Calcium-Phosphate Combination Enhances Spinosad Production in *Saccharopolyspora Spinosa* via Regulation of Fatty Acid Metabolism

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Research Article

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Abstract

Phosphate concentration above 10 mM reduces the production of many secondary metabolites; however, the phenomenon is not mechanistically understood yet. Specifically, the problem of phosphorus limitation in antibiotic production remains unresolved. This study investigates the phosphorus inhibition effect on spinosad production and alleviates it by calcium and phosphate supplementation to fermentation media. Furthermore, we examined the mechanism of fatty acids induced increase in polyketides production. NaH_2PO_4 was found to be the most effective phosphate. Under the optimal phosphate condition, the maximal spinosad production reached 520 mg/L, showing a 1.65-fold increase over the control treatment. In the NaH_2PO_4-$CaCO_3$ system, the de novo fatty acid biosynthesis was significantly downregulated while spinosad biosynthesis and β-oxidation were upregulated. The coordination of de novo fatty acid biosynthesis and β-oxidation promoted intracellular acetyl-CoA concentration. The results demonstrate that NaH_2PO_4-$CaCO_3$ combined addition is a simple and effective strategy to alleviate phosphorus inhibition effect through the regulation of fatty acid metabolism and accumulation of immediate precursors. This information improves our understanding of phosphates' influence on the large-scale production of polyketides.

Introduction

Spinosyns, produced by *Saccharopolyspora spinosa*, are macrocyclic lactones with a special structure consisting of a 21-carbon tetracyclic parent nucleus attached to rhamnose and famolose, respectively (16). Notably, *S. spinosa* can produce > 25 structurally different spinosyns (1). The major structural differences are the C6 methylation on the tetracyclic ring or O- and N-methylation of the glycosyl group (25). The mixture of spininosyn A and spininosyn D, which is the most abundant and effective, is called spinosad (24). Due to specific and high insecticidal activity, wide insecticidal spectrum, and less environmental pollution, > 100 kinds of production processes and strains of spinosad have been registered worldwide; it was also awarded the Presidential Green Chemistry Challenge Award in 1999 (4, 17).

The polyketone structure of spinosad, a highly effective green insecticide (3), is synthesized from repetitive condensation reactions by multifunctional enzymes called type I polyketide synthases (PKSs) (26). Several studies have elucidated the primary biosynthetic pathway and related genes of spinosad in *S. spinosa* (7), including the synthesis and cyclization of the 21-carbon polyketone chain (11), and the synthetic linkage and modification of rhamnose (13) and famolamide (6). Biosynthesis of spinosad involves twenty-three genes dispersed in two clusters: spnA, spnB, spnC, spnD, and spnE produce polyketone synthetase; spnG, spnH, spnI, spnK, gtt, gdh, epi, and kre are involved in the synthesis and methylation of rhamnose; spnF, spnJ, spnM, and spnL participate in the post-ring modification of lactone polyketones, and spnN, spnO, spnP, spnQ, spnR, and spnS encode for the synthesis and ligation of florosamine (12). Although the gene modification and medium formulation optimized strategies improve spinosad production (20), the metabolites yield remains mediocre (100–550 mg/L).

Manipulation of the medium formulation was also reported to improve the yield of secondary metabolites (21). For instance, optimization of the seed medium for spinosad fermentation through response surface analysis increased the yield by 86.88% (18). Likewise, supplementation with several inorganic salts, optimization of fermentation medium, and controlling dissolved phosphorus can improve the spinosad yield by 2.07 times (29). The addition of 30 g/L camellia oil to medium improved spinosad production up to 520 mg/L (8). However, so far, there is a lack of understanding about the effect of phosphate on the gene expression and intermediates of the spinosad biosynthesis pathway. Notably, phosphate concentration > 10 mM significantly reduces the production of many secondary metabolites (14); accordingly, the current study aims to reduce the inhibition effect of phosphorus in spinosad production using advanced fermentation technology for better yield.

The phosphate/calcium carbonate buffer system (9) forms a slightly soluble calcium phosphate salt, which maintains the normal growth of bacteria and promotes the synthesis of antibiotics. We speculated that this combined system can slowly dissolve the fermentation broth phosphorus limiting its inhibitory effect in the early stage fermentation while relieving the phosphorus deficiency in the later stage at least to some extent. The negative regulation of phosphate is thought to be associated with the adaption of primary and secondary metabolism to the culture conditions (15). For instance, excess inorganic phosphate in fermentation broth significantly inhibits the synthesis of rifamycin in *Nocardia Mediterranean* but improves biomass and lipid content (19). Overall, a metabolic explanation from the perspective of β-oxidation, de novo fatty acid synthesis, and spinosad biosynthesis can better explain the influence of phosphate and phosphate/calcium carbonate buffer system on spinosad production.

In this work, we used the phosphate/calcium carbonate buffer system in the fermentation medium of *S. spinosa* to enhance spinosad production. Phosphate concentration and addition time were optimized to improve secondary metabolite production. Based on metabolic enzyme activity and gene transcription analysis, we propose the regulatory role of fatty acid metabolism on spinosad biosynthesis in *S. spinosa*. The combined phosphate/calcium carbonate buffer system can significantly limit the inhibitory effects of phosphorus on spinosad production.

Materials And Methods

Microorganism and culture conditions

S. spinosa strain 1733 was used in this study. The solid medium containing 10 g glucose, 1 g beef extract, 5 g yeast extract, 3 g tryptone, 2 g MgSO4, 20 g agar per liter of distilled water (pH was adjusted to 7.4 with 1 N NaOH) was used for plate culture at 28 ℃. The fermentation experiment was carried out as follows: A seed culture was prepared by inoculating *S. spinosa* into a 250 mL Erlenmeyer flask containing 50 mL of seed medium (glucose 5 g/L, beef extract 1 g/L, yeast extract 5 g/L, tryptone 5 g/L, $MgSO_4$ 2 g/L and pH 7.4), which was incubated for 3 d at 28 ℃ with constant shaking at 180 rpm. Next, 8 mL of the seed culture was transferred into the 40ml fermentation medium (glucose 6 g/L, yeast extract 6 g/L, corn steep powder 10 g/L, cake powder 15 g/L, cottonseed meal 10 g/L, $FeSO_4$ 0.05 g/L, soybean

oil 6 g/L, $CaCO_3$ 5 g/L and pH 7.4). The culture (fermentation) was carried out at 28°C and 180 rpm for 10 d. The fermentation medium was supplemented with different concentrations of phosphates (NaH_2PO_4, Na_2HPO_4, KH_2PO_4, and K_2HPO_4) at different time points to study the effects of phosphates on spinosad production. Each experiment was carried out in triplicates under identical conditions, and standard deviations were calculated.

Growth and spinosad estimation

4 mL of fermentation broth was centrifuged at 4000 rpm for 10 min; the obtained mycelium was re-suspended in 6 mL of 0.1 M HCl with vigorous stirring. Next, this solution was centrifuged at 3000 rpm for 10 min, and dry cell weight (DCW) was measured after drying to a constant weight at 65 ℃. For spinosad extraction, fermentation cultures were mixed with 4× volume of ethanol (28). Then, the mixture was sonicated > 1 h at RT and filtered through a 0.22 μm micro-membrane for HPLC (Waters Symmetry C18 column, 4.6×250 mm, 5 μm) analysis at 254 nm. 10 μL mixtures were separated using buffer A (45% methanol, 45% acetonitrile, 10% 260 mM ammonium acetate) as a mobile phase at a flow rate of 1.0 mL/min. Spinosad was quantified using a regression line generated from a commercially available standard (Nongle Bioproduct Co., Ltd OF Shanghai) dissolved in methanol. Each treatment was performed in triplicates to calculate the standard deviations.

Determination of extra- and intracellular phosphate concentration

1 mL of cell cultures were chilled on ice immediately after collection and then centrifuged at 4000 rpm for 10 min to obtain 700 μL supernatant and 0.1 g cell pellet. The supernatant was filtered by a 0.22 μm aqueous phase filter and stored at − 80°C until used for extracellular phosphate content estimation. The cell pellets were immediately frozen in liquid nitrogen and ground into powder. From these, the intracellular phosphate was extracted using 300 μL 0.2 mol/L HCl and centrifugation at 12000 rpm and 4 ℃ for 10 min. The collected supernatant was filtered by a 0.22 μm aqueous phase filter and the intra- and extracellular phosphate contents were determined using the phosphate content detection kit (Solebul Reagent, China), according to manufacturer's instructions. The kit is based on the ammonium molybdate spectrophotometry method (22).

RNA extraction and cDNA synthesis

For transcriptional studies, 1 mL of fermentation broth, collected at 120, 144, 168, 192, 216, and 240 h, were centrifuged at 12000 rpm for 10 min to obtain the bacterial cell pellets. These were quickly frozen in liquid nitrogen and ground into powder. Then 0.2 g of bacterial dry powder was incubated with 1 mL Trizol reagent (Thermofid, USA) for 5 min. The mixture was centrifuged at 12000 rpm for 10 min to collect the supernatant, which was mixed with 0.2 mL chloroform. This mixture was centrifuged at 12000 rpm for 10 min and the obtained supernatant was mixed with 0.4 mL isopropanol. The mixture was centrifuged at 12000 rpm for 10 min and the collected precipitation was mixed with 1 mL 75% ethanol. Again, the supernatant was collected after centrifugation at 7500 rpm for 5 min. After drying at RT, the supernatant was dissolved in 50 μL RNase-free water and stored at -80℃ for later use. The

concentration, purity, and integrity of RNA were determined by ultraviolet spectrophotometry and gel electrophoresis.

For cDNA synthesis, the extracted RNA (described above) was reverse transcribed using HiScript III 1st Strand cDNA Synthesis Kit (Novezan, China) in a total volume of 20 µl containing 2 µl of 10 × RT buffer, 2 µl HiScript III Enzyme Mix, 10 µl total RNA and 6 µl RNase-free H2O. After evenly mixing, the reactions were performed as follows: incubation for 15 min at 37℃, and then reaction termination by heating at 85 ℃ for 5 sec. The products of reverse transcription reaction were stored at − 80℃ until used.

Quantitative reverse transcriptase PCR (RT-qPCR)

Triplicate independent RT-qPCR assays were performed using the LightCycler 480 (Roche Diagnostics GmbH, Germany). ACEQ Universal SYBR qPCR Master Mix Kit (Novizan, China) was used to monitor the real-time amplification of the qPCR product following the manufacturer's protocol. The reaction was performed in 20 µl containing 10 µl of 2× qPCR Master Mix, 0.4 µl each primer, 1 µl cDNA and 8.2 µl RNase-free ddH_2O. After gentle mixing, the PCR reactions were performed as follows: initial denaturation at 95 ℃ for 5 min; 40 cycles of 10 s denaturation at 95 ℃; 30 s annealing, and final extension at 72 ℃. The target genes spnA, spnJ, spnG, spnO, spnP, gtt, fadA, fadE, fadG and fadI were amplified accordingly. The used primer sequences are listed in Table 1 (23). CT values of the target genes were normalized to 16S rRNA of *S. spinosa*, and the results were analyzed using the 2 − CT method.

Extraction and HPLC analysis of intracellular acetyl-CoA

Intracellular acetyl-CoA were extracted as described previously with slight modifications (2). Briefly, 1 mL cell cultures were collected at different fermentation stages and centrifuged at 12000 rpm for 10 min to collect the bacterial pellets. These were quickly frozen in liquid nitrogen and ground into powder. 0.2 g of precisely weighed dry powder was stored at -80 ℃ for analysis. 1 mL of 10% (m/v) precooled trichloroacetic acid was added to the dry powder, swirled for 30 s, and then centrifuged at 12,000 rpm at 4℃ for 10 min. The collected supernatant was filtered by a 0.22 µm aqueous phase filter for analysis. The standard for analysis was prepared by dissolving 1.0 mM acetyl-CoA in 100 mM sodium acetate (pH 4.0), which was stored at -80 ℃ until use.

HPLC method was employed to estimate the acetyl-CoA content as described previously with slight modifications (5). The samples and standards were filtered through 0.22 µm micro-membrane filters and analyzed using HPLC (Plastisil ODS C18 column, 4.6×250 mm, 5 µm) at 254 nm. Buffers A (0.2 M sodium dihydrogen phosphate) and B (0.25 M sodium dihydrogen phosphate and 20% acetonitrile) were used as the mobile phase solvents. 10 µL samples were separated on the HPLC column with a flow rate of 1.0 mL/min. The analytes were eluted with a gradient as follows: 90% buffer A (10% buffer B) for 5 min; 90–60% buffer A (40% buffer B) in 15 min, and then 60–50% buffer A (50% buffer B) in 20 min. Standards of acetyl-CoA were used to find its retention time (17.5 min).

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 8, t-test Statistical significance was accepted for $p < 0.05$. All experiments were conducted in triplicates. Data are presented as mean values with standard deviations (SD).

Results

Effect of phosphate and calcium carbonate content on spinosad production and cell growth

First, we examined the effect of the different kinds of inorganic phosphates in the initial fermentation stage and different ratios of phosphate and calcium carbonate in the fermentation culture. After the end of fermentation, spinosad production and biomass were compared and comprehensively analyzed among different treatments. Four kinds of phosphate (K_2HPO_4, KH_2PO_4, Na_2HPO_4, NaH_2PO_4) and different supplemental amounts (0, 2.5, 5, 10, 20 L, and 40 mmol/L) were tested.

As shown in Fig. 1, in the presence of 5g/L calcium carbonate, both potassium phosphate and sodium salts slightly promoted the growth of bacterial biomass. An increase in phosphate concentration increased the spinosad yield; however, a phosphate concentration > 20 mM began to inhibit the spinosad biosynthesis. NaH_2PO_4 was the best phosphate salt and the optimum concentration was 20 mM, which increased spinosad production by 49%. When the calcium carbonate content was reduced by ten times and the phosphate was only 5 mM, the spinosad production decreased, but the bacterial weight increased significantly. In the absence of calcium carbonate, the production of spinosad in the phosphorus alone group was significantly lower (0.4–0.6 times) than the phosphorus-calcium combined group.

Effect of phosphate addition time on spinosad production and cell growth

Previous studies showed that phosphate addition time can influence polyketides biosynthesis. NaH_2PO_4 (20 mM) was added to the spinosad fermentation media at 0, 24, 48, 72, 96, and 120 h to find a suitable addition time (Fig. 2). The bacteria grew rapidly and accumulated well at the initial stage. On day 3, when the bacteria biomass reached the maximum value, spinosad biosynthesis began and reached maximum by the 10th d of fermentation. In the presence of calcium carbonate, supplementation with NaH_2PO_4 at different fermentation stages increased the production of spinosad. Importantly, phosphate supplementation on the 4th d of fermentation resulted in maximum biosynthesis of spinosad (520 mg/L), which was 1.65 times higher than that of the control group (without phosphorus and calcium), 11% higher than the 0 h treatment group. Concerning the bacterial biomass, in absence of calcium carbonate, the bacterial growth rate, in turn, bacterial biomass was significantly higher in the experimental group supplemented with phosphate than the other groups. The increase in spinosad production was consistent with the decrease in the bacterial biomass, suggesting the significant effect of phosphate on the growth of spinosad-producing bacteria.

20 mM NaH_2PO_4 added at 96 h was found to be the optimal phosphate addition condition. In the subsequent experiments, this condition was applied to investigate the positive effect of the phosphate-

calcium system on spinosad production. The phosphate consumption curve shown in Fig. 2.C indicates both intra- and extracellular phosphorus deficiency in the control group at 96 h of fermentation. On the contrary, in the experimental group with added phosphate, extracellular phosphorus concentration increased; also, the intracellular phosphorus concentration increased to ~ 12 mM within 48 h and then remained stable for the later period. In the later fermentation period, when the dissolved phosphorus becomes insufficient, a part of the NaH_2PO_4-$CaCO_3$ system as sparingly dissolved salt can slowly release phosphorus into the fermentation broth to maintain the metabolic needs of the bacteria.

Effect of phosphate addition on transcriptional regulation of spinosad biosynthetic genes

Concerning the genes of the spinosad biosynthetic pathway, NaH_2PO_4-$CaCO_3$ supplementation upregulated the expression of all detected genes compared with the control group (Fig. 3). The spnA gene showed the maximum upregulation (4–5 times). The mRNA level of the genes related to the spinosad biosynthesis reached the maximum at 192 h and then began to decline. The decline in the later stage could be due to the mRNA degradation caused by bacterial decay with the extension of fermentation time. Compared with the control group, the calcium alone group did not show a significant difference in the gene transcription level with the fermentation time. However, gene transcription was inhibited to varying degrees in the phosphorus alone group. Interestingly, the addition of 5 g/L $CaCO_3$ significantly improved the transcription of these genes. We found that though 20 mM NaH_2PO_4 could increase the bacterial biomass, it inhibited the biosynthesis of secondary metabolites unless 5 g/L $CaCO_3$ was added to the fermentation broth. Overall, the increase in spinosad yield in the NaH_2PO_4/$CaCO_3$ system can be attributed to the transcriptional upregulation of spnA encoding polyketone synthase.

NaH_2PO_4-$CaCO_3$ increased intracellular acetyl-CoA content

Acetyl-CoA, an intermediate in glycolysis, is the immediate precursor of malonyl-CoA. Besides, it is the main product of β-oxidation. Malonyl-CoA, an intermediate for de novo fatty acid synthesis, is the immediate precursor of spinosad biosynthesis. Next, we used HPLC to examine the dynamic changes in intracellular acetyl-CoA content (Fig. 4). The concentration of intracellular acetyl-CoA was higher in the NaH_2PO_4-$CaCO_3$ group than in the control group at each fermentation stage after phosphorus supplementation. However, towards the end of fermentation, the acetyl-CoA concentration became similar in the two groups. The acetyl-CoA content reached 2.13 nmol/mg DCW at 168 h and then decreased to ~ 0.37 nmol/mg DCW at the end of fermentation in the NaH_2PO_4-$CaCO_3$ group. Whereas, in the control and the calcium alone groups, the acetyl-CoA concentration remained at a lower level of 0.25–0.40 nmol/mg DCW. Compared to the control group, the intracellular acetyl-CoA content was even lower in the phosphate alone group at 144, 168, 192, 216, and 240 h.

Gene expression assays of de novo fatty acid synthesis and β-oxidation

To understand the relationship between the main metabolism of fatty acids and the secondary metabolism of spinosad biosynthesis, we examined the transcription levels of target genes involved in

these metabolic pathways using RT-qPCR; genes fadG and fadI are involved in de novo fatty acid synthesis and fadA and fadE are involved in β-oxidation (Fig. 5). Notably, after phosphorus supplementation, the gene transcription levels of fadG and fadI showed a rapid response and decreased to 0.02 times in the NaH_2PO_4-$CaCO_3$ group than in the control group. The calcium alone group did not show a significant difference compared with the control group (without phosphorus and calcium), while the gene transcription increased by 1.4–2.1 times in the phosphorus alone group. In contrast, transcription of fadA and fadE were significantly upregulated in the NaH_2PO_4-$CaCO_3$ group compared to the control group, which is consistent with the upregulation of acetyl-CoA content.

Discussion

Previous studies have shown that phosphate concentration in fermentation medium significantly affects spinosad production (10). This is attributed to the coordinated regulation of primary and secondary metabolism of the bacteria to adapt to various vegetative environments (14). However, the specific regulatory mechanism of phosphorus signal transduction in microorganisms is obscure because of its intricate interrelation with other trophic environmental pressures, such as carbon source decomposition repression and nitrogen limitation. Here, we examined the possible mechanism behind this phenomenon in *S. spinosa* by analyzing the transcriptional levels of genes related to spinosad biosynthesis, β-oxidation, and de novo fatty acid biosynthesis and their relationship with acetyl-CoA concentration.

During spinosad fermentation, the addition of 5 mM phosphate promoted the growth of bacteria by upregulating primary metabolism, which in turn inhibited the biosynthesis of spinosad. However, a combined supplement of 5 mM phosphate and 5 g/L $CaCO_3$ decreased the bacterial biomass but improved spinosad yield compared with the phosphorus alone group. Possibly, the two salts formed a complex that limited the amount of dissolved phosphorus in the fermentation system, which in turn relieved the phosphorus inhibition effect on spinosad production. Furthermore, an optimal combination of 20 mM NaH_2PO_4-5 g/L $CaCO_3$ significantly improved spinosad production.

In the Na_2HPO_4/$CaCO_3$ fermentation system, the genes related to spinosad biosynthesis, especially the gene encoding for polyketone synthase, were significantly up-regulated, which was accompanied by a significant increase in intracellular acetyl-CoA content. Overall, this change promoted the production of spinosad. Although the increased expression of polyketone synthase would consume more acetyl-CoA for spinosad biosynthesis, the intracellular acetyl-CoA concentration in the Na_2HPO_4/$CaCO_3$ combined group remained higher during the whole fermentation process. The highest acetyl-CoA concentration (2.04 nmol/mg DCW) was about 3.6 times more than that of the control group (0.56 nmol/mg DCW) after 168 h of fermentation. Notably, the precursor acetyl-CoA is not only the upstream precursor of de novo fatty acids synthesis but also the main product of fatty acid β-oxidation (27). The addition of 5g/L $CaCO_3$ after phosphorus addition increased the intracellular acetyl-CoA content of S. spinosa, which can be attributed to inhibition of fatty acid synthesis due to lack of active acetic acid units. Meanwhile, the upregulation of the β-oxidation pathway also promotes the generation of acetyl-CoA, which is a favorable condition for

the polymerization of macrolides. Concisely, the Na_2HPO_4-$CaCO_3$ combination reduces fatty acid metabolism while increasing acetyl CoA accumulation, which allows the efficient expression of polyketone synthase and the synthesis of the polyketone chain.

In conclusion, in this study, we demonstrated that the combination of 20 mM NaH_2PO_4-5 g/L $CaCO_3$ effectively promoted the synthesis of spinosad in *S. spinosa*; calcium carbonate could alleviate the barrier of phosphorus limitation. The addition of calcium carbonate after phosphorus supplementation at 96 h of fermentation increases intracellular acetyl-CoA content by coordinating the fatty acid metabolism and upregulating the spinosad biosynthesis pathway (Fig. 6). The phosphate/calcium combination strategy relieves the phosphorus inhibition effect on spinosad production and can also be applied to increase the fermentation yield of other macrolide antibiotics.

Declarations

Ethical Approval

This article does not contain any studies with human participants or animals.

Authors Contributions

CP performed the data analysis and wrote the first draft of the manuscript. M-YW and CP performed the experiments and data interpretation. M-RW and W-XD provided technical assistance to CP and J-FH. J-YL supervised the experimental design, data analysis, manuscript writing, and revision. All authors read and agreed on the final text.

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Competing Interests

All authors have no conflict of interest to declare.

Availability of data and materials

All datasets generated for this study are included in the article/Supplementary Material.

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Tables

Table 1 is not available with this version.

Figures

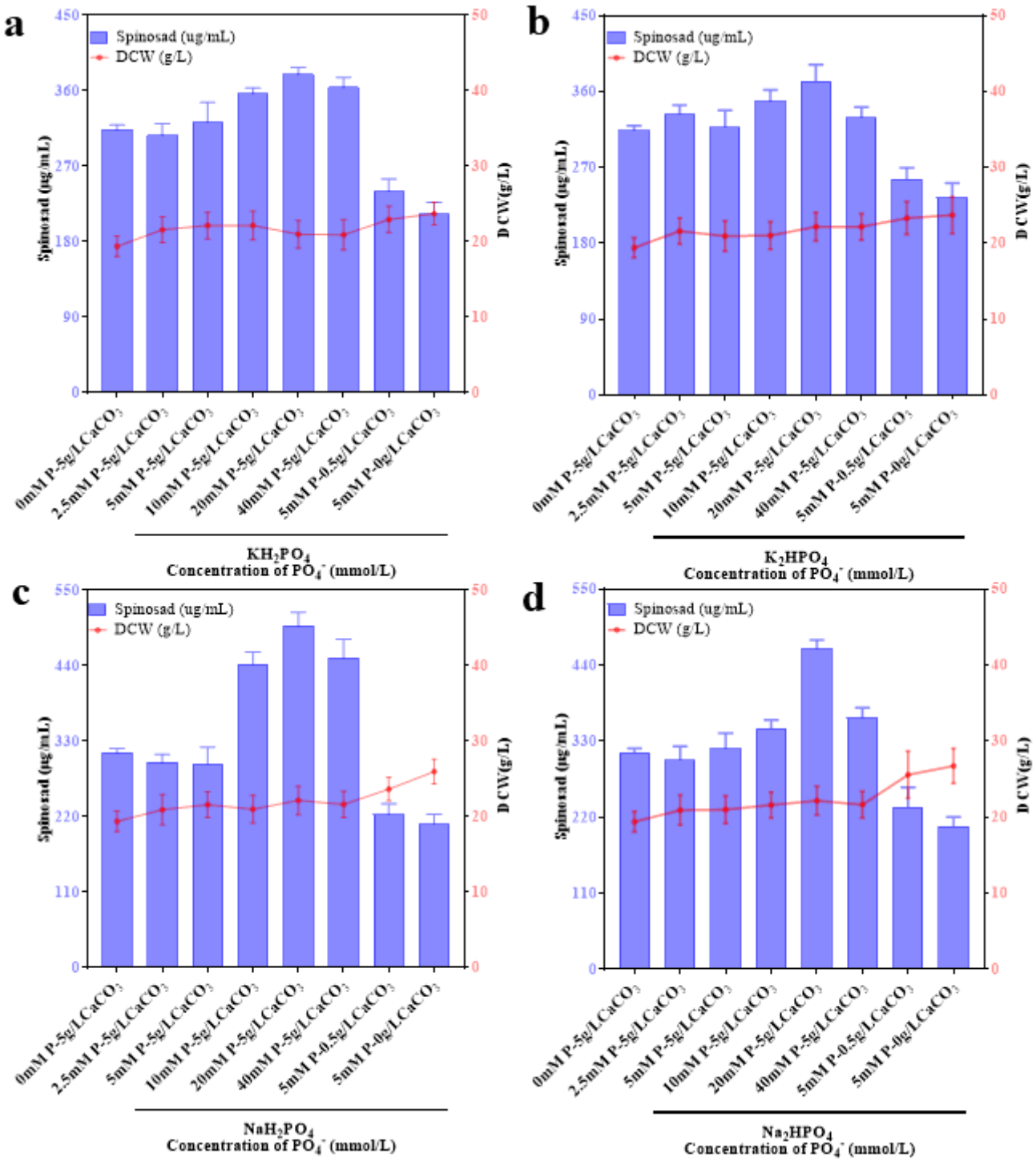

Figure 1

The effect of different phosphate and calcium carbonate concentrations on spinosad production and bacterial growth. (a) KH2PO4; (b) K2HPO4; (c) NaH2PO4; (c) Na2HPO4.

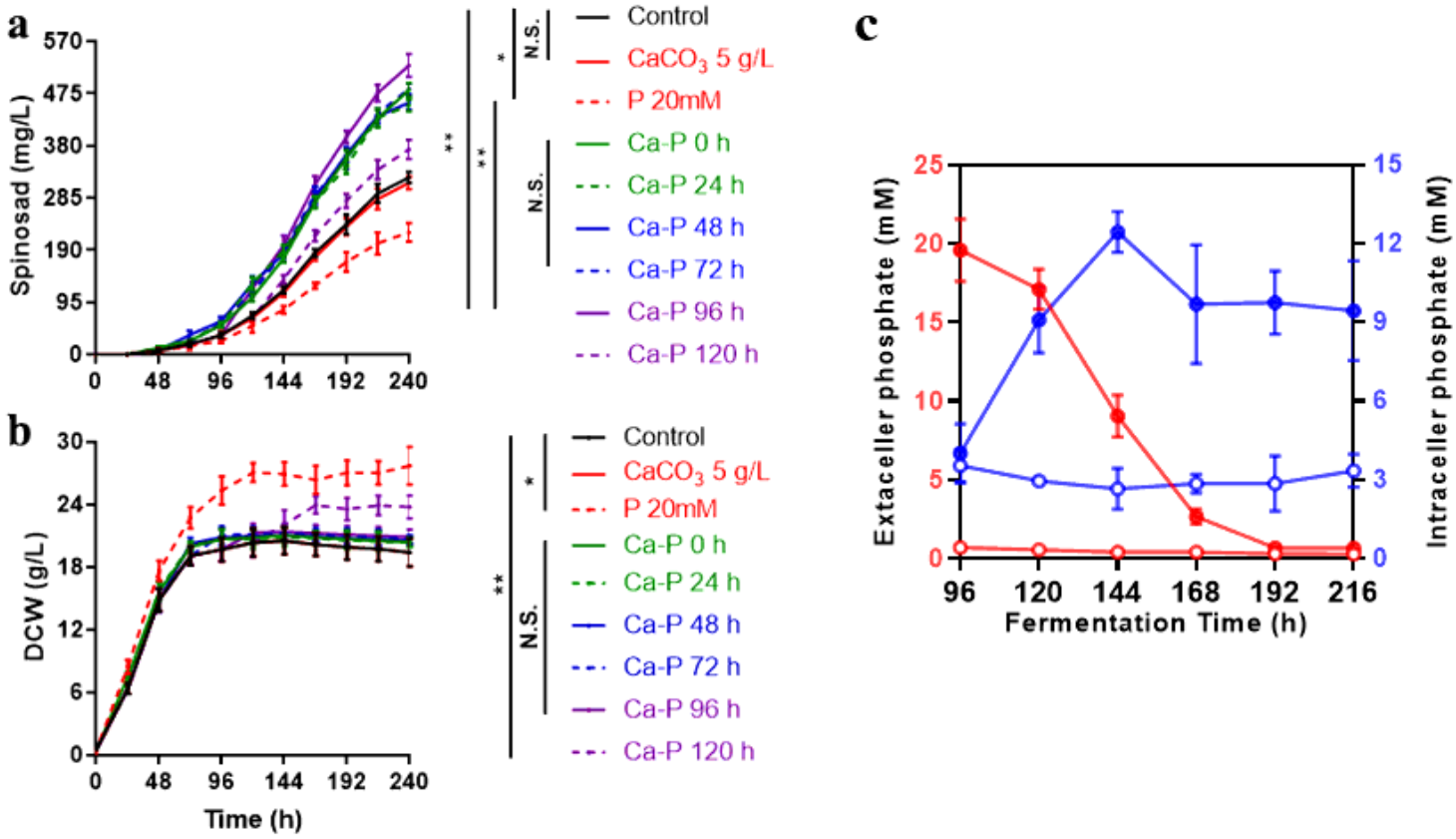

Figure 2

The effect of phosphate addition time on spinosad production and bacterial growth. (a) Spinosad production-time curve; (b) Biomass-time curve; (c) Intracellular and extracellular phosphate concentration-time curve.

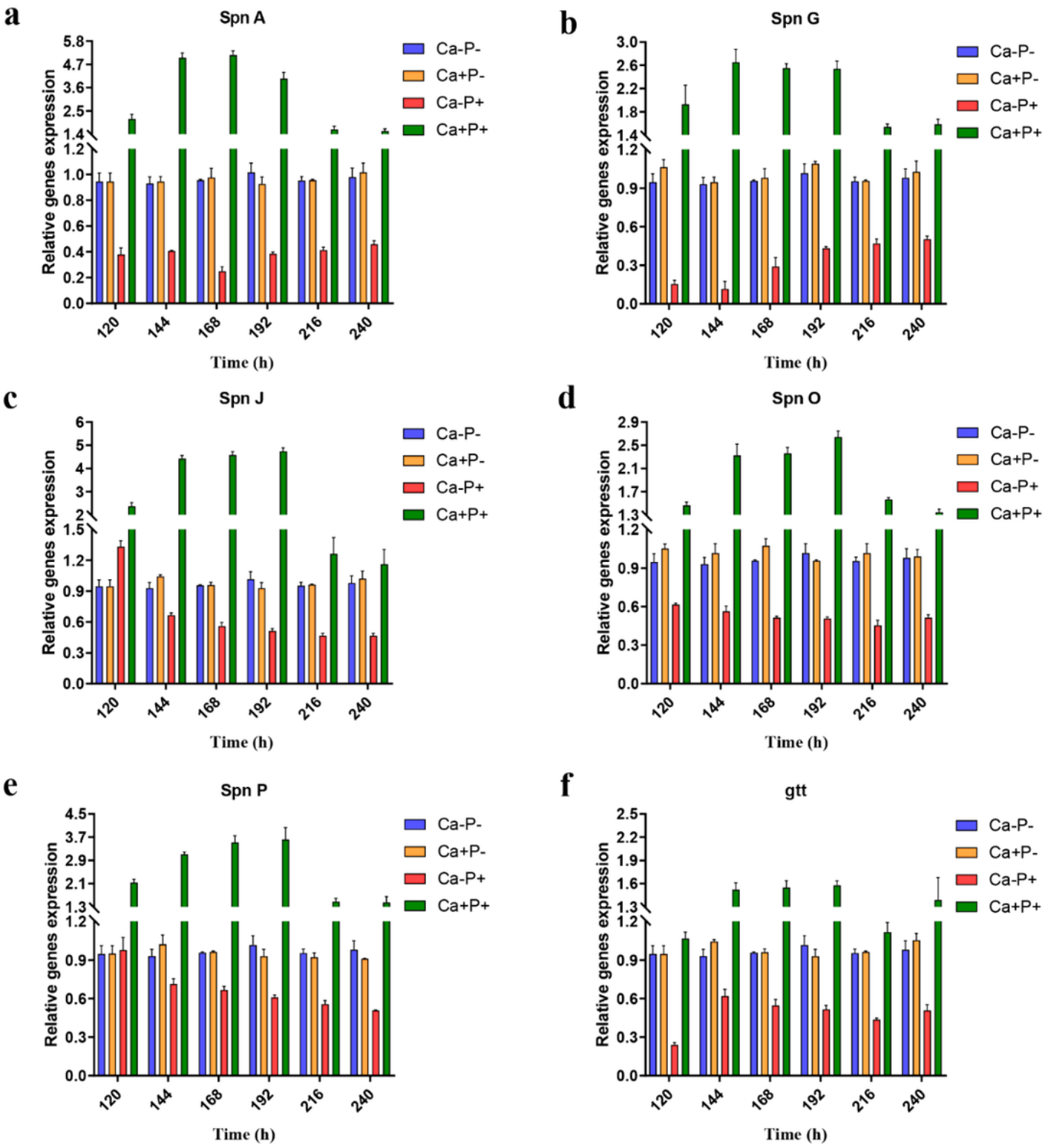

Figure 3

The effect of Na2HPO4-CaCO3 fermentation system on the transcription of spinosad biosynthesis-related genes. (a) SpnA; (b) SpnG; (c) SpnJ; (d) SpnO; (e) SpnP; (f) gtt.

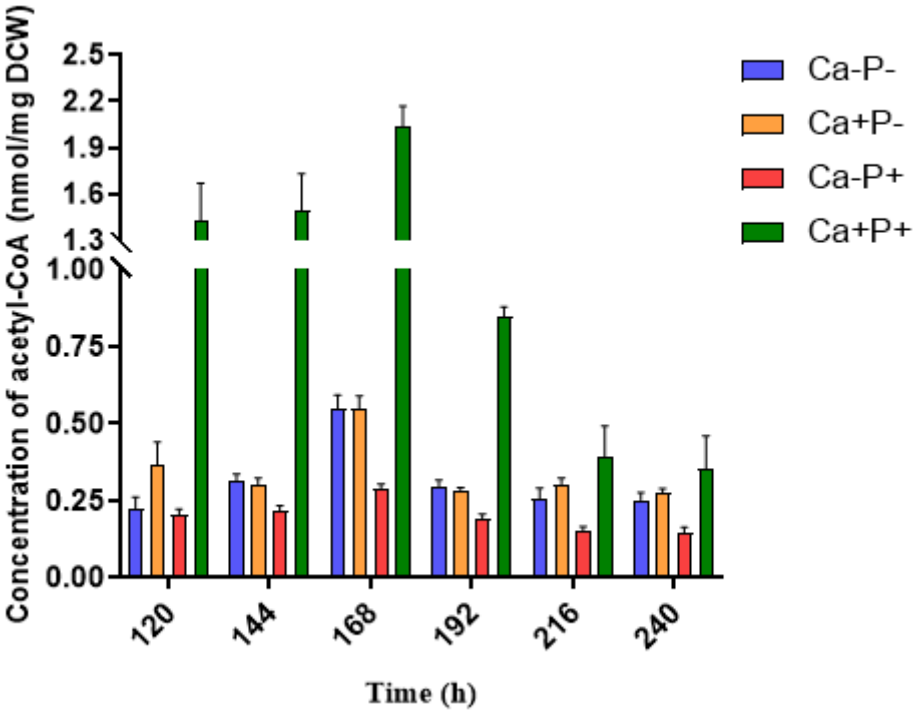

Figure 4

The effect of Na2HPO4-CaCO3 fermentation system on the accumulation of acetyl-CoA.

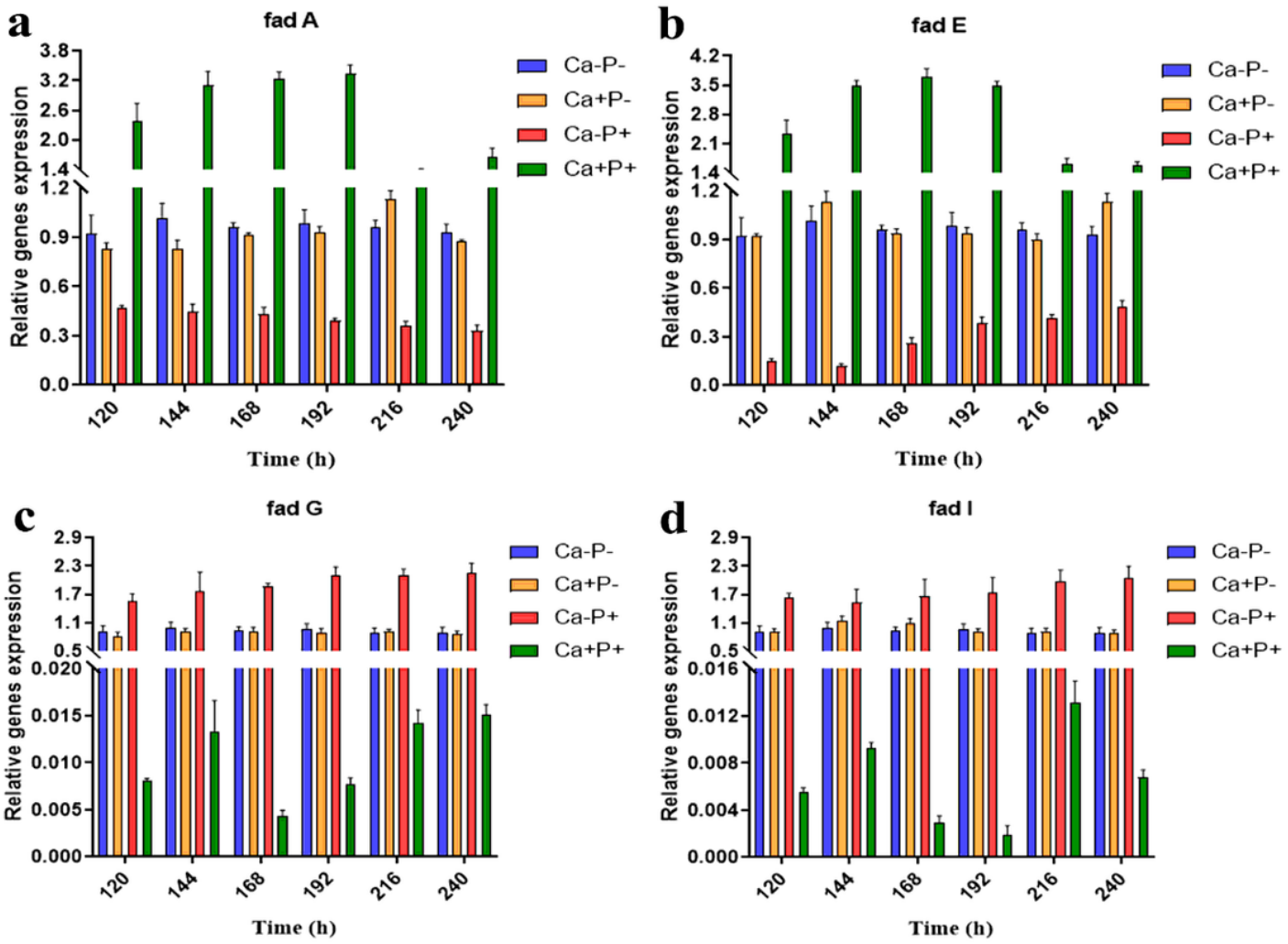

Figure 5

The effect of Na2HPO4-CaCO3 fermentation system on the transcription of genes involved in fatty acid de novo synthesis and β oxidation. (a) FadA; (b) FadE; (c) FadG; (d) FadI.

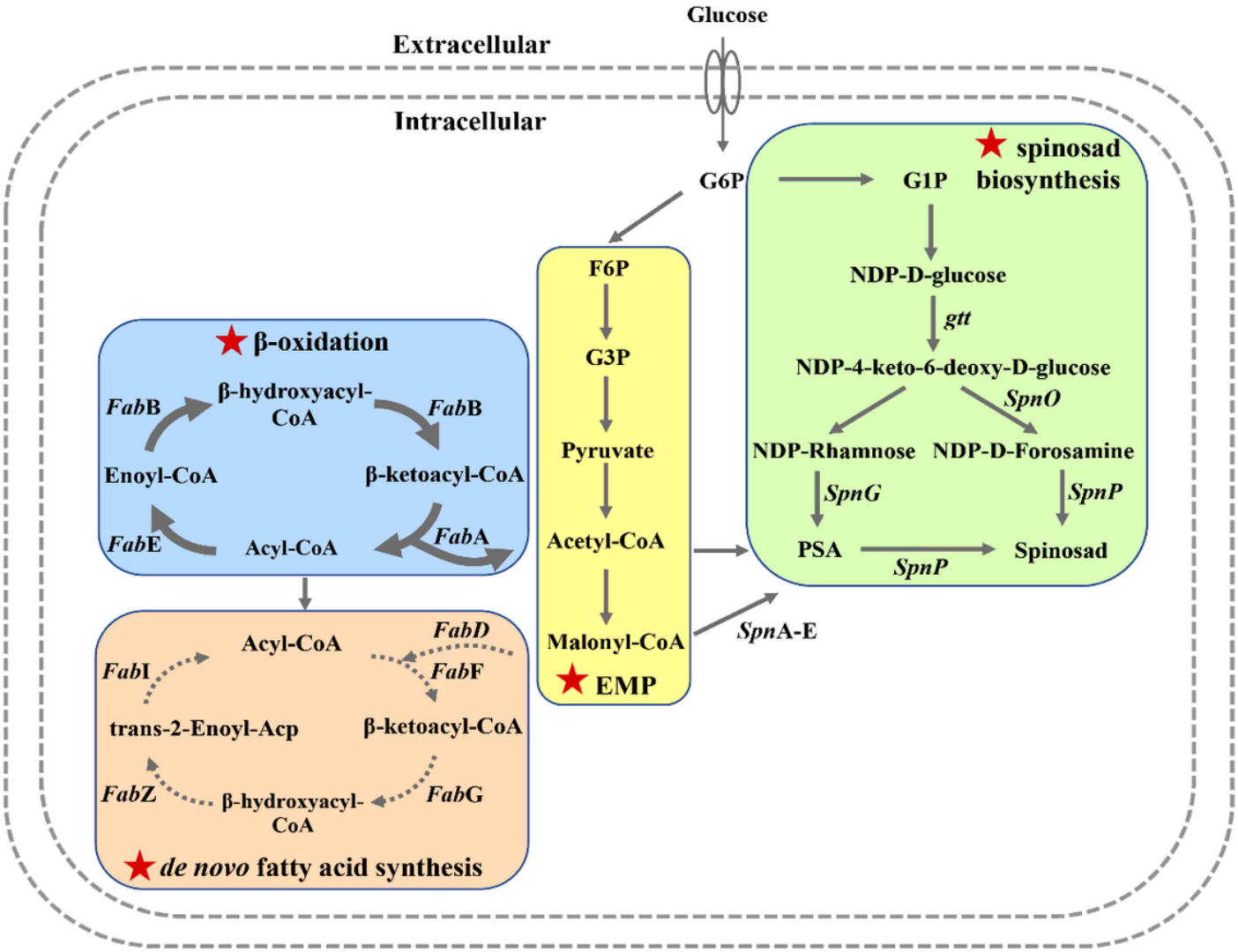

Figure 6

Regulatory mechanism of Na2HPO4-CaCO3 fermentation system to promote spinosad biosynthesis. A decrease or increase in the thickness of the path line denotes downregulation or upregulation of the corresponding pathway.