nm.

Statistical analysis

All fermentation experiments were conducted at least in triplicate, and data were calculated to obtain the mean value and standard deviation. The *t* test was carried out with Data Processing System (DPS) 7.05 to evaluate the significance of difference at the 95% confidence level.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

X. Wei and Y. Liu designed this study, and contributed reagents and materials. D. Zou, L. Li, Y. Min and A. Ji conducted the experimental work. D. Zou analyzed the data. X. Wei, Y. Liu and D. Zou wrote and revised the manuscript. All authors read and approved the final manuscript.

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Figures

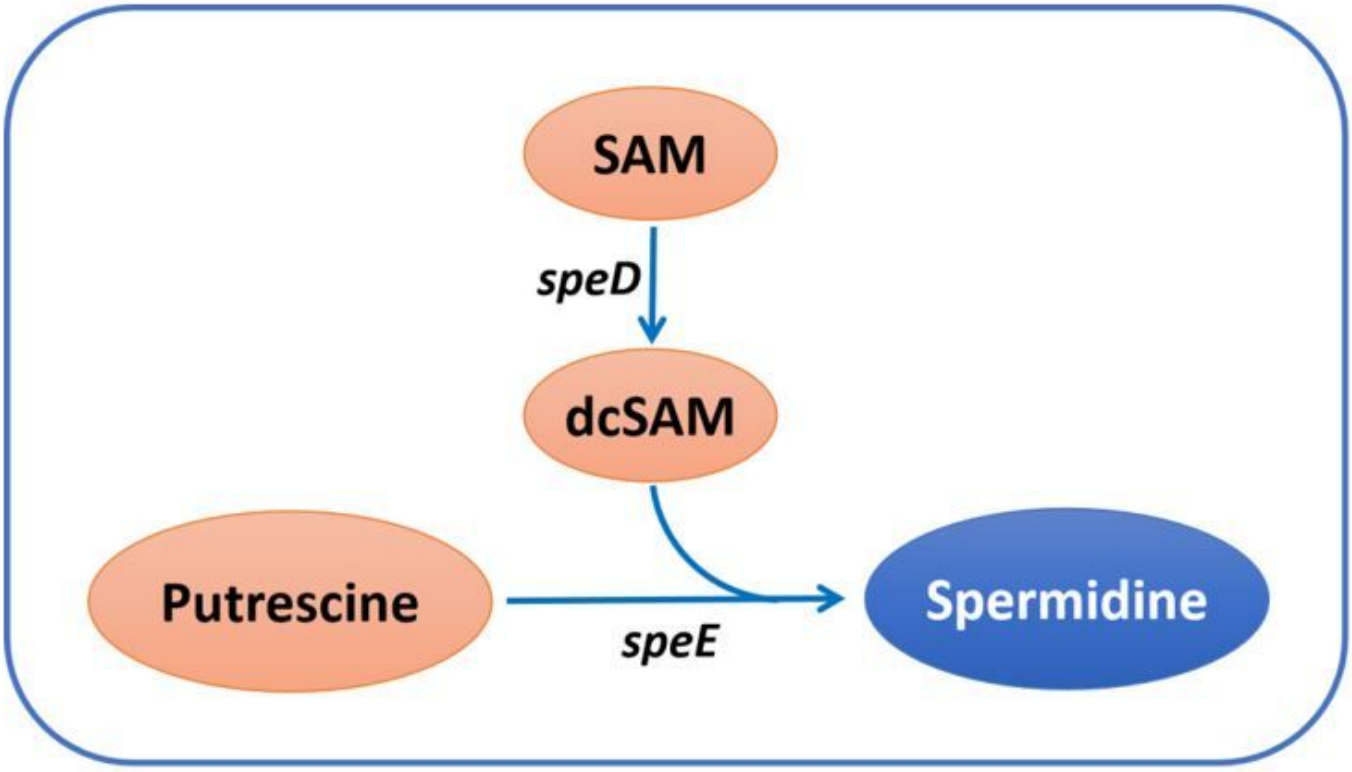

Figure 1

The key synthesis pathway of spermidine in B. amyloliquefaciens. Metabolite and gene abbreviations are as follows: SAM (S-adenosylmethionine), dcSAM (decarboxylated SAM), speD (SAM decarboxylase gene), speE (spermidine synthase gene).

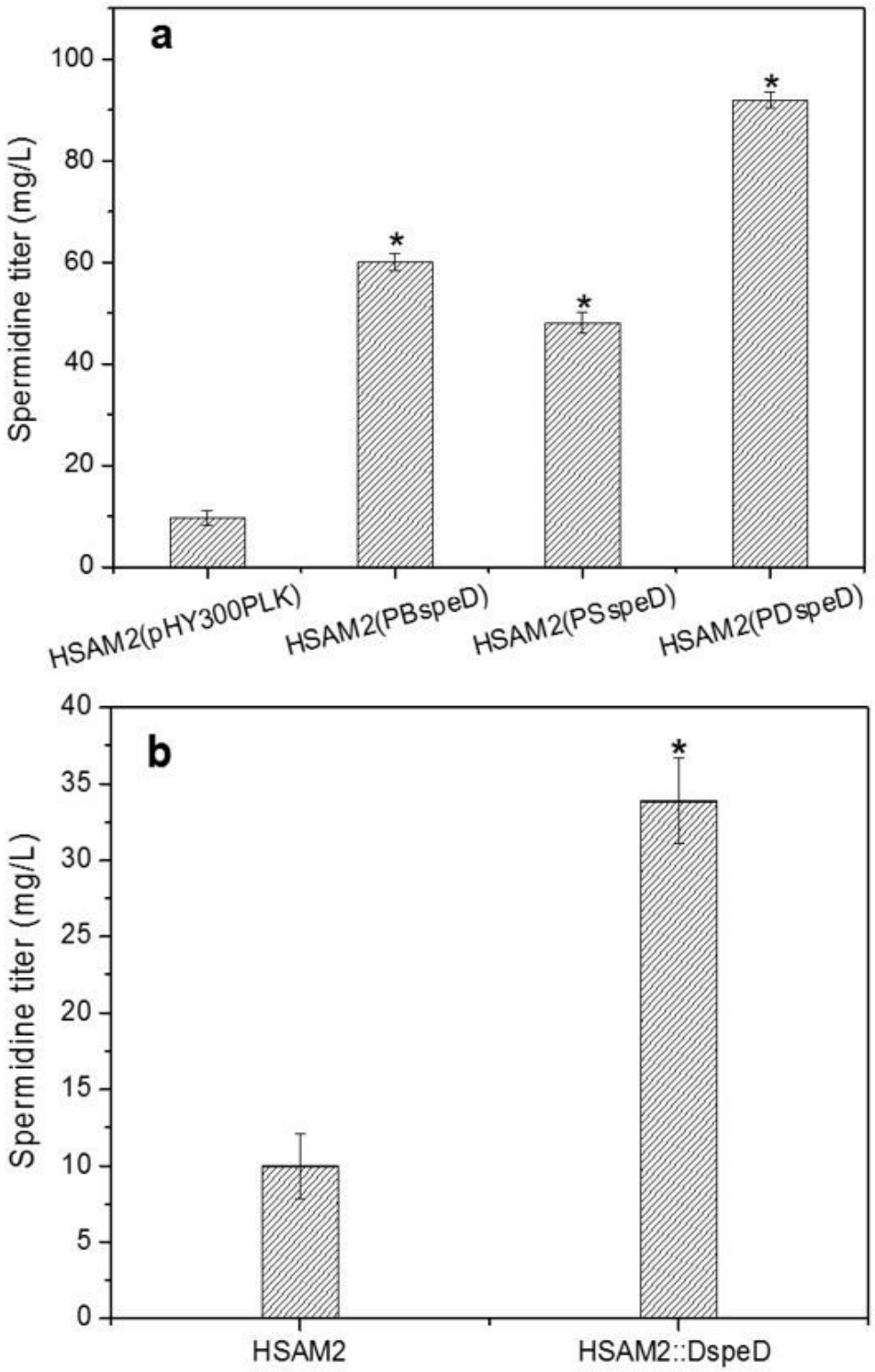

Figure 2

The effect of expressing speD genes on spermidine production. a: The effect of plasmid-based expression of different speD genes on spermidine production. b: The effect of integrative expression of speD gene on spermidine production. Asterisks show the signifcant diference ($p < 0.05$) compared with the control.

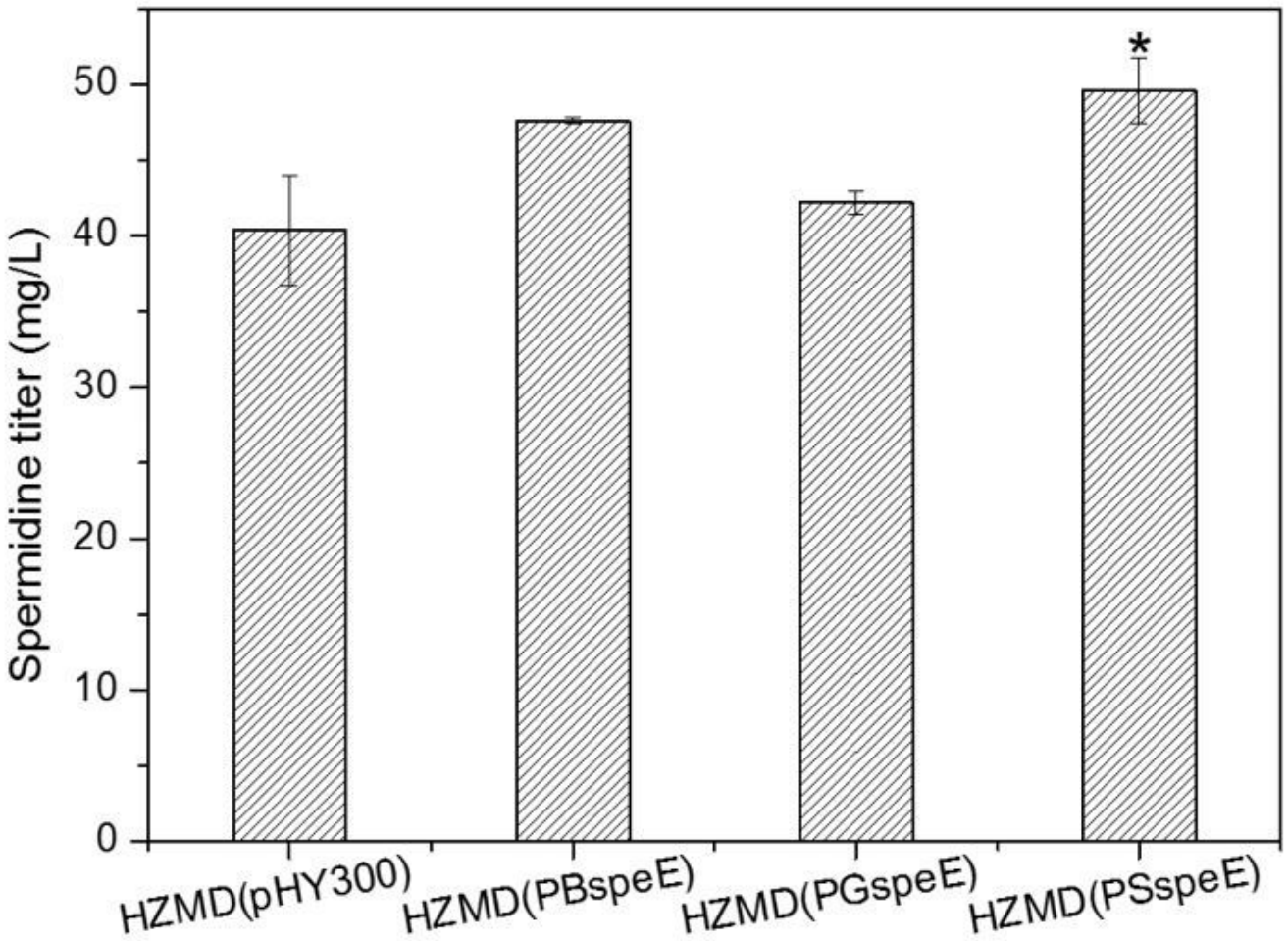

Figure 3

The effect of expressing different speE genes on spermidine production. Asterisks show the signifcant diference ($p < 0.05$) compared with the control.

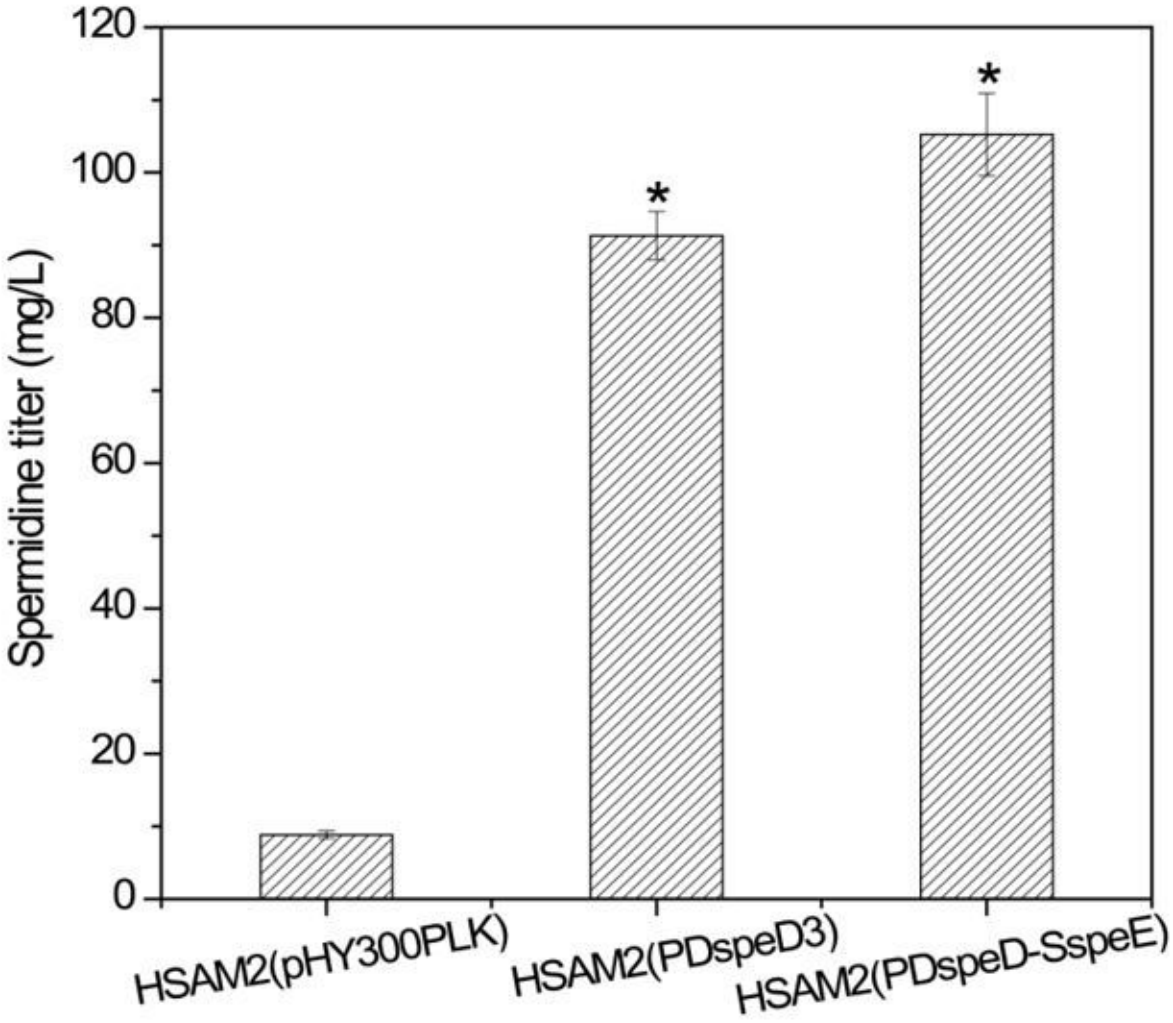

Figure 4

The effect of co-expressing speD and speE on spermidine production. Asterisks show the signifcant diference ($p < 0.05$) compared with the control.

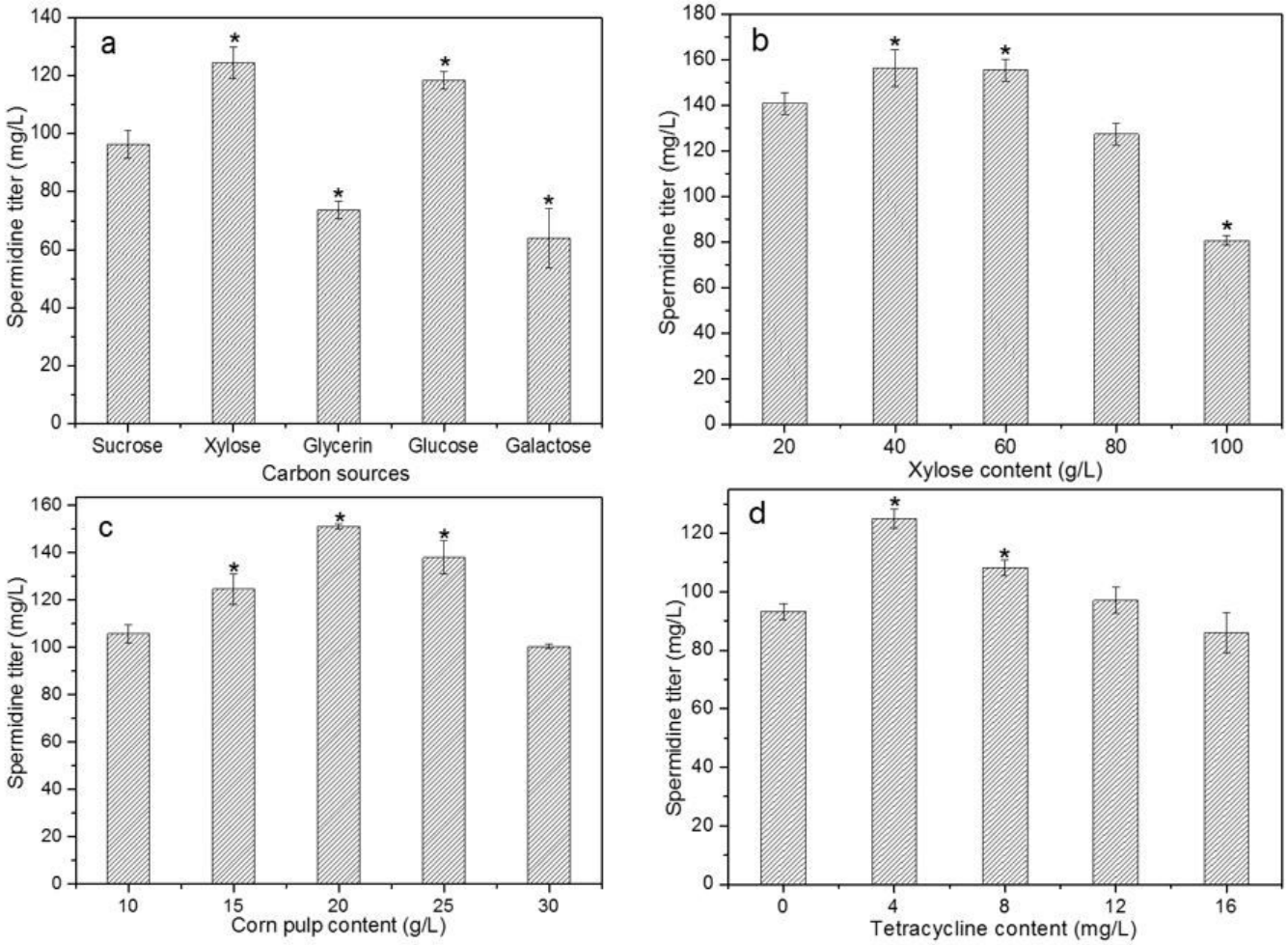

Figure 5

Optimization of fermentation medium. a: Effects of carbon sources. b: Effects of xylose contents. c: Effects of corn pulp contents. d: Effects of tetracycline contents. Asterisks show the signifcant diference ($p < 0.05$) compared with the control.

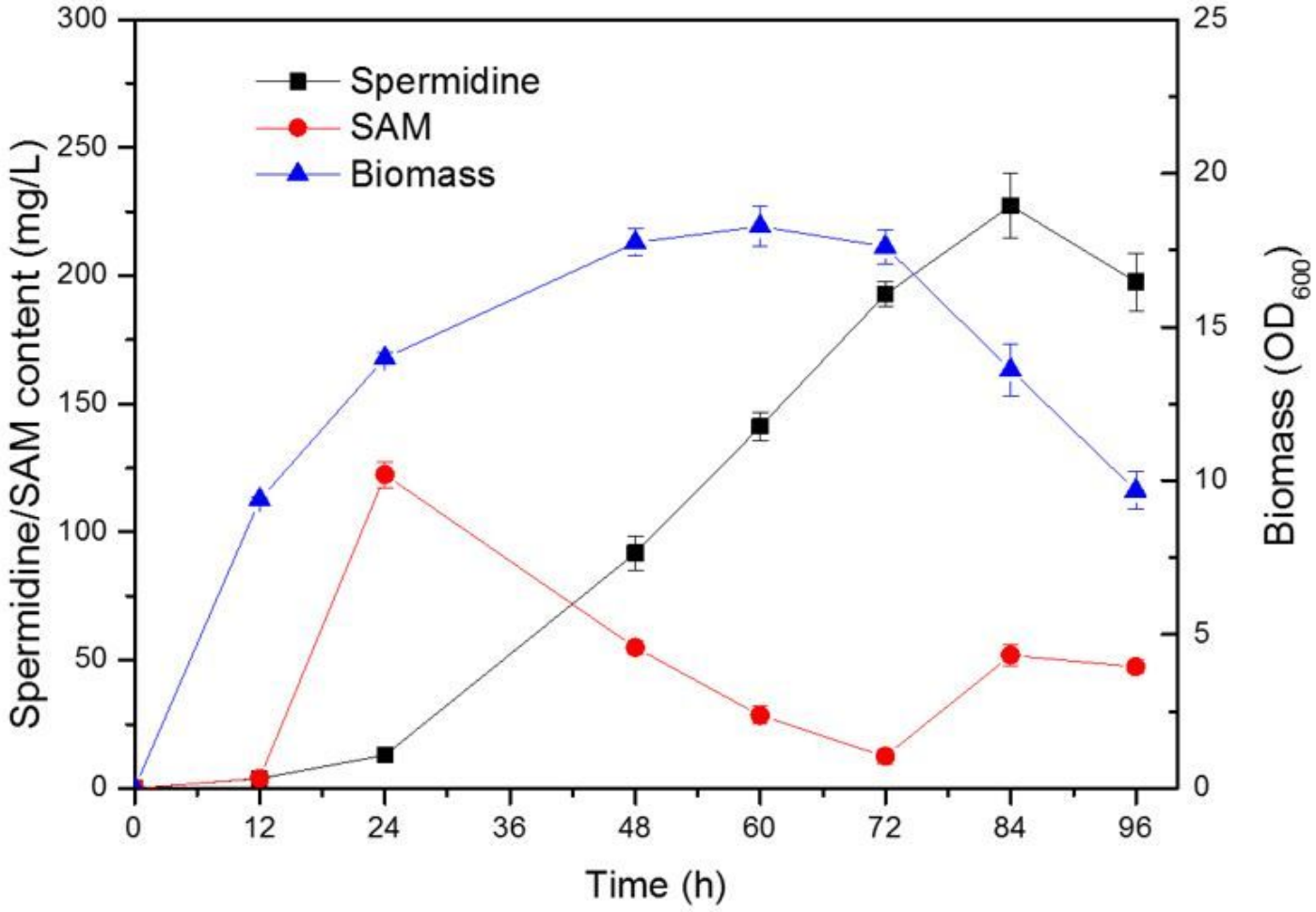

Figure 6

Time profiles of spermidine production in HSAM2(PDspeD-SspeE).

Supplementary Files

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- Supplementarymaterials.docx