

Study on Mechanism of γ -PGA and Nattokinase Produced by *Bacillus Subtilis* Natto Based on RNA-Seq Analysis

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Research

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Abstract

Poly- γ -glutamic acid (γ -PGA) and nattokinase (NK) are the main substances produced by *Bacillus subtilis natto* in solid-state fermentation and have wide application prospects. We found that our strains have higher nattokinase activity when soybean is used as a substrate to improve the yield of γ -PGA. Commercial production of γ -PGA and nattokinase requires an understanding of the co-production mechanism. Here, we monitored the metabolites of the fermentation process firstly, analyzed the transcriptome of *Bacillus subtilis natto* when co-producing γ -PGA and NK, and obtained the maximum γ -PGA yield ($11.39 \pm 0.38\%$, w/w) and highest activity of NK during fermentation. By comparing the up-regulated genes and down-regulated genes of key enzymes and product-related metabolic pathways for genetic engineering, the co-production mechanism of γ -PGA and nattokinase can be summarized. This study firstly provides new insights into the co-production mechanism of γ -PGA and nattokinase by *Bacillus subtilis natto*, and reveals potential molecular targets for promoting the co-production of γ -PGA and nattokinase.

Introduction

Poly- γ -glutamic acid (γ -PGA) is a natural multifunctional biopolymer composed of D- and/or L-glutamic acid units that are connected by γ -amide bonds (Ashiuchi et al., 2002). It is within the molecular weight rang of 10 to over 1000 kDa, and mainly synthesized by microbial fermentation (Ashiuchi M et al., 2001; Zhao C et al., 2013). With the predominant characteristics such as non-toxic, biocompatibility and biodegradable (Ashiuchi et al., 2002), Poly- γ -glutamic acid has the functions of thickening, gelation, emulsification, film formation, moisturizing and adhesion, etc (Otani Y et al., 1996). It is gradually being used in the fields of cosmetics, food processing, agriculture, medicine and environmental protection (Zhang D et al., 2012; Lim S M et al., 2012; Yu Y et al., 2016; Yu J et al., 2020).

Nattokinase (NK) is a biological enzyme extracted from fermented soybeans (Sumi H et al., 1987). Its structure is a linear amino acid chain without space folding, produced in the form of a signal peptide, a propeptide and a mature peptide, with the molecular weight of 27.7 KDa and 275 amino acid residues (Yanagisawa Y et al., 2010). The catalytic triad of the NK enzyme is Ser-His-Asp (Dabbagh F et al., 2014). The characteristic substrate is fibrin, its thrombolytic effect is much stronger than the snake venom fibrinolytic enzyme, urokinase (UK) and lumbrokinase. The fibrinolytic activity of NK is 4 times higher than that of plasmin, and the molecular weight is much smaller than urokinase, lumbrokinase, and can be absorbed by the intestine (Kapoor R et al., 2013; Qing C et al., 2017; Man L L et al., 2019). Thus, nattokinase as a subtilisins, which is currently considered as a potential drug for preventing and treating cardiovascular diseases (Lee B H et al., 2015; Wu H et al., 2019).

B. subtilis natto has the ability to produce both γ -PGA and nattokinase efficiently (Ogawa Y et al., 1997; Wang C et al., 2009). In *B. subtilis natto*, γ -PGA is encoded by the synthesis genes *pgsB*, *pgsC*, *pgsA*, and *pgsE* (Ashiuchi M et al., 1999; Ashiuchi M et al., 2003), whereas nattokinase is synthesized by the gene *aprN*, first called subtilisin NAT has 1143 base pairs was first discovered in 1992 (Nakamura T et al.,

1992). While both γ -PGA and NK have competitive metabolic pathways, it would be of great economic benefit if γ -PGA and NK could be co-produced simultaneously by *B. subtilis natto*. γ -PGA is an important component of natto, which can promote the utilization of nutrients. It can retain the moisture of the solid medium, and increase the NK production under solid state fermentation furtherly (Nie G et al., 2015). Accordingly, it is considered that co-production of NK and γ -PGA under the condition of minimizing other by-products should be an interesting work. However, lots of studies have investigated the fermentation of γ -PGA and nattokinase, less attention has been given to the simultaneous fermentation of γ -PGA and NK by *B. subtilis natto*. Liquid-state fermentation has been successfully used in co-production strategies to generate high value-added bioproducts (Qin G et al., 2008). Solid-state fermentation is widely used in the production of γ -PGA and nattokinase. For example, γ -PGA and fibrinolytic enzyme were produced simultaneously from *Bacillus subtilis GXA-28* by solid-state fermentation (Zeng W et al., 2013). Another successful example of a co-production strategy is the use of *B. subtilis natto* to simultaneously produce γ -PGA and nattokinase by solid-state fermentation (Nie G et al., 2015).

In this study, we will explore the molecular mechanism of co-production of γ -PGA and nattokinase by transcriptome analysis to assess the fermentation process and the differences in gene expression in: 6 hours (production time of NK), 9 hours (production time of γ -PGA) and 24 hours (the highest activity time of NK). The effects of the concentration of metabolites on γ -PGA and nattokinase co-production by *B. subtilis natto* will be systematically investigated. Then, the up-regulated and down-regulated genes will be analyzed to identify the key ones. Combined with the main metabolic pathways, such as carbohydrate metabolism, the potential target genes that can improve the activity of nattokinase and the yield of γ -PGA are observed. To the best of our knowledge, it is the first report that revealed the mechanism of co-production of γ -PGA and nattokinase. The results of this study will promote us to better understand the mechanism by which soybean fermentation produces γ -PGA and nattokinase, and lays the foundation for the transformation of high-yielding strains for the co-production of γ -PGA and nattokinase.

Materials And Methods

2.1 Microorganisms, media, and cultivation conditions.

B. subtilis natto was used throughout the experimental study and was cultivated in the medium consisting of the following: 20g soybean, 1.5g saccharose, 0.4g glutamate, 0.025g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.025g $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 0.05g CaCl_2 . The seed culture (5%, v/v) was transferred into 50 mL of the fermentation medium in 250 mL flasks. The fermentation was carried out at 37°C and static culture for 24 h. The cultures were diluted to 5×10^5 cells per ml and harvested to prepare total RNA. The soybeans were cleaned and soaked in the water overnight at 24°C and the soaking water was discarded. Twenty grams soaked soybeans were placed in 100 ml erlenmeyer flask covered with gauze and sterilized at 121°C for 20 min. The soybeans were inoculated with 1×10^5 CFU/g of *B. subtilis natto*. The fermentation process was carried out at 37°C for 36 h in oxygen limitation conditions (cap tighten). All experiments were performed independently in triplicate, and the reported results represent the averages for three replicate experiments.

2.2 Determination of γ -PGA and nattokinase

The cells in the fermentation broth were harvested by centrifugation (4 °C, 8,000 × g for 20 min) and washed three times with PBS (pH 7.0). The concentration of γ -PGA was determined by the photometric cetyltrimethylammonium bromide (CTAB) assay (Kanno A et al., 1995; Zhang Q Q et al., 2012; Da Silva S B et al., 2014). The water-insoluble complex is formed by γ -PGA and CTAB, which causes the turbidity of the solution to increase. Samples taken from shake flask were centrifuged (16,000 × g, 10 min, 4°C). The turbidity at 400 nm was measured so that the γ -PGA in the supernatant was quantified. For this purpose, 100 μ L 0.7 M CTAB in 2% NaOH were added to 100 μ L sample or standard solution in a 96-well microtiter plate (Zeng W et al., 2012). After 3 min of incubation the turbidity was measured in a Synergy MX microplate reader.

The activity of NK was determined by Tos-Arg-OMe (TAME) method. TAME can be cleaved to Tos-Arg and CH₃OH by NK. Then, CH₃OH is oxidized to CH₂O by KMnO₄, and the chromotropic acid reacts with it to form a blue-violet compound, which has a sensitive UV-visible absorption peak at 574 nm (Chen C et al., 2014). In brief, 0.1 mL of TAME solution (0.1 mol · L⁻¹), 0.1 mL of phosphate buffer (pH 8.0), and 0.1 mL of enzyme solution were mixed in color comparison tubes and kept in a water bath (37°C) for 30 min. Then, 0.2 mL of TCA (15%) was added to terminate the reaction, and 0.1 mL of KMnO₄ (2%) was added into the tubes and shaken for 2 min. NaSO₄ (0.1 mL, 10%) was added to reduce excess KMnO₄. Chromotropic acid (0.4%, 4 mL) was added to terminate the reaction by keeping the mixture in boiling water bath for 25 min, followed by ice-water bath for 10 min. The sample was obtained and its UV-vis absorbance spectrum was recorded at 574 nm using a Perkin-Elmer Lambda UV-vis spectrophotometer.

2.3 RNA isolation and RNA-seq

Total RNA was extracted from 3 samples cultured to 6th h, 9th h and 24th h using an RNA prepure Cell/Bacteria Kit (Tiangen Biotech Co, Ltd, Beijing, China), and quality inspection was performed using the 2100 Bioanalyzer (Lu Y et al., 2018). The qualified RNA samples were digested with 10U DNaseI (Takara, Japan) at 37°C for 30 minutes. Ribo-Zero™ Magnetic Kit (Gram-Negative Bacteria or Gram-positive Bacteria) (Epicentre, USA) is used to remove rRNA after DNase digestion of RNA. After Ribo-Zero Reaction Buffer and Ribo-Zero rRNA Removal Solution (Gram-Negative Bacteria or Gram-positive Bacteria) were added, the volume was made up to 40 μ L and reacted at 68°C for 10min. The sample was then placed at room temperature for 5 minutes. The processed RNA was added to the pre-washed magnetic beads, mixed thoroughly immediately, placed at room temperature for 5 minutes, and then reacted at 50 °C for 5 minutes, immediately placed on a magnetic stand for more than 1 minute, the supernatant was sucked, and water was added to 180 μ L 3 M Sodium Acetate, Glycogen (10 mg/ml) and 600 μ L of absolute ethanol were added and placed at - 20°C for more than 1 hour, centrifuged to obtain a precipitate, and dissolved in water to form rRNA-depleted RNA.

2.4 RNA-Seq data analysis

The RNA library construction of samples at three time points (6th h, 9th h and 24th h) was completed by the Shanghai Human Genome Research Center. 2 x 150 bp paired-end sequencing was performed using the Illumina X10 (Illumina, San Diego, CA, USA). Sequence reads from all samples were cleaned using the FASTX toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). After adaptor trimming and quality trimming, the clean reads were mapped to the *B. subtilis natto* genome using Bowtie2 (`-very-fast-local`) (Langmead and Salzberg, 2012). The reads number of each gene was firstly transformed into RPKM (Reads Per Kilo bases per Million reads) (Mortazavi et al., 2008), then the MARS model (MA-plot-based method with the random sampling model) in the DEGseq package (Wang et al., 2010) was used to calculate the gene expression difference between each two samples. We simply defined genes with at least 2-fold change between two samples and FDR (false discovery rate) less than 0.001 as differential expressed genes.

Up-regulated or down-regulated genes of *B. subtilis natto* in culture was filtered using $FDR \leq 0.05$, fold change ≥ 2 , or $FDR \leq 0.05$, fold change ≤ -2 . The filtered genes were associated with the Gene-ontology (GO) terms and the Kyoto Encyclopedia of Genes and Genomes (KEGG) functions. Fischer's exact test was used and restricting analysis to functional groups with more than 2 genes. Enrichment score greater than or equal to 1 for GO term and pvalue less than 0.05 for KEGG functions were set as the stringency. For KEGG, the color of the element appearance was set by $FDR \leq 0.05$, fold change ≥ 2 , or $FDR \leq 0.05$, fold change ≥ -2 .

Results And Discussion

3.1 Co-producing of γ -PGA and Nattokinase

In this study, the biomass of *B. subtilis natto*, the yield of γ -PGA and the activity of nattokinase were determined. The growth curve, the γ -PGA yield and nattokinase activity curve was obtained under this condition. It can be found that the logarithmic growth phase mainly occurs between 6 h and 24 h through analysis of the growth curve analysis in the soybean culture medium. When γ -PGA was produced at 9th h, CFU decreased slightly, and then slowly resumed growth at about 24th h, but this growth process was slow. When it grows to 36th h, the biomass can basically be reached the same level as the 16th h. Therefore, the period from 24 h to 36 h is considered the growth recovery period, and the change in biomass from 36 h to 40 h is negligible. The biomass CFU of the bacteria at 6th h, 9th h, and 24th h are 1.2×10^5 , 1.6×10^6 , 1.0×10^8 , respectively.

In the previous work, *B. subtilis natto* could produce a high-yield of γ -PGA and nattokinase in the solid-state fermentation, respectively (Mohanraj R et al., 2019; Lan G et al., 2020). In this study, soybean was used as basic substrate for co-producing γ -PGA and nattokinase based on this phenomenon. Carbon sources and sodium glutamate which added to the substrates could improve γ -PGA and nattokinase production obviously (Zeng W et al., 2013; Sha Y et al., 2019). Wherein selecting sucrose as the carbon source based on the previous study that glucose, sucrose and fructose have similar effects on γ -PGA production (Shi F et al., 2006). Thus, *B. subtilis natto* was cultured in sucrose and sodium glutamate medium, and cultured at 37 °C to monitor the growth during fermentation (Fig.1). After 40 hours of

continuous monitoring of fermentation, we found that nattokinase was produced at 6th h and γ -PGA was produced at 9 th h.

As shown in Fig.2, the maximum γ -PGA yield ($11.39 \pm 0.38\%$, w/w) was obtained when 50 g of sucrose per kilogram of substrates were added. The number of viable cells increased rapidly after 4 h, reached the highest 5.13×10^9 CFU/g at 24 th h, and remained steady thereafter. This implied that the present co-production conditions had no adverse effect on the growth of bacteria. Interestingly, the time courses of γ -PGA yield and nattokinase activity showed synchronism, they increased rapidly between 6 and 36 h, then reached the maximum yield ($11.39 \pm 0.38\%$, w/w) and maximum activity (1388 U/g). Then, the nattokinase activity remained relatively constant after 24 h, but the concentration of γ -PGA decreased markedly after 36 h. This might be caused by γ -PGA depolymerase, which was excreted abundantly by *B. subtilis natto* during the late stationary phase (Kimura K et al., 2010). These suggested the co-production of γ -PGA and nattokinase by using the current medium components. Overall consideration of the γ -PGA yield, NK and the cost of extraneous nutrients, 5% (w/w) sucrose, 4% (w/w) glutamate, and 37°C were selected for further study.

By analyzing the change curve of γ -PGA concentration and nattokinase activity in different time periods, it is found that the synthesis and accumulation of γ -PGA mainly occur in the logarithmic growth phase, which is consistent with the study of Mahaboob Ali AA (Mahaboob Ali AA et al., 2020). At 36 h, the number of viable bacteria reached the highest value in solid-state fermentation; within 12 h to 20 h was the stable period; after 20 h to 40 h was the decay period. From the relative activity curve of NK, it can be found that as the bacteria grow, nattokinase is gradually synthesized, which is consistent with Gao Z's research (Gao Z et al., 2018). As the concentration of γ -PGA increases, the viscosity of the fermentation broth will gradually increase, making RNA extraction very difficult (Shih L et al., 2001). Therefore, the 6th, 9th and 24h samples were used to analyze the transcription of *B. subtilis natto* in this study.

3.2 Analysis of transcriptome of *B. subtilis natto*

During our research, it was found that adjusting the content of sucrose and sodium glutamate in the medium does not affect the production time of γ -PGA and NK, which may be related to the expression of related genes during the fermentation. Therefore, to analyze the interaction in the process of co-production of γ -PGA and nattokinase systematically, the global transcriptional regulation of *B. subtilis natto* in different fermentation periods was performed by RNA-seq. More than 30 million high-quality base pairs reads were generated in each samples. In addition, saturation analysis showed that sequencing depth was saturated (Fig.3).

Three stages of data from *B. subtilis natto* was analyzed respectively. Post-alignment was performed subsequently and the aligned reads are mapped to their corresponding genomic reference, which is used to construct genes that are significantly different between samples (Table. 1). In total, more than 3800 protein-coding genes were expressed in the fermentation process, accounting for 99.19% of the total 4023 genes. Significantly expressed genes are mainly obtained through DEGseq software analysis (Fig.

7). In order to further study gene expression profiles, we analyzed the genes that were significantly expressed at three time points. In general, compared with the 6th h, 316 and 564 genes were significantly up-regulated and down-regulated at the 9th h, respectively; while at the 24th h, 1547 and 1138 genes were significantly up-regulated and down-regulated, respectively. On the contrary, compared with 9th h, only 1534 and 1060 genes were significantly up-regulated and down-regulated at 24th h. The above results indicate that the transcription level changes slightly during the early NK production, while the expression difference after the production of γ -PGA is significant (Table 1).

Table 1. Significant Difference Gene Statistics among Samples

	up	down
6th hvs9th h	316	564
6th hvs24th h	1547	1138
9th hvs24th h	1534	1060

Note: Statistical list of significantly down-regulated gene numbers between samples.

Specific pathways are grouped based on the KEGG database among the differentially expressed genes. The percentage of genes involved in carbohydrate metabolism is up-regulated, including the PTS system, pyruvate metabolism and other processes. Many metabolic pathways are significantly affected by co-production environment, such as amino acid metabolism and carbohydrate metabolism. Many of these functionally related genes are up-regulated, indicating that these pathways are activated under co-production conditions to provide sufficient substrate and energy to maintain cell survival.

Table 2. Important expression-changed genes under different times

Gene name	Gene annotation	6th h RPKM	9th h RPKM	24th h RPKM
<i>AprE</i>	subtilisin AprE	53.89	1677.84	26090.62
<i>glnA, GLUL</i>	glutamine synthetase	1313.58	5606.32	308.68
<i>gudB, rocG</i>	glutamate dehydrogenase	1.95	25.45	49.05
<i>pgsB</i>	poly-gamma-glutamate synthase PgsB	25.62	1462.01	47.47
<i>pdhC</i>	pyruvate dehydrogenase E2 component	3.43	23.92	2681.45
<i>pdhD</i>	dihydrolipoamide dehydrogenase	8.11	25.41	2760.24
<i>gltD</i>	glutamate synthase small subunit	100.37	1465.77	99.54
<i>gutB</i>	glutamate synthase large subunit	122.12	1179.40	104.15
<i>aspB</i>	Aspartate aminotransferase	732.16	1620.20	216.93

Compared with 6th h, amino acid synthesis-related genes, especially the related to glutamate, aspartic acid, histidine and serine metabolism were significantly differentially expressed at 9th h and 24th h (Table 1). Glutamate-oxoglutarate amidotransferase (GOGAT) and glutamate dehydrogenase (GDH) are essential for the synthesis of glutamate (Cao M et al., 2018). In this study, the gene *glnA* encoding GOGAT was up-regulated. However, the two genes encoding GDH, *gudB* and *rocG*, showed opposite transcriptional changes. Changes in the transcription levels of the genes indicated that Glutamine synthesis is not inhibited by nattokinase synthesis. Under the co-production environment, γ -PGA production is related to the up-regulation of genes related to the synthesis of glutamate and the down-regulation of γ -PGA degrading enzymes directly. The genes encoding these proteins are also present in sodium. In *B. subtilis natto*, we found that the three *ycg MNO* genes are up-regulated in the co-production environment, which is consistent with the results in *Bacillus subtilis* (Sha Y et al., 2019). That may be the reason for the co-production of γ -PGA and NK simultaneously in soybean medium. Based on the analysis of the above results, the co-production of γ -PGA and the activity of nattokinase were improved in *B. subtilis natto* through several metabolic patterns.

To analyze the regulation of *B. subtilis natto*, we focused on three pair-wise comparisons: as can be seen in Fig 5, 249 genes were up-regulated between 6h and 9h, and 119 genes were down-regulated between 6h and 9h. The significant difference gene for amino acid metabolism were down-regulated from 6h to 9h, probably because NK synthesis rate is affected by γ -PGA synthesis (Wang L et al., 2020), and the significant difference gene for amino acid metabolism were significantly up-regulated from 9th h to 24th h. This is consistent with the highest nattokinase activity at 24 hours during fermentation. 118 genes were up-regulated between 9h and 24h, and 289 genes were down-regulated between 9th h and 24th h.

This may be related to the inhibition of the expression of γ -PGA synthesis gene when the synthesis rate of nattokinase is high.

In order to study the functional information of these DEGs, the genes are classified according to COG function (Fig.6). The figure shows that most of the functional classes contain both up-regulated and down-regulated genes, indicating that *B. subtilis natto* needs to balance metabolic pathways in soybean culture to maintain cell survival. In general, under co-production conditions, far more genes are induced to express than suppressed genes. Many metabolic pathways will be significantly affected by the co-production conditions, such as amino acid metabolism, energy metabolism, carbohydrate metabolism. Many of these functionally related genes are up-regulated, indicating that these pathways are activated under co-production conditions to provide sufficient substrate and energy to maintain cell survival.

When *B. subtilis natto* was cultivated in soybean medium, the expression level of genes related to energy and carbon metabolism is greatly affected by the environment of co-production (Fig. 4). Among of the 124 energy and carbon metabolism-related genes, 100 genes were significantly down-regulated and 24 genes were significantly up-regulated when 9th h vs. 6th h. In addition, genes related to energy conversion, such as BSNT_RS01180 (NAD(P)H-quinone oxidoreductase) and BSNT_RS20605 (cytochrome bd ubiquinol oxidase), have been induced to express. In addition to the above genes, genes encoding γ -PGA and NK synthesis related genes should be up-regulated and expressed separately to improve the ability of cells to produce these two substances. However, it is unexpected that the *pgsB* and *aprN* genes in *B. subtilis natto* are not up-regulated in response to the co-production environment, and the result is consistent with *Corynebacterium glutamicum* and *Bacillus licheniformis* (Xu G et al., 2019; Cai D et al., 2017). Although this result is inconsistent with our expectations, this response to co-production seems to be similar in all three *Bacillus* strains.

The production of γ -PGA requires the participation of glutamate dehydrogenase (GDH). The experiment shows that the gene expression level of glutamate dehydrogenase is significantly increased from 6h to 9h, which is consistent with the previous reports (Unrean P et al., 2013). The expression level of *pgsB* is continuously increasing, consistent with the synthesis of γ -PGA (Cao M et al., 2011). However, the expression level of glutamate transferase gradually decreased at three time points, which means that in addition to the synthesis of γ -PGA from endogenous glutamate, the synthesis of endogenous glutamate was also added. This phenomenon is related to glutamate-dependent strains that require a large amount of exogenous glutamate to be added, while the synthesis of endogenous glutamate only accounts for 10% (Zhang D et al., 2012). The expression of *aprN* gradually increased at three time points, proving that NK is not restricted by co-production conditions during fermentation. At the same time, a large number of amino acids are used in the process of cell synthesis of nattokinase, and pentose phosphate pathway is one of the key (Chen P T et al., 2006).

The first enzyme of pentose phosphate pathway is an advantageous condition for the production of nattokinase. In the co-production process, the synthesis of γ -PGA and nattokinase is affected by the synthesis pathways of substances other than the two as by-products. Polysaccharides are one of the by-

products, not only the production of γ -PGA and nattokinase is affected, but also the viscosity of the fermentation broth is affected, which affects the metabolism of bacteria. It can be seen from the Fig. 6 that with the production of γ -PGA, the gene expression of polysaccharide biosynthetic protein begins to decrease after 9 hours. It can be proved that the production of polysaccharides is not a key factor affecting the viscosity of the fermentation broth in solid-state co-production fermentation. However, if the multi-polymerization gene is down-regulated, whether other by-products will be affected. This triggered whether the down-regulated expression of polysaccharide genes caused the up-regulated expression of the synthesis of γ -PGA and nattokinase (Yu W et al., 2017; Feng J et al., 2015).

3.3 Identification of key modules for co-production of γ -PGA and nattokinase

The differentially expressed genes involved in central metabolism are shown in Fig. 10. When *B. subtilis natto* is cultivated in soybean medium, most genes related to Carbohydrate cycle, Amino acid synthesis and the γ -PGA synthesis were found to be upregulated, whereas genes related to glutamate degradation were downregulated.

The gene expression levels of 9h and 6h were compared. The results showed that the expression levels of glucose dehydrogenase, dehydrogenase acetate, γ -PGA synthase and isocitrate synthase in glycolysis pathway were all down-regulated. The expression levels of genes such as acid dehydrogenase are all down-regulated. Acetyl-CoA is completely oxidized through the TCA cycle, and intermediates required for ATP production and other synthetic pathways, such as amino acid biosynthesis, are synthesized (Kongklom N et al., 2015). Glutamic acid is the precursor for the synthesis of γ -PGA and is mainly generated from α -ketoglutarate from the TCA cycle (Hachiya T et al., 2019). It means that γ -PGA produced at 9h is almost supplied by exogenous glutamic acid. The gene for glucose 6-phosphate dehydrogenase was down-regulated, indicating that the PPP metabolic pathway during the synthesis of nattokinase was inhibited slightly due to the synthesis of γ -PGA. When the gene expression levels of 24th h and 9th h was compared, glyceraldehyde triphosphate dehydrogenase and pyruvate dehydrogenase were down-regulated. During this period, the gene expression levels of glutamate dehydrogenase and γ -PGA synthase were up-regulated, which proved that the synthesis of γ -PGA was not affected by the synthesis of NK in Fig. 7. These results indicate that the higher yield and productivity of γ -PGA are affected because of the systematic enhancement of glutamic acid synthesis. In recent years, researchers have proposed that the supply of glutamate in cells is improved through several strategies to improve the production of γ -PGA, which is suitable for a wide range of compounds produced through glutamate-intensive pathways (Zhang D et al., 2012). In the pentose phosphate pathway, it is mainly the synthesis of nattokinase and polysaccharides. The polysaccharide synthesis gene was down-regulated during the fermentation process, which proved that the synthesis of polysaccharide was inhibited from the synthesis of nattokinase and γ -PGA. This phenomenon is beneficial to the main fermentation product. In contrast, there were no significant differences in transcript level of genes involved in the glucose transport and the ammonium transport pathway in this study which may be that have relatively little influence on the glutamate dependence in *B. subtilis natto* (Tang B et al., 2015).

Combined with the fermentation curve, it can be seen that the NK activity stabilized after 24h, while the yield of γ -PGA reached the highest at 36h. Subsequently, a part of the γ -PGA is degraded due to the γ -PGA depolymerase, which is largely excreted by *B. subtilis natto* in the later stage of fixation (Zeng W et al., 2013; Sha Y et al., 2019).

3.4 Analysis of pentose phosphate pathway (PPP), tricarboxylic acid cycle (TCA) and glycolysis metabolism

Metabolomics analysis of strains containing newly generated promoter sequences of different strengths revealed the key metabolites most related to the synthesis of γ -PGA and NK. Metabolite analysis was performed based on previous studies (Mitsunaga et al., 2016, Fathima et al., 2016, Fathima et al.) were detected 93 metabolites as part of central carbon metabolism, including pentose phosphate pathway (PPP), tricarboxylic acid cycle (TCA) and Glycolysis intermediates as well as amino acids and nucleotides (Fig. 6).

The most striking metabolites from central carbon metabolism are ribose-5-phosphate (R5P), pyruvate and dihydroxyacetone phosphate (DHAP), as well as the gene expression of acetyl-CoA (Cai D et al., 2017), γ -PGA, glutamate and the immediate precursor of 2-oxoglutaric acid hardly changes in concentration. The homeostasis of these metabolites can be explained by their outstanding functions in carbon and nitrogen metabolism (Tian G et al., 2017). Serine and R5P are two metabolites directly derived from glycolysis intermediates glycerol 3-phosphate and glucose-6-phosphate (G6P). Therefore, the decrease in the concentration of serine and R5P metabolites can be explained by the higher demand for the TCA cycle. The supply of precursors for γ -PGA production was restricted by TCA activity. The results indicate that citrate synthase, aconitase and malate dehydrogenase form a protein complex that catalyzes the sequential reactions of the TCA cycle. In addition, 2-oxoglutarate dehydrogenase complex and glutamate synthetase are affected by these protein-protein interactions (Meyer et al., 2011). NADPH acts as a cofactor for glutamate synthesis. Therefore, the higher demand for glutamate also leads to higher demand for NADPH in the co-production and fermentation of γ -PGA and NK. Here, the gene expression levels of NAD^+ and NADP^+ are also significant. Since the applied metabolite extraction method is not suitable for studying the redox state of cells, the increase in the concentration of NAD^+ may be due to the higher demand of NAD(P)H to increase the new synthesis of glutamate.

In addition, phosphoenolpyruvate (PEP) is the metabolite most associated with γ -PGA and NK synthesis. The expression level of PEP gene was up-regulated with the increase of γ -PGA production rate and growth rate. In addition to PEP, further intermediates of central carbon metabolism also increase with the yield of γ -PGA (Najar I N et al., 2015). Glutamine is a metabolite that is negatively related to the higher rate of γ -PGA production. It is a substrate for glucose re-synthesis to glutamate, whose concentration is closely related to the demand for glutamate. Proline and NADP^+ concentrations are affected by the increased demand of glutamate for higher γ -PGA production, as shown by the gene expression levels of these metabolites. Succinic acid is another metabolite, indicating that the requirement for glutamate has changed, because the carbon flux at the branch point of 2-oxoglutarate can point to glutamate or

succinate. The obtained positive load value of succinic acid indicates that the weaker relationship between the concentration of succinic acid and the production rate of γ -PGA is emphasized. The comparison of the concentrations of 2-oxoglutarate, succinic acid and glutamate (Fig. 10) shows this connection.

3.5 Analysis of γ -PGA synthase genes

Existing research results show that the mechanism of high yield of γ -PGA is complicated in co-production fermentation, involving the supply of substrate glutamate, the transport and secretion metabolism of γ -PGA, energy metabolism, and glutamate metabolism. The biosynthesis and regulation of γ -PGA, the two-component system and other complex related pathways may be regulated by a variety of regulatory factors, which is a very complex system. Therefore, the transcriptome of this strain was compared under co-production conditions by RNA-seq. The RNA-seq library was constructed mainly from samples at three time points: 6th h (production of nattokinase); 9th h (production of γ -PGA); 24th h (the highest activity of nattokinase). Such as *glt ABC* (co-coding glutamate- α -ketoglutarate amidotransferase), *glt P* (glutamate transporter) and *ycg MNO*, etc. are all up-regulated, while *gud B* (coding glutamate dehydrogenase) is down-regulated, and changes in the expression levels of these genes directly or indirectly promote the increase of glutamate production, so that γ -PGA can produce high yields in a co-production environment. The γ -PGA synthesis genes are *pgs B*, *pgs C*, *pgs AA* and *pgs E*, among which *pgs B* and *pgs C* are mainly responsible for the synthesis of γ -PGA, and *pgs AA* and *pgs E* are responsible for transporting the synthesized γ -PGA outside the cell (Feng J et al., 2015). A gene, *pgd S*, mainly encodes γ -D /L glutamyl hydrolase, which facilitates the release of γ -PGA (Candela and Fouet 2006; Shih and Van 2001; Shi et al., 2007). The transcription of tricarboxylic acid cycle (TCA) related enzymes from α -ketoglutarate to oxaloacetate is inhibited (Yeh C M et al., 2010; Scoffone V et al., 2013), and the transcription level of α -ketoglutarate to proline synthase is significantly increased, indicating that *B. subtilis natto* has a proline response co-production mechanism. In addition, the synthesis of TCA-related enzymes from α -ketoglutarate to oxaloacetate is inhibited, thereby ensuring a large amount of α -ketoglutarate to synthesize proline.

3.6 Amino acid metabolism of co-production γ -PGA and nattokinase

During fermentation, Glu serves as the starting molecule leading to the synthesis of Asp and Gln via the reactions mediated by aspartate aminotransferase and glutamine synthetase, respectively. Therefore, the use of Glu can be avoided when Asp or Gln in the cell is directly supplied from the medium. In response to an elevated pool of Gln, a moderately high level of glutamate synthase is present in *B. subtilis natto*, thereby resulting in an increased synthesis of Glu (Schreier 1993). Taken together, it indicates that Glu plays a determining role in NK Synthesis (Chen P T, 2006). The highest NK-yielding pathways catabolize glycerol into ATP and NADPH using glycolysis and the pentose phosphate pathway (PPP), and produce NK and CO₂ as by-product (Rajasekar V et al., 2015; Larroche C et al., 1999). Synthesis pathways of NK use the incomplete tricarboxylic acid (TCA) cycle and do not produce any fermentative by-products. The reverse reaction between PEP and pyruvate and two anaplerotic reactions are inactive for NK synthesis.

However, these reactions are activated when biomass is produced (Fig. 2). The production of NK requires both the oxidative and non-oxidative branch of the PPP to produce precursors for the appropriate amino acids, while the production of biomass does not utilize the oxidative branch of the PPP (Ju S et al., 2019). These results indicate that cell growth and the synthesis of enzymes and γ -polyglutamic acid follow different paths and do not interfere with each other.

Conclusion

In conclusion, in the solid-state fermentation process of γ -PGA and nattokinase, we found that *B. subtilis natto* can produce nattokinase at the same time without affecting the production process of γ -PGA. We use the fermentation method of Japanese natto to produce γ -PGA in a targeted manner, and to produce nattokinase while minimizing by-product output. As a result, the maximum NK activity was 1388 U/g substrates obtained at 24 h, and 318.1 g/kg substrates of γ -PGA obtained at 36 h under sterilized condition. In conclusion, our results provide the first insights into the co-production mechanism in *B. subtilis natto* during γ -PGA and NK producing fermentation. Genes involved in carbohydrate metabolism, amino acid metabolism and γ -PGA synthesis pathways were identified by comparing the transcriptome data of strains cultivated. In this study, RNA-seq was used to explore the potential factors, metabolites and metabolic pathways for the synthesis of recombinant γ -PGA and nattokinase, and initially replaced the metabolic mechanism of the co-production of γ -PGA and nattokinase. The successful completion of the research can guide the molecular improvement of the *B. subtilis natto* genome, which is the basis for the propagation of *B. subtilis natto* with high-efficiency and large-scale production of target metabolites.

Declarations

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Figures

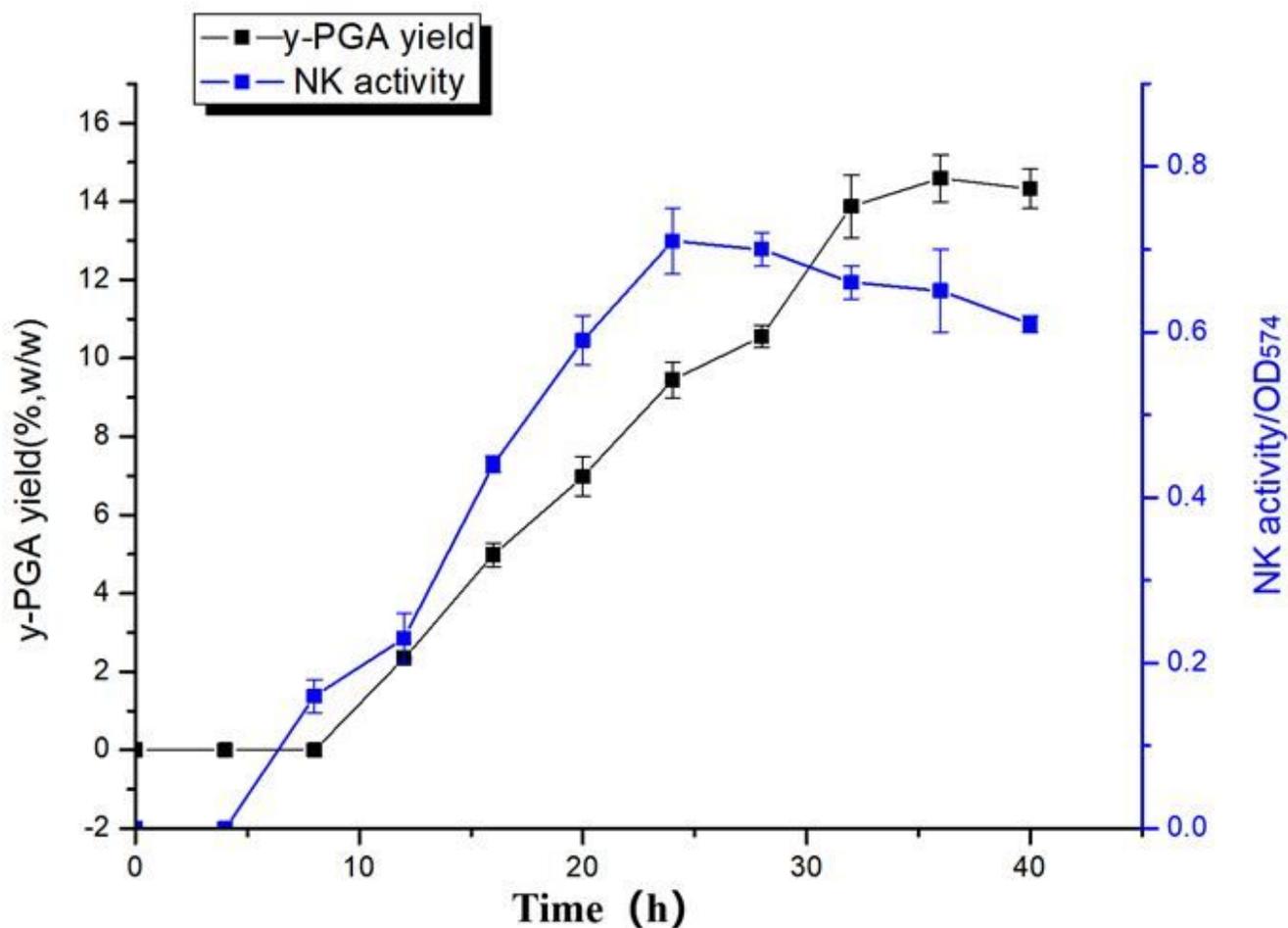


Figure 1

Time courses of γ -PGA yield, nattokinase activity from soybean under solid-state fermentation in 250 ml flask

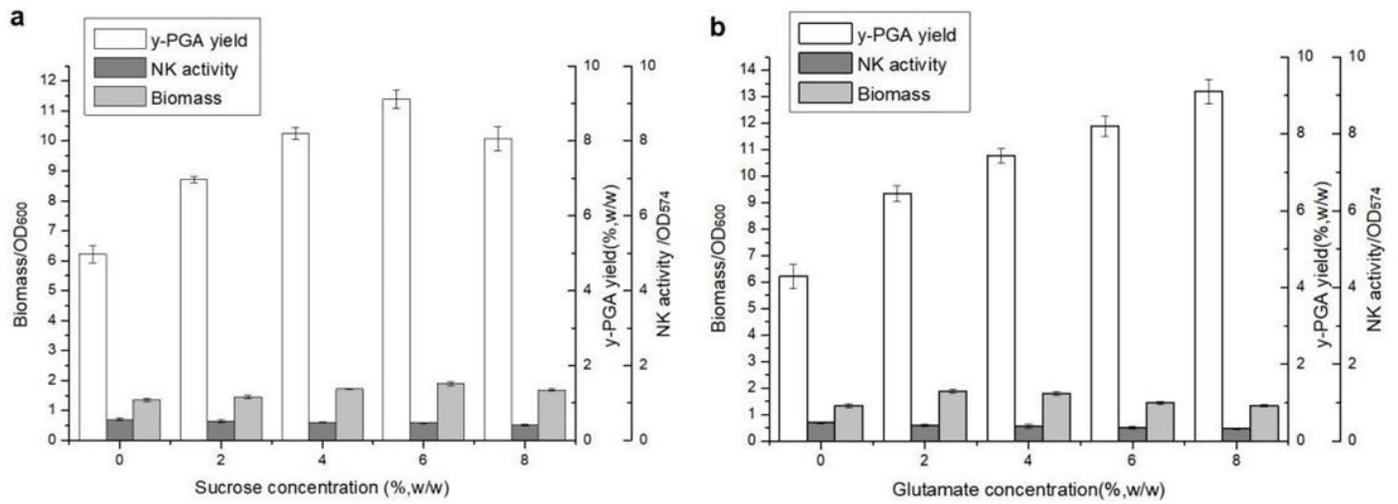


Figure 2

Effect of different sucrose concentration (a) and glutamate concentration (b) on γ -PGA yield, NK and biomass of *B. subtilis natto*.

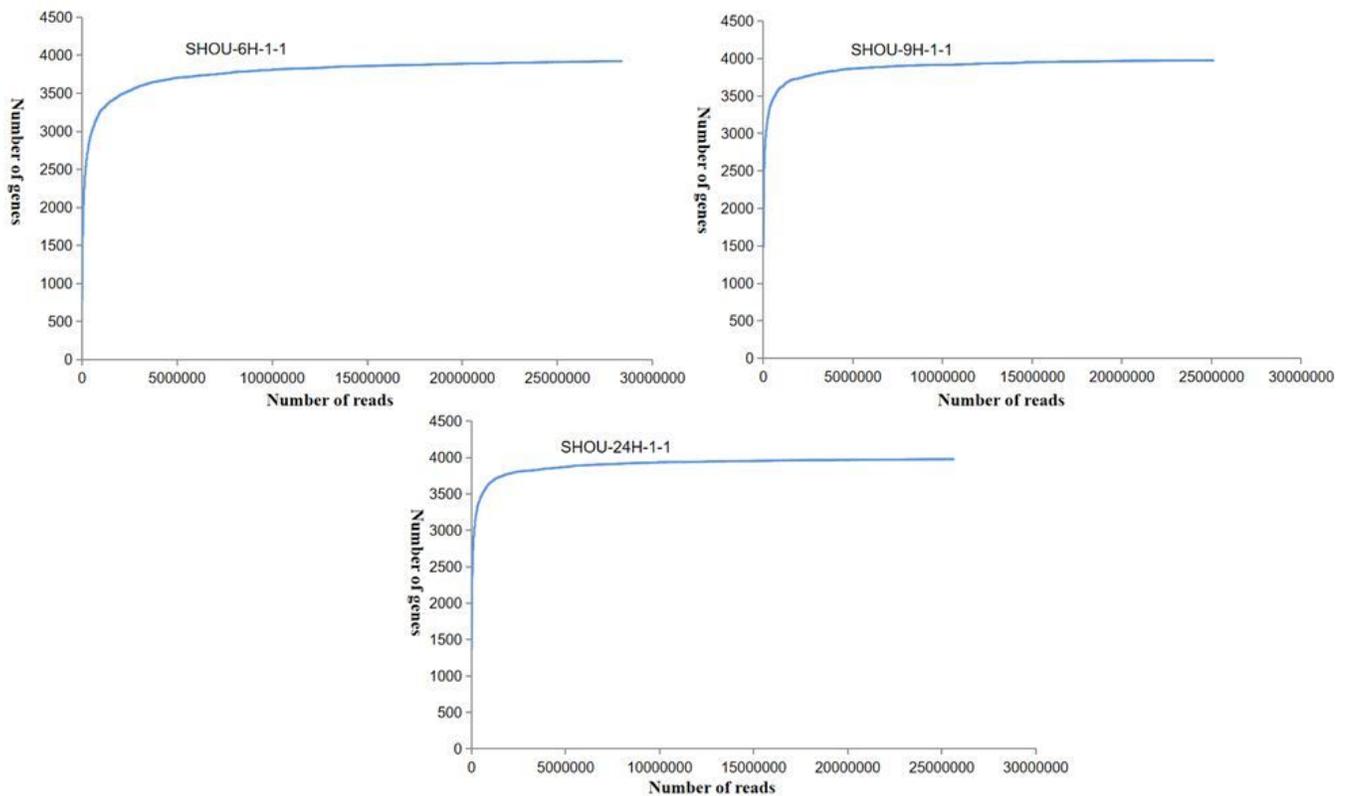


Figure 3

Saturation analysis of sequencing for 6th h, 9th h, 24th h: *B. subtilis natto*: by RNA-seq.

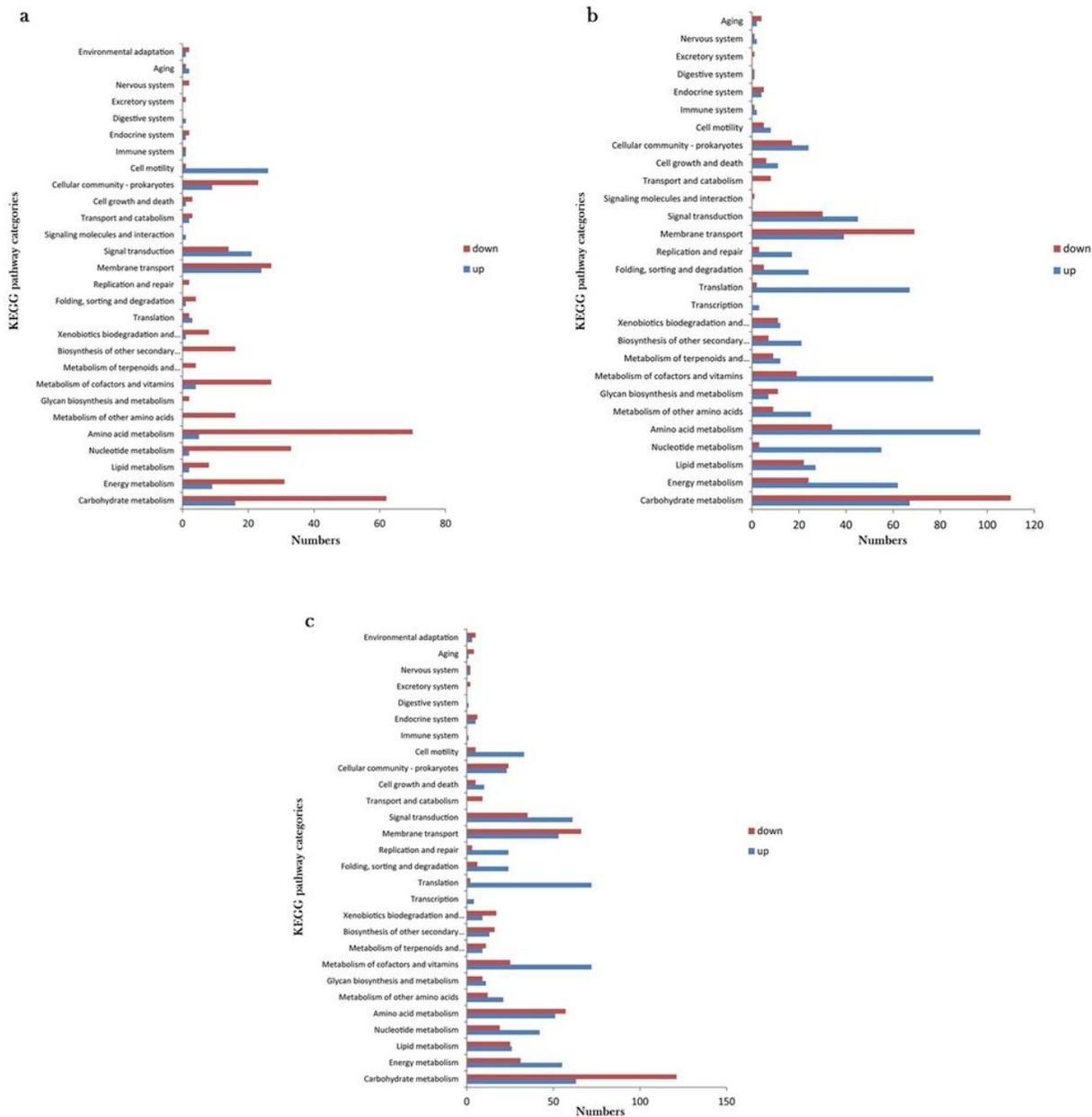


Figure 4

The distribution of up-regulated and down-regulated genes was compared by three pairs of KEGG pathway categories. a The strains of *B. subtilis* natto grown on soybean medium were compared with 6th h and 9th h. b. The strain of *B. subtilis* natto grown on soybean medium was compared with 9th h and 24th h. c. The strain of *B. subtilis* natto grown on soybean medium were compared with 6th h and 24th h.

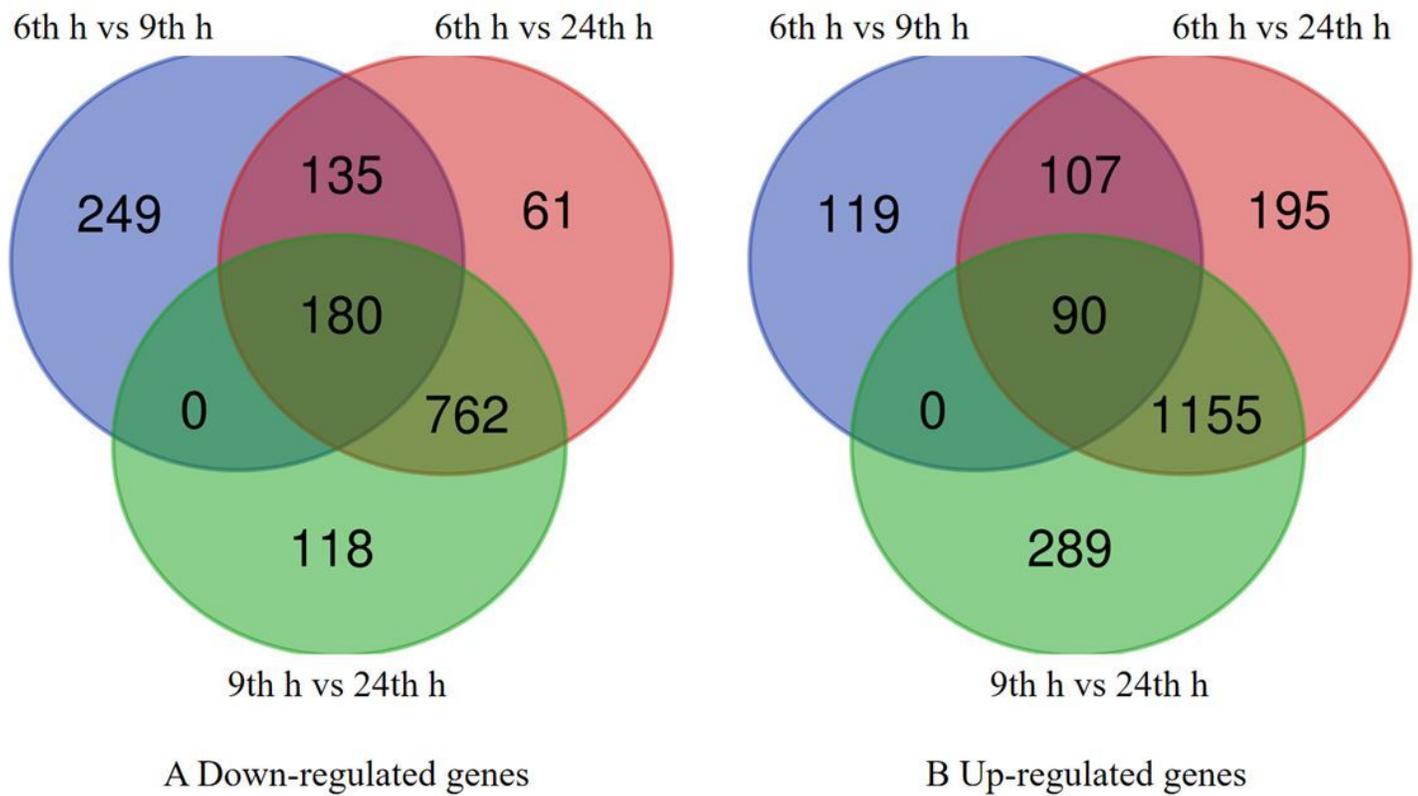


Figure 5

Venn diagrams of the number of genes differentially altered in the three pair-wise comparisons.

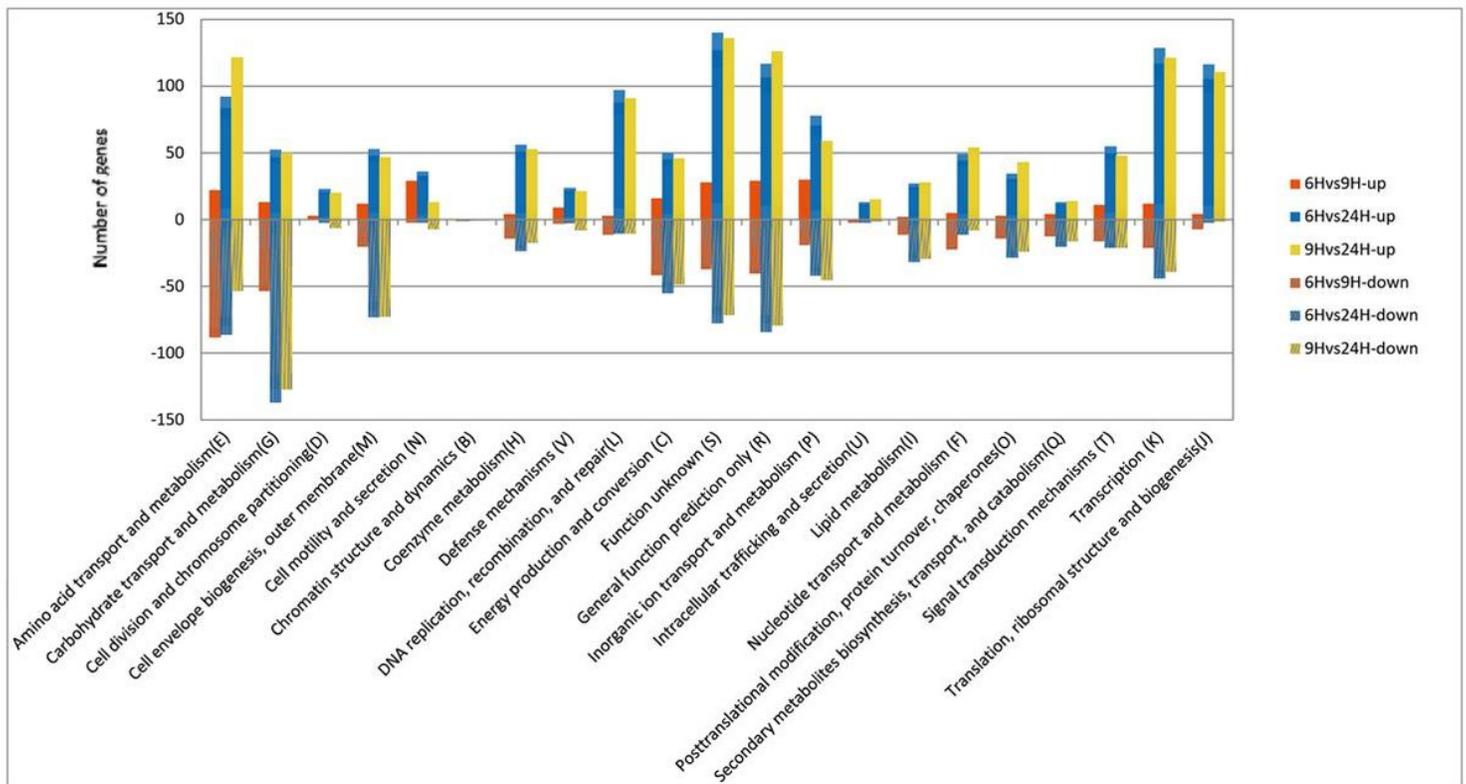


Figure 6

Cluster of Orthologous Groups (COG) functional categories of genes induced and repressed under different conditions. Each bar represents the number of DEGs in each category in the *B. subtilis natto* genome. Orange bars indicate genes repressed at 9th h relative to 6th h; The diagonal orange bars indicates 9th h significantly up-regulated genes relative 6th h . Blue bars indicate genes repressed at 24th h relative to 6th h, and The diagonal Blue bars indicates 9th h significantly up-regulated genes relative 6th h. Yellow bars indicate genes repressed at 24th h relative to 9th h, and the diagonal yellow bars indicates 24 th h significantly up-regulated genes relative 9th h.

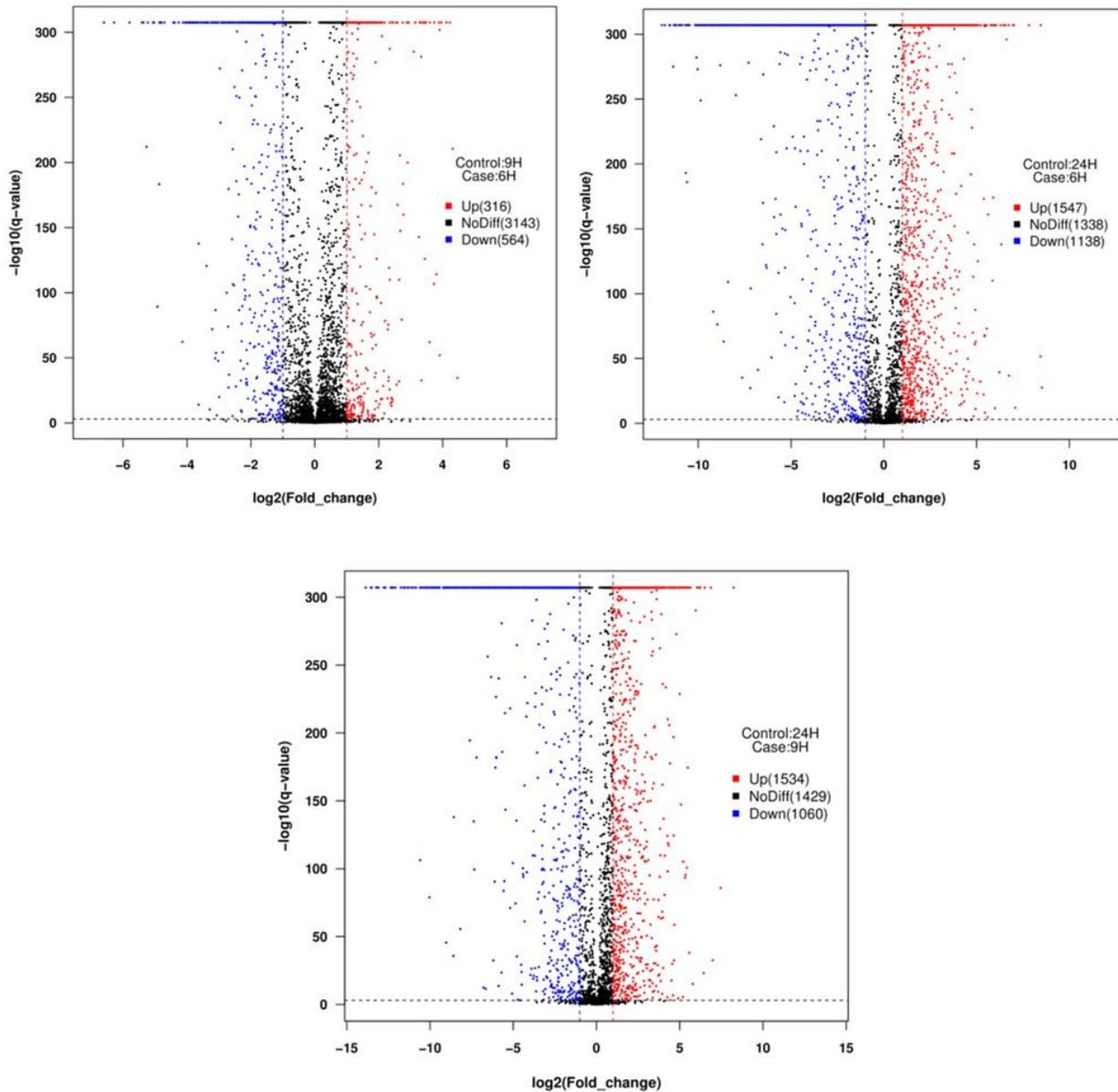


Figure 7

MA plots for differential gene expression. Significantly up and down differentially expressed genes are highlighted in red and blue dots, respectively.

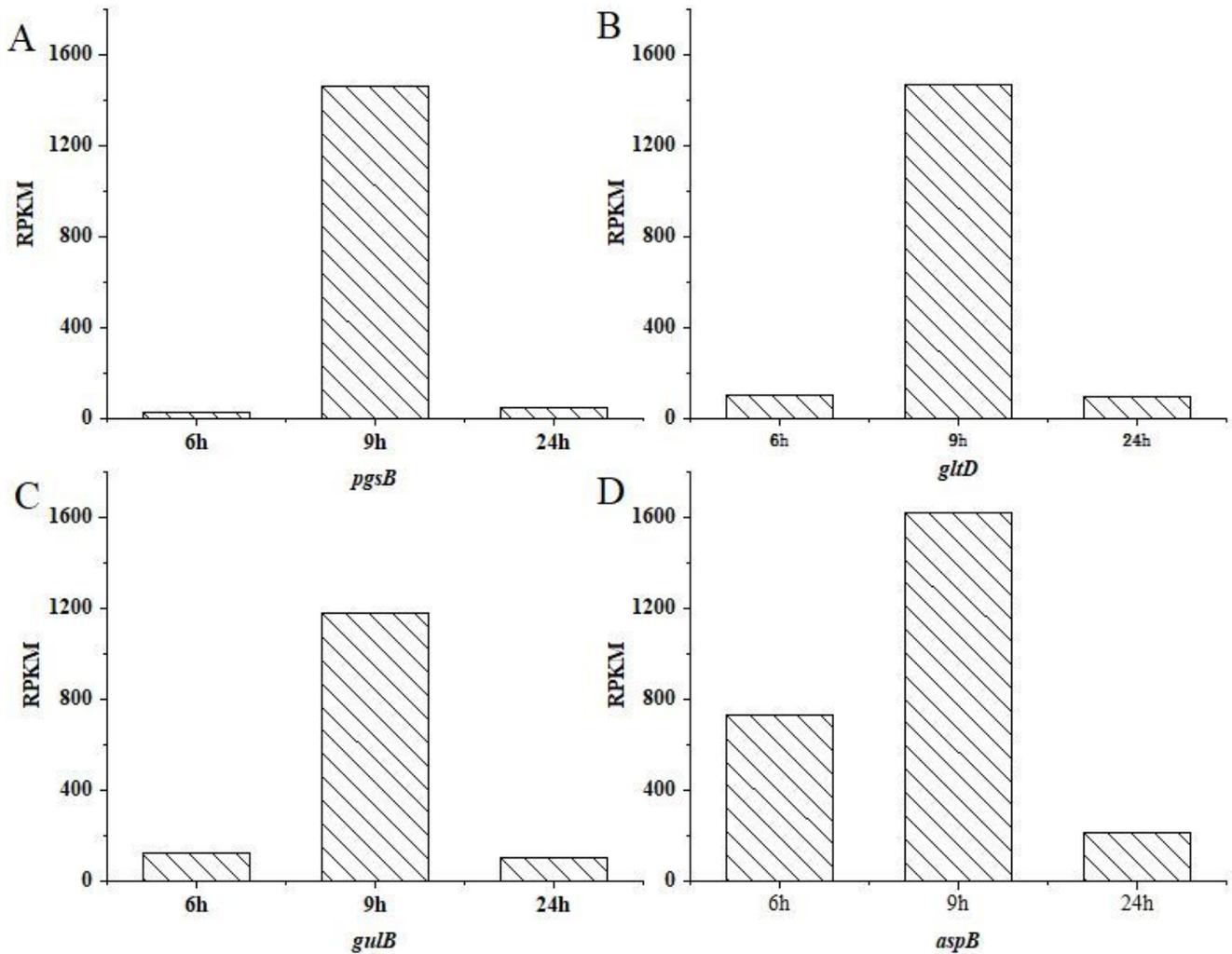


Figure 8

The expression level (RPKM) of *pgsB* (poly-gamma-glutamate synthase PgsB), *gltD* (glutamate synthase small subunit), *gulB* (glutamate synthase large subunit) and *aspB* (Aspartate aminotransferase) at 6th h, 9th h and 24th h were used to measure expression in *B. subtilis natto*.

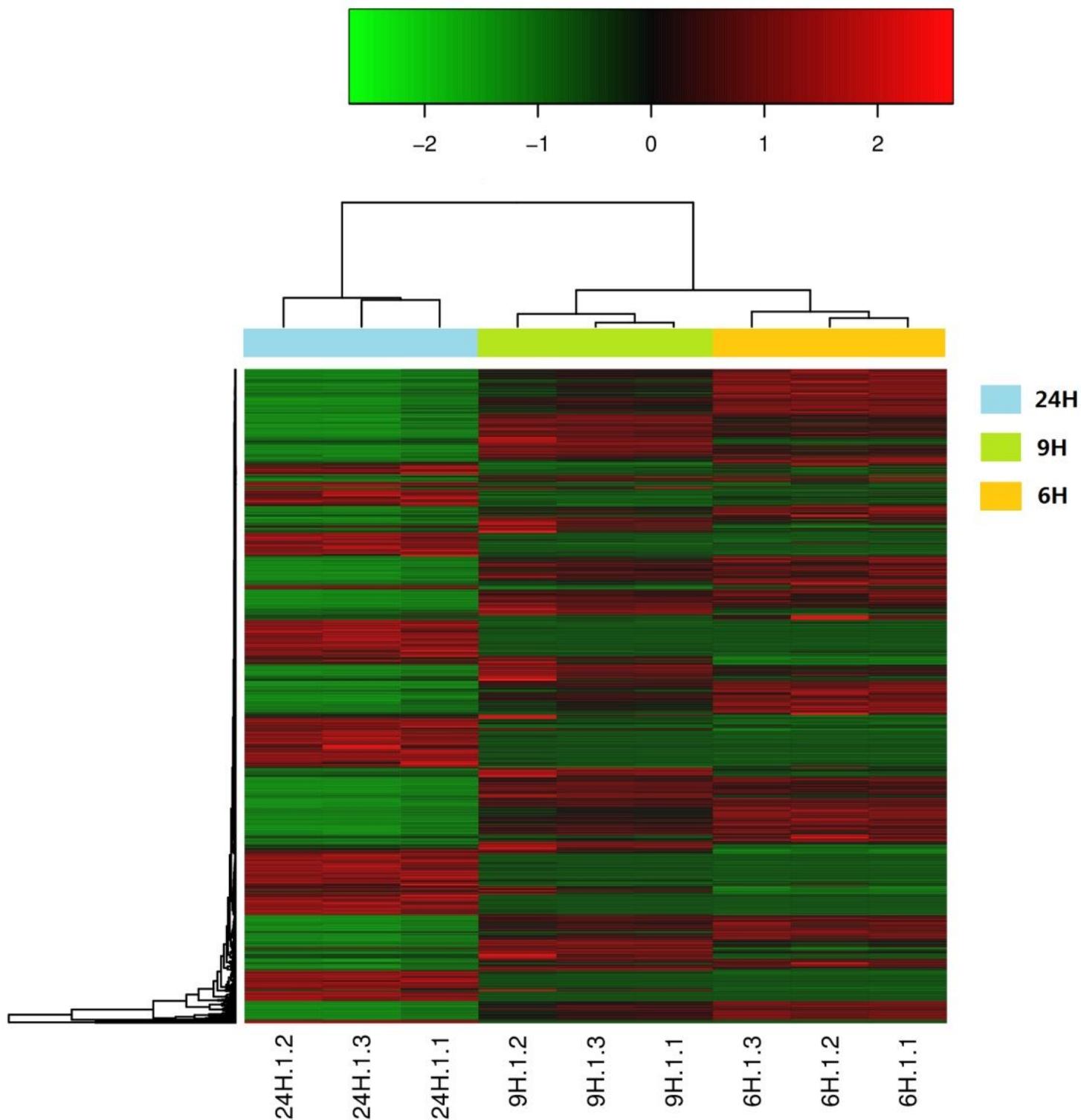


Figure 9

B. subtilis natto genes differential expression in co-fermentation of the whole soybean, in duplicated experiments, which show consistency in regulation of gene expression. Each horizontal line represent the expression level of a gene. Red and green indicate up- and down-regulated genes, respectively. In the culture, the most transcripts of *B. subtilis* natto were down-regulated. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

