Integrating Metabolomics and Statistical Analysis for Enhanced Serine Alkaline Protease Production in *Bacillus subtilis*

Hossein Sedighikamal; Shohreh Mashayekhan*; Reza Roostaazad

Department of Chemical and Petroleum Engineering, Sharif University of Technology, PO Box: 11365-11155, Tehran, Iran

Abstract:

Using quantitative analysis of intracellular metabolites is a valuable method for understanding the biochemical processes in cellular systems. This study aimed to objectively evaluate quenching–extraction techniques for intracellular metabolite profiling of *Bacillus subtilis* expressing serine alkaline protease (SAP) and to link metabolome data with fermentation optimization. Among the tested methods (cold methanol, cold ethanol, and an acetonitrile:chloroform mixture combined with fast filtration), cold methanol yielded the most efficient and reproducible extraction .Metabolomics analysis identified glucose-6 phosphate (G6P) ,fructose-6 phosphate (F6P) ,serine, and glycine as the most influential intracellular metabolites .Using the Box–Behnken design , different concentrations of the identified metabolites were systematically varied ,and the model predicted optimal levels) G6P 0.52 mM ,F6P 0.054 mM ,serine 1.72 mM ,glycine 0.07 mM (that enhanced SAP activity by nearly ,.300%reaching 1530.33 U/mL compared to the control .These results establish a validated workflow that couples metabolomics with statistical design for enhanced protease production ,offering both methodological and industrial relevance.

Keywords: Serine alkaline protease; *Bacillus subtilis*; Quenching; Extraction; Intracellular metabolomics

* Corresponding Author:
mashayekhan@sharif.edu
Tel.: (98) 21-6616 6414

1. Introduction

Bacillus subtilis bacteria are incredibly important in the field of biotechnology due to their versatile characteristics and beneficial properties [1-3]. They play a significant role in the production of various enzymes, such as amylases, proteases, and lipases, as well as antibiotics like bacitracin and subtilin, which have been harnessed for therapeutic uses [4]. The production of these compounds is facilitated by the bacteria's ability to form endospores, allowing them to survive harsh conditions and be easily harvested for industrial applications [3,4]. Furthermore, *B. subtilis* is well-known for its ability to degrade organic matter through the secretion of extracellular enzymes, contributing to nutrient cycling in natural ecosystems. Its effectiveness as a biocontrol agent against plant pathogens stems from its production of lipopeptides, which exhibit antimicrobial activity, as well as its capacity to induce systemic resistance in plants, thereby enhancing crop resilience and yield [2-4]. As research continues to uncover the complex regulatory networks and metabolic pathways of *B. subtilis*, its role in synthetic biology and the development of bio-based products is expected to expand, positioning it as a cornerstone organism in future biotechnological innovations [5].

Serine alkaline proteases (SAPs) play a crucial role in various industrial applications. According to reports [6], the global SAP market reached to USD 895.6 million by the end of 2023 and is expected to reach a valuation of 1.6 billion USD by 2033. Another crucial aspect of SAP lies in its applications in various industrial processes. These enzymes have a high stability and activity at alkaline pH levels (pH 8–10), which makes them suitable for use in alkaline detergents [7]. When compared to other types of proteases, such as acid or neutral proteases, SAPs stand out for their specific activity under alkaline conditions. While acid proteases are effective in low pH environments, limiting their utility in many industrial applications, SAPs can operate efficiently in a broader range of alkaline contexts [7]. In detergent formulations, alkaline proteases aid in the removal of tough protein stains from fabrics. SAPs efficiently break down proteins present in stains like blood, egg, or grass, ensuring their effective removal during washing [3,6]. This effectiveness in stain removal not only improves the quality of the cleaning process but also reduces the need for harsh chemicals and high-temperature washing, thus promoting environmental sustainability and energy conservation [8,9].

Despite their many advantages, the application of SAPs is not without limitations. Scale-up of SAP enzyme production from laboratory to industrial levels can pose operational challenges, including the optimization of fermentation conditions and downstream processing. One of the primary challenges in expressing SAP is achieving high yields while maintaining enzyme stability and activity [6,8,9]. Factors such as pH, temperature, and nutrient availability must be meticulously controlled, as even minor fluctuations can adversely affect enzyme production [10]. Moreover, the need for robust downstream processing strategies cannot be overstated. The purification of SAP often involves complex separation techniques that can become cost-prohibitive at larger scales. Techniques such as chromatography and ultrafiltration require precise optimization to ensure high purity

and yield, which can be further complicated by the presence of protease inhibitors or other contaminants in the fermentation broth [11].

To address these challenges and enhance enzyme expression, the incorporation of advanced tools like metabolomics has become increasingly necessary [12]. Metabolomics provides valuable insights into the metabolic pathways and regulatory networks involved in enzyme production, allowing for the identification of key metabolites that may enhance or inhibit protease synthesis. By analyzing the metabolic profiles of different microbial strains under various fermentation conditions, optimal growth conditions and nutrient formulations that promote higher yields could be pinpointed [13,14]. Nevertheless, analyzing and quantifying intracellular metabolites presents significant challenges due to their rapid turnover rates and diverse chemical properties. Therefore, the development of reliable and precise methods for quenching cellular metabolism and extracting metabolites is crucial for accurately investigating the in vivo metabolic state of cells under specific conditions. The effectiveness of these methods directly influences the variety and concentration of metabolites that can be detected in metabolomics studies [15].

Cold methanol quenching is a commonly employed technique for halting cellular metabolism in microbial cells. This approach has also been utilized in studies involving various bacteria, such as *Lactobacillus bulgaricus*, *Lactococcus lactis*, and *Escherichia coli*, as well as filamentous fungi like *Aspergillus niger* and *Penicillium chrysogenum* [16-20]. However, unlike yeast cells, bacterial cells often experience leakage of intracellular metabolites during this quenching process, complicating accurate evaluations of their intracellular concentrations [20,21]. As an alternative, a rapid filtration system paired with a combined quenching and extraction method has been shown to effectively minimize the loss of intracellular metabolites [22-23]. An optimal combined quenching and extraction technique should not only halt biological reactions within cells but also extract the maximum number of metabolites in an unbiased and nondestructive manner. McCloskey et al. reported a pressure-driven fast filtration system followed by cold extraction, which serves as a rapid sampling and quenching method for *E. coli* cells [24]. This method is noted for its speed and efficacy in providing an accurate snapshot of intracellular metabolism while mitigating matrix effects from the growth media.

Various researchers have suggested different solvent extraction methods, such as cold methanol for *P. chrysogenum*, boiling ethanol for *Monascus ruber* and *E. coli*, as well as methanol/chloroform/water mixtures for yeast, perchloric acid and potassium hydroxide for *S. cerevisiae*, *A. niger*, and *E. coli* [25]. While many of these techniques are tailored for specific classes of metabolites and microorganisms, they are often applied in different contexts without adequate validation for the specific conditions and organisms being studied, potentially leading to biased results. Therefore, it is crucial to validate sampling, quenching, and extraction protocols for each microorganism and metabolite of interest [20-25].

Applying intracellular metabolomics, several studies have shed light on the significance of specific intracellular metabolites in SAP production of *B. subtilis* [26-28]. One such study conducted by Pal et al. [26] investigated the role of the intracellular metabolite glutamine in protease production. They found that the level of intracellular glutamine positively influenced protease synthesis by providing the necessary nitrogen for protein synthesis and increasing the availability of glutamate, which acts as a precursor for the enzyme. Moreover, they identified that higher intracellular glutamine levels were associated with improved alkaline protease production. However, one limitation of this study is that it focused solely on the role of glutamine, without considering potential interactions with other intracellular metabolites that may also influence protease production. Another study by Pramastya et al. explored the impact of pyruvate, a critical glycolytic intermediate, on SAP production in *B. subtilis*. The results showed that increasing intracellular pyruvate levels significantly enhanced protease expression [27]. They hypothesized that pyruvate acts as a signaling molecule that activates regulatory pathways controlling protease synthesis. Furthermore, their results indicated that pyruvate supplementation could effectively improve overall protease productivity. However, the exact mechanism by which pyruvate enhances protease production needs further investigation [28].

This study was aimed at enhancing the titter of *B. subtilis*, a strain recognized for its capacity to produce SAP. To achieve this objective, a metabolomics approach was utilized, which allows for a comprehensive analysis of the metabolic profile of the organism in question. The first step was to establish a reliable quenching–extraction protocol to ensure efficient and reproducible recovery of intracellular metabolites in *Bacillus subtilis* .The focus of this optimization was exclusively methodological, intended to improve the accuracy of metabolomics analysis rather than to directly enhance protease production. Following sample preparation, we employed liquid chromatography-mass spectrometry (LC-MS) ,for the quantification of metabolites. This method allowed us to measure the levels of key intracellular metabolites in *B .subtilis* ,providing insights into their roles and impacts on the production of SAP .Once the optimal method was identified ,we integrated the metabolome data with statistical analysis techniques. This analytical approach enabled us to identify significant metabolites and their interactions within the metabolic network of *B. subtilis*. By understanding these relationships, we could make informed decisions regarding the optimization of the fermentation medium. Specifically, we sought to identify which nutrients or conditions could be manipulated to enhance the production yield of SAP.

2. Materials and Methods

2.1. bacterial strain and medium

In this study, *B. subtilis* (ATCC 6633), a strain known for its ability to produce SAP, was used. The bacteria were retrieved from storage in a deep freezer with a temperature below -80 ± 2.5 °C, thawed in a baffled Erlenmeyer flask (Corning, US) and then sub-cultured in minimal medium. Each liter of minimal medium consisted of 2.10 g of $NaH_2PO_4 \cdot 2H_2O$, 2.10 g of $(NH_4)_2SO_4$, 12.00 mg of EDTA, 0.20 g of $MgCl_2 \cdot 6H_2O$, 2.30 mg of $ZnSO_4 \cdot 6H_2O$, 1.50 mg of $CaCl_2 \cdot 3H_2O$, 4.00 mg of $FeSO_4 \cdot 5H_2O$, 0.10 mg of Na_2MoO_4, 0.15 mg of $CuSO_4 \cdot 3H_2O$, 0.50 mg of $CoCl_2 \cdot 7H_2O$, 2.00 mg of $MnCl_2 \cdot 4H_2O$, and 4.50 g of glucose as a carbon source adjusted to pH 7.2 ± 0.2. All

chemical reagents and culture medium nutrients were procured from Sigma-Aldrich, unless otherwise stated. To quantify the cell concentration, a spectrophotometer (Perkin Elmer, US) was used at an optical density of 600 nm (OD_{600}) for the samples collected hourly. The primary seed culture with initial OD_{600} of 0.05 ± 0.01 was incubated overnight at 37 ± 0.5°C and 300 rpm in a 250 mL baffled Erlenmeyer shake flask (with working volume of 50 ± 1 mL) in a shaker incubator to prepare seed culture. Subsequently, the seed culture was transferred to a new 2000 mL Erlenmeyer shake flask (with working volume of 500 ± 5 mL) with fresh minimal medium to initiate a secondary seed culture with initial OD_{600} = 0.05 ± 0.01 using the same cultivation procedure [24]. Also, to quantify the cell dry weight (CDW), 1 mL of fermentation broth was centrifuged at 15000 × g for 10 min at 4℃ and the microtube containing the cell pellet were dried in an oven (Memmert, Germany) at 70 ± 2 ℃ until a constant weight was recorded)These samples were not used for further analysis including metabolomics([25]. For all quenching/extraction methods, the samples were sourced from the same shake flask, and each quenching/extraction procedure was conducted using five separate shake flasks as biological replicates.

2.2. Sample processing

In 2000 mL shake flasks, samples were collected in logarithmic growth phase (OD_{600} = 2.5 ± 0.1) to extract the intracellular metabolites) Although OD 2.5 = $_{600}$is often considered late-log, in this cultivation system the growth curve confirmed that cells were still in exponential phase at this point; therefore, samples were collected at OD $_{600}$ 2.5 ≈to represent actively growing cells.(To ensure no cellular stress or metabolic changes, the samples were transferred into plastic centrifuge tubes within 10 seconds, formerly pre-cooled with liquid nitrogen to immediately halt enzymatic processes with careful agitation to prevent freezing and metabolite leakage. Subsequently, the supernatant was filtered using a vacuum-assisted filtration system to separate the cells from the medium. The cell pellet on filter was transferred into different extraction solutions pre-cooled at - 40°C, followed by immediate freezing of the samples in liquid nitrogen. The samples underwent five cycles of freeze-thaw (freezing in liquid nitrogen and thawing in an extraction solution at -40°C), with each thawing period lasting less than 15 seconds while being gently shaken. The freeze-thaw cycle numbers were selected based on optimization trials and prior studies ,[25 ,24 ,16] which demonstrated that this number ensures efficient metabolite extraction while avoiding cellular rupture. Afterward, to remove the cellular debris, the vacuum-assisted filtration was carried out once more and the samples were stored at deep freezer (-80 ± 2.5 °C) and protected from light for further analysis (see workflow in Fig. 1).

2.3. Enzyme activity

The activity of alkaline protease enzymes was measured using the method outlined by Saleem et al. [29] and Sun et al [30]. This method defines one unit of enzyme activity as the amount of enzyme that releases 1.0 µg of tyrosine from casein per minute at 45°C and pH 10.0 ± 0.1, using a 10.0 g/L casein solution. A standard curve was created with L-tyrosine standard solutions ranging from 0.0 to 80.0 µg/mL. Folin's reagent was used to react with L-tyrosine, forming a blue compound, and the absorbance was measured at 680 nm. The enzyme activity of the samples can be calculated according to Eq (1):

$$Enzyme\ activity = (A \times V) / (4 \times D \times m \times 10). \quad (1)$$

Here, A is the enzyme activity of the final dilution compared to the standard curve (U/mL), V indicates the volume used to dissolve the samples (mL), D stands for the dilution factor of the enzyme liquid of alkaline protease, m represents the mass of the samples (g), 4 represents the total volume of the reaction mixture, and 10 is the reaction time.

2.4. Analytical techniques

The intracellular metabolites were quantified using liquid chromatography-mass spectrometry (LC-MS), utilizing a Waters LC system with XSelect HSS T3 column (75 mm × 4.6 mm × 2.5 μm). The mobile phases consisted of buffer A (5.0 mM tributylamine, 15.0 mM acetic acid, 2.5% methanol, and 3% 2-propanol) and buffer B (2-propanol) with a specific gradient over different time points: 0 min, 0% B; 4 min, 0% B; 8 min, 2.5% B; 9.0 min, 6.5% B; 10.5 min, 6.5% B; 11 min, 11.5% B; 14.0 min, 12% B; 14.5 min, 28.5% B; 15.5 min, 54% B; 21.0 min, 54% B; 22 min, 0% B; 26 min, 0% B; 34 min, 0% B [25,31]. The flow rate varied throughout the analysis. The flow rate was 0.50 mL/min, 0.20 mL/min, and 0.50 mL/min, respectively. The mass spectrometry was carried out in negative ion mode using electrospray ionization (ESI) with specific settings on a Qtrap1 5500 system from AB SCIEX.

2.5. Energy change quantification

The energy change of a cell can be effectively quantified by analyzing the levels of various adenine nucleotides, adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP). These nucleotides are fundamental to cellular metabolism, acting as key players in energy production and transfer within the cell. The adenine energy change (AEC) ratio can be expressed mathematically with the Eq. (2) [32]:

$$AEC = (ATP+½ADP)/ (AMP+ADP+ATP) \quad (2)$$

In this equation, (ATP+½ADP) signifies the total high-energy phosphate content available in the cell. ATP is often referred to as the "energy ratio" of the cell, and it is the primary molecule that stores and transfers energy necessary for cellular activities. (ATP+½ADP) is a method of accounting for the energetic contribution of ADP, as it can, under certain conditions, be converted back into ATP through phosphorylation. (AMP + ADP + ATP) sums the total adenine nucleotide pool present in the cell. This total reflects the overall availability of adenine nucleotides, which are crucial for various biochemical reactions, including those involved in energy production, signal transduction, and nucleotide synthesis. In actively growing and non-stressed cells, the AEC typically falls between 0.80 and 0.95 [32]. In the field of metabolomics, monitoring the levels of adenine nucleotides and calculating the AEC provides valuable insights into the energetic status of cells, enabling evaluation of cellular metabolism, energy dynamics, and potential dysfunctions that may arise in various physiological or pathological stress [32].

2.6. Quenching and extraction methods

Table 1 provides an overview of various joint quenching-extraction solutions employed for the analysis of intracellular metabolites from *B. subtilis*, which were previously separated using a pressure-driven fast filtration system. The solutions highlighted include cold ethanol (CE), cold methanol (CM), and a cold mixed solvent of acetonitrile and chloroform (CMAC). Each of these solutions has been selected for their efficacy in preserving metabolic integrity during extraction, ensuring that subsequent analyses yield reliable and reproducible results. The importance of temperature and the specific volume percentages of these solvents in water for injection (WFI) is crucial, as they directly influence the extraction efficiency and the stability of the metabolites [19,20,23,25].

2.7. Data reproducibility

In microbial metabolomics, the robustness of each extraction technique can be effectively gauged through its reproducibility, which is often assessed using the percent relative standard deviation percentage of RSD):

$$percentage\ of\ RSD = 100\times \frac{\sigma}{\mu} \quad (3)$$

Where σ represents the standard deviation and μ denotes the average of the set of values. A lower percentage of RSD indicates that the extraction method consistently yields similar results across multiple experiments, thereby enhancing confidence in the data's validity.

2.8. Experimental design and data analysis

Response surface methodology (RSM) was employed to design experiments and analyze the effects of the selected parameters. The parameters studied are the selected metabolites achieved based on metabolomics results, while the SAP activity is the responses measured. A Box–Behnken design was utilized to evaluate each parameter to identify the optimal conditions for higher SAP titer. The Box–Behnken experimental design was implemented using Design Expert software)version(13 . The experiments were conducted with 5 replicates at the center point to estimate the error sum of squares, based on the quadratic model generated by the RSM software. Each factor was assigned at three levels, −1 (low), 0 (intermediate), and +1 (high). The predicted activity was calculated using the following second-order polynomial Eq. (4):

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (4)$$

Where Y represents the predicted response and X_1, X_2, and X_3 correspond to independent variables, and β terms represent the intercept ,linear ,quadratic ,and interaction coefficients of the second-order polynomial model .The model's significance was evaluated by ANOVA ,and $R^2$and adjusted R^2were used to assess goodness of fit. Statistical analyses and graphical representations were generated using Design-Expert software, with interactions between the variables and responses illustrated through three-dimensional surface plots.

2.9. *Statistical Analysis*

Each experiment conducted for enhanced enzyme activity optimization was designed using the "Design Expert 13" software employing the RSM method. Furthermore, each test related to cell culture was performed in triplicate. All results are presented as the mean of the replicates ± standard deviation (SD). A *p*-value of less than 0.05 was regarded as statistically significant.

3. Results and Discussion

3.1. Quenching Efficiency

The efficiency of quenching solutions, including CE, CM, and CMAC, was evaluated based on AEC ratio. Fig. 2 illustrates the AEC ratio for different quenching solutions. Results showed that CE and CM were successful in deactivating cellular metabolism, as evidenced by AEC values within a physiologically relevant range (AEC ≈ 0.904 and 0.899, respectively). This aligns with findings from other recent research that have also highlighted the advantages of CE and CM as quenching agents. For instance, de Jonge et al. [20] reported that in *P. chrysogenum*, CM treatment resulted in minimal changes to key metabolites involved in glycolysis and the TCA cycle, validating its effectiveness in preserving metabolic integrity.

In contrast, CMAC solution was found to be less effective in halting cellular metabolisms (AEC ≈ 0.455). These findings highlight the significant impact of the choice of quenching-extraction solution on the accurate quenching of cellular metabolism. This observation is supported by findings of Lu et al. [32], which demonstrated that CMAC not only failed to adequately suppress enzymatic activity but also introduced solvent effects that interfered with downstream metabolite extraction. These discrepancies raise concerns about the applicability of CMAC in metabolomics studies, particularly when precise quantification of metabolites is essential.

3.2. Data reproducibility

The reproducibility of each quenching-extraction solution was evaluated by calculating average of percentage of RSD for biological sample replicates. According to Fig. 3, the average percentage of RSD for all metabolites varied depending on the quenching-extraction solution. The average RSDs for quantified metabolites were 25% for CM, 32% for CE, and 28% for CMAC. Notably, metabolites extracted using the CM solution exhibited mostly low RSDs compared to the other solutions. These findings show that CM is the most reliable solution for extracting metabolites with high reproducibility, surpassing the other methods tested. Conversely, the results of this study, which examined an effective method for sampling the metabolome of *B. subtilis*, significantly diverged from those of other gram-negative bacteria, such as *K. oxytoca* [34] and *E. coli* [35]. In those cases, CMAC, perchloric acid, and potassium hydroxide were more effective. These discrepancies may be attributed to fundamental differences in cell wall structure and membrane composition among these organisms [32]. Therefore, it is crucial to evaluate the metabolome sampling technique tailored to the specific organism in question. Another comparative study by

Lagzian et al. [36] examined the impact of different quenching-extraction protocols on the metabolomic profiling of *B. subtilis* under various growth conditions. Their results indicated that the use of acetonitrile as a quenching agent yielded a richer array of secondary metabolites. These findings on quenching-extraction solutions efficiency and reducibility underscore the complexity of metabolome analysis. Each quenching-extraction solution indeed presents a unique set of advantages and disadvantages, depending on the physical and chemical properties of the metabolites under study and the microorganisms as well [32-36].

The optimization of quenching–extraction methods played a critical role in establishing the reliability of the metabolomics data used in this study. In microbial metabolomics, inadequate quenching can result in continued enzymatic activity or metabolite leakage, leading to misleading interpretations. By employing cold methanol combined with fast filtration, intracellular metabolites could be preserved in a reproducible manner, ensuring that the metabolite profiles truly reflected the physiological state of *B .subtilis* during the logarithmic growth phase. This methodological rigor is particularly important when linking metabolite abundance to protease synthesis. For example, the consistent detection of elevated glucose 6-phosphate, fructose 6-phosphate, serine, and glycine under optimal conditions provided a solid foundation for subsequent statistical modeling. The Box–Behnken design then confirmed that supplementation with these metabolites significantly enhanced SAP production. Therefore, the optimization of quenching not only strengthened the accuracy of metabolite quantification but also validated the observed correlations between intracellular metabolic state and protease synthesis.

3.3. Metabolome compositions of B. subtilis

Fig. 4 shows the metabolites quantified from the Embden-Meyerhof-Parnas (EMP) pathway, the pentose phosphate pathway (PPP), and the tricarboxylic acid cycle (TCA). While these metabolites may not encompass the full metabolome of *B. subtilis*, they do represent the majority of the most abundant metabolites in terms of molar concentration and play a crucial role in central metabolism. The metabolome of *B. subtilis* shows a significant composition of amino acids, sugar phosphates, organic acids, and nucleotides. With amino acids constituting 58% of its metabolome, *B. subtilis* appears to prioritize protein synthesis and cellular growth. This is particularly interesting when compared to *E. coli*, which also exhibits a rich amino acid profile but allocates a more balanced proportion of metabolites [37]. This study has shown that *E. coli* has a more diversified metabolome that includes a higher percentage of organic acids compared to *B. subtilis*, reflecting its different ecological niches. The emphasis on amino acids in *B. subtilis* could indicate a robust mechanism for dealing with nutrient availability, as amino acids are critical not only for protein synthesis but also for various signaling pathways and metabolic functions [4,37].

In addition to the amino acid dominance in *B. subtilis*, according to Fig. 4, the presence of sugar phosphates and organic acids suggests a well-developed glycolytic pathway and robust energy metabolism. In comparison, *S. cerevisiae* also displays a high concentration of sugar phosphates, particularly during fermentation processes

where glucose is abundant. However, *S. cerevisiae* tends to have a more complex network of organic acids like acetic acid and succinic acid [38]. Also, Fig. 4 shows among sugar phosphates and organic acids, reduced glutathione, D-glucose 6-phosphat (G6P), and D-fructose 6-phosphate (F6P) are the most abundant intracellular metabolites. The comparison of metabolome data from *E. coli* and *B. subtilis* during aerobic growth reveals significant differences in their glucose catabolic pathways. While *E. coli* exhibits a more streamlined glycolytic approach favoring rapid ATP production, *B. subtilis* demonstrates metabolic flexibility, utilizing the pentose phosphate pathway to balance energy production and biosynthetic needs. These metabolic distinctions are supported by various studies that highlight the regulatory mechanisms and environmental adaptability of these organisms [37,38].

The F6P/G6P ratio in *B. subtilis* is 0.35 while in *E. coli* it is 0.10, as reported by McCloskey et al. [24]. The higher F6P/G6P ratio in *B. subtilis* indicates a more balanced utilization of glucose through alternative pathways, including PPP. This suggests that *B. subtilis* may prioritize the generation of reducing equivalents (NADPH) and precursors for biosynthesis over rapid ATP production. The increased ratio aligns with findings from studies that highlight the metabolic flexibility of *B. subtilis*, which can switch between different pathways depending on nutrient availability and cellular demands. For instance, Smith et al. [39] has indicated that under aerobic conditions, *B. subtilis* can effectively utilize the PPP to not only generate energy but also to produce ribose-5-phosphate for nucleotide synthesis, suggesting a more integrated approach to carbon metabolism.

Moreover, Fig. 4 displays the molar concentration of nucleotides per gCDW. Among them, ATP, guanosine triphosphate (GTP), and uridine triphosphate (UTP) have the highest intracellular level. The observation that *B. subtilis* exhibits high intracellular levels of ATP, GTP, and UTP is noteworthy when considering the energy and signaling pathways within microbial cells. In *B. subtilis*, ATP serves as the primary energy ratio, while GTP and UTP play crucial roles in protein synthesis and RNA metabolism, respectively. Comparatively, *E. coli* maintains high levels of ATP, but its intracellular concentrations of GTP and UTP differ based on growth conditions [40]. In the case of *S. cerevisiae*, however, their GTP and UTP concentrations can be influenced by the availability of carbon sources [40]. This comparison underlines the diversity in nucleotide metabolism among microorganisms like *B. subtilis*, *E. coli*, and *S. cerevisiae*, revealing that while ATP remains a common energy ratio, the regulation and significance of GTP and UTP vary considerably based on the organism and its environmental condition.

It should be noted that intracellular metabolite analysis in this study was performed exclusively at the logarithmic growth phase**)** OD **,(0.1 ± 2.5 ≈** $_{600}$when cells exhibit active metabolism and stable growth. The rationale for this choice was to obtain reproducible metabolite profiles with minimal variability caused by stress responses. As such, the dynamics of metabolites across different growth stages were not investigated here. Nevertheless, enzyme activity monitoring (Fig. 6) revealed that SAP production increased significantly during the late exponential and stationary phases, suggesting a temporal separation between metabolite abundance profiling and peak protease

synthesis. Future work will therefore focus on time-resolved metabolomics to directly connect intracellular metabolite fluctuations at distinct growth stages with protease production behavior.

3.4. Enhanced SAP production

The five most abundant metabolites identified in the previous step through metabolomics— G6P, F6P, serine, glycine, and threonine—are presented in Table 2. These metabolites were selected to conduct a RSM design (Box-Behnken) aimed to study their role in enhancing the fermentation process of *B .subtilis* for SAP production .)No OVAT) One Variable At a Time (experiments were performed ,and the Box–Behnken design of RSM was directly applied for statistical optimization.(.

Applying RSM, the importance of the experimental model's impact on SAP activity is clearly demonstrated by the results presented in Table 3, with a very low *p*-value of less than 0.002, ensuring high credibility. Furthermore, the lack-of-fit *p*-value of 0.761 surpasses the significance threshold of 0.05, suggesting that the model fits well. According to the *p*-values, it can be concluded that threonine is the only term that does not have a significant effect on the activity of SAP produced by *B. subtilis* (*p*-value ≈ 0.15). The *F*-values for the linear terms of G6P, F6P, serine, and glycine are 1.16, 0.86, 7.05, and 5.48, respectively. This means that the accumulation of alkaline protease is predominantly influenced by serine, followed by glycine, G6P, and F6P. Additionally, when considering both the *F*-value and *p*-value of the linear and quadratic terms, it is apparent that the impact of serine and glycine on SAP titer is more significant compared to G6P and F6P. Kocabaş. [41] suggested that serine significantly enhances SAP production because of its role in forming phosphoserine intermediates, directly impacting protease expression pathways. Arifoglu [42] identified glycine's role in stabilizing the metabolic network during SAP production, emphasizing its dual role in nucleotide synthesis and protein stabilization. This result confirmed the substantial influence of the factors identified in the metabolomics study on SAP expression. Also, Çalık et al. [43], emphasizes that while G6P and F6P maintain cellular energy balance, their over-supplementation may divert resources away from protease biosynthesis toward biomass accumulation.

Furthermore, the adj- R^2 of the model is 0.9478, showing a robust correlation between the actual and predicted values. The pred-R^2 value is estimated to be 0.8870, indicating a slight difference from the adj- R^2. This suggests that the regression model effectively explains the manufacturing process. The coefficient of variation, at 7.23%, falls below the 10% threshold, signifying high reliability and precision in the experiment.

The response surface plots for the interaction of G6P and F6P, as well as G6P and serine, in three dimensions (Fig. 5) show clear and steep trends, indicating a significant impact on SAP titer. Fig.5A demonstrates a significant interaction (*p*-value < 0.05) between G6P and serine, with an evident elliptical pattern suggesting a strong interaction effect. In Fig. 5B, the plot for G6P and F6P also displays an elliptical trend, indicating some level of interaction. While the regression model for this combination shows a *p*-value > 0.05, not quite reaching

significance, there is still a noticeable interaction effect present. The remaining four combinations do not exhibit a significant interaction effect. The experimental model effectively captures the variations in SAP enzyme activity produced by *B. subtilis* under different conditions, making it useful for predicting enzyme activity in *B. subtilis* fermentation. Furthermore, the ANOVA results for the regression model suggest that the prediction equation can be formulated as Eq. (5):

$$SAP\ (U/mL) = 1779.1 + 35.0\ G6P - 78.14\ F6P + 20.08\ Ser + 68.94\ Gly + 92.87\ G6P\ F6P - 119.1\ G6P\ Ser - 0.68\ G6P\ Gly + 10.0\ F6P\ Ser + 62.14\ F6P\ Gly - 45.21\ Ser\ Gly - 221.0\ G6P^2 - 342.8\ F6P^2 - 362.4\ Ser^2 - 352.8\ Gly^2 \quad (5)$$

RSM has demonstrated its effectiveness in optimizing the production of microbial-synthesized products such as enzymes, pigments, and pectin oligosaccharides [30]. Based on the prediction equation (5), the optimal points can be identified and it is recommended to add G6P at a concentration of 0.52 mM, F6P at 0.054 mM, serine at 1.72 mM, and glycine at 0.07 mM to the basal medium. All other variables for fermentation verification were maintained at constant levels. The chosen concentrations are supported by prior studies, enhancing SAP yields. Yuan et al. [43] found that serine supplementation had a direct positive effect on SAP yield by facilitating carbon flux toward amino acid biosynthesis. They reported an optimal range of 1.5–2.0 mM. Çalık et al. [41] highlighted that G6P supplementation should not exceed 0.6 mM to avoid excessive glycolytic flux that competes with protease biosynthesis pathways. Çalık et al. [44] demonstrated that F6P supplementation enhances the availability of NADPH for biosynthetic reactions, indirectly supporting SAP production. However, the concentration must be optimized; excessive F6P (> 0.1 mM) was shown to divert carbon flux toward biomass and energy generation, reducing protease-specific yield. According to Arifoğlu [42] study, while glycine supplementation supports purine biosynthesis (which is critical for nucleotide generation in energy-intensive SAP secretion), higher glycine levels (> 0.1 mM) caused feedback inhibition in purine metabolism, reducing overall efficiency.

Fig. 6 illustrates the growth curve and SAP activity of control (minimal medium + 4.5 g/L glucose) and optimal (minimal medium + 4.5 g/L glucose + supplements) during the validation experiment. In the initial phase (0–24 hours), the *B. subtilis* population increased gradually, while enzyme activity remained negligible, indicating a lag phase as the strain adapted to the new medium. After 26 hours, the bacterial population in the fermentation broth began to grow rapidly. By approximately 70 hours, the growth curve reached stationary phase, with an OD_{600} of about 4.9–5.0 under optimized conditions, compared to 4.0–4.1 in the control experiment. Concurrently, SAP activity increased sharply, coinciding with the accumulation of secondary metabolites. By 94 hours, SAP activity peaked at 1530.33 U/mL, representing a ≈ 300% increase compared to the control experiment. This result closely matched the predicted value of 1606.91 U/mL from Equation (5), validating the reliability of the mathematical model developed in this study. The accuracy of the predicted model in comparison to the experimental results demonstrates that nearly all data points fall within the ±5% error margin, indicating that the proposed model is appropriately accurate.

From an economic perspective, the feasibility of metabolite supplementation is an important consideration for industrial application. The optimized concentrations identified in this study were relatively low, thereby limiting cost implications. Serine and glycine are inexpensive and commercially available amino acids, making them viable supplements for large-scale fermentation. However, sugar phosphates such as glucose 6-phosphate and fructose 6-phosphate are less economically attractive for direct supplementation due to their higher cost. In practice, metabolic engineering strategies to enhance endogenous pools of these sugar phosphates in *B .subtilis* may provide a more cost-effective alternative. Therefore, while the present results demonstrate the positive effect of metabolite supplementation on SAP production, large-scale implementation would likely require a hybrid strategy combining low-cost amino acid supplementation with targeted metabolic pathway optimization.

4. Conclusion

The study focused on conducting a quantitative analysis of intracellular metabolites in *B. subtilis* (ATCC 6633) to better understand the biochemical processes within cellular systems. Various quenching-extraction techniques, such as cold methanol, cold ethanol, and cold mixed acetonitrile: chloroform, were tested and evaluated for their effectiveness in extracting and quantifying metabolites. Based on factors such as quenching efficiency and experimental reproducibility, the pressure-driven fast filtration system combined with cold methanol quenching-extraction solution was determined to be the most suitable method for *B. subtilis* metabolome analysis. Amino acids, nucleotides, and carbon metabolism intermediates were among the quantified metabolites. The most abundant intracellular metabolites in *B. subtilis* were found to be G6P, F6P, ATP, UTP (from PPP, EMP, and TCA), serine, and glycine. Using the results from the metabolome analysis, the Box–Behnken design was employed to identify significant factors influencing fermentation conditions. The optimized conditions - with concentrations of G6P at 0.52 mM, F6P at 0.054 mM, serine at 1.72 mM, and glycine at 0.07 mM in the basal medium - were predicted to maximize alkaline protease enzyme activity. The approximated alkaline protease enzyme activity was 1606.91 U/mL, with experimental validation yielding a result of 1530.33 U/mL. These results provide important information for improving the yield and effectiveness of alkaline protease, which could lead to significant practical and economic advantages in industrial settings.

Statements & Declarations

Acknowledgments

The authors acknowledge the grant funded by Sharif University of Technology, Iran.

Funding

The funding provided by Council of Scientific and Industrial Research, Sharif university of technology, Iran.

Author contributions

HS conducted the manuscript, wrote the main manuscript text, and prepared the figures included in the manuscript. SM and RR conceptualized the topic, and reviewed and edited the manuscript.

Data availability

All the data used in this study is available from the corresponding author upon request.

Competing interests

The authors declare no conflict of interest with regard to the content of this review article. The authors have no relevant financial or non-financial interests to disclose.

References

[1]. Fessia, A., Sartori, M., García, D., et al. “In vitro studies of biofilm-forming Bacillus strains, biocontrol agents isolated from the maize phyllosphere”, Biofilm, 4, 100097 (2022). https://doi.org/10.1016/j.bioflm.2022.100097

[2]. Tsotetsi ,T ,.Nephali ,L ,.Malebe ,M ,.et al“ .Bacillus for Plant Growth Promotion and Stress Resilience : What Have We Learned ,”?Plants (Basel). 11(19):2482 .(2022) doi/10.3390 :plants11192482.

[3]. Chen, T., Haris U., Stanley B., et al. "Developing Bacillus subtilis as cell factory for the production of the natural biocontrol compound pulcherrimin.", Bioresource Technology 427 (2025): 132433. https://doi.org/10.1016/j.biortech.2025.132433

[4]. Cui, W., Han, L., Suo, F., et al. “Exploitation of Bacillus subtilis as a robust workhorse for production of heterologous proteins and beyond”, World J Microbiol Biotechnol. 34:1-19 (2018). https://doi.org/10.1007/s11274-018-2531-7

[5]. Akinsemolu, A.A., Onyeaka, H., Odion, S., et al. “Exploring Bacillus subtilis: Ecology, biotechnological applications, and future prospects”, J Basic Microbiol. e202300614 (2024). https://doi.org/10.1002/jobm.202300614

[6]. Alkaline Proteases Market Size, Share, Growth, and Industry Analysis, By Types (Serine Protease, Metalloprotease), By Applications Covered (Detergents, Pharmaceuticals, Leather Industry, Meat Tenderizers, Protein Hydrolyzates, Food Products, Waste Processing), Regional Insights and Forecast to 2033. Global Growth Insights (2025). https://www.globalgrowthinsights.com/market-reports/alkaline-proteases-market-106062#menu1

[7]. Golani ,M ,.and Hajela, K“ .Purification and characterization of lipase from a new strain Staphylococcus argenteus MG2) MTCC ,”(12820 Brazilian Journal of Science .(2024) 18–1 3:5 .DOI : https://doi.org/10.14295/bjs.v3i5.547 .

[8]. Singh, S., Bajaj, B.K. “Potential application spectrum of microbial proteases for clean and green industrial production”, Energy Ecol Environ. 2:370-386 (2017). https://doi.org/10.1007/s40974-017-0076-5

[9]. Shankar, R., Upadhyay, P.K., Kumar, M. “Protease enzymes: highlights on potential of proteases as therapeutics agents”, Int J Pept Res Ther. 27(2):1281-1296 (2021). https://doi.org/10.1007/s10989-021-10167-2

[10]. Sujitha, P., Shanthi, C. “Importance of enzyme specificity and stability for the application of proteases in greener industrial processing-a review”, J Clean Prod. 138915 (2023). https://doi.org/10.1016/j.jclepro.2023.138915

[11]. Srivastava, N., and Sunil K., K. "Advances in Microbial Alkaline Proteases: Addressing Industrial Bottlenecks Through Genetic and Enzyme Engineering.", Applied Biochemistry and Biotechnology (2025): 1-36. https://doi.org/10.1007/s12010-025-05270-9

[12]. Praveen, M., & Brogi, S“ .Microbial Fermentation in Food and Beverage Industries : Innovations ,Challenges ,and Opportunities .Foods) ”Basel, Switzerland .(2025) 114 ,(1)14 ,(https://doi.org/10.3390/foods14010114

[13]. Ohno, S., Uematsu, S & ,.Kuroda, S .“Quantitative metabolic fluxes regulated by trans-omic networks” .The Biochemical journal804–787 ,(6)479 , (2022) .https://doi.org/10.1042/BCJ20210596

[14]. Ghafari, N., & Sleno, L“ .Challenges and recent advances in quantitative mass spectrometry-based metabolomics .”Analytical science advances ,(5-6)5 ,e2400007 .(2024) https://doi.org/10.1002/ansa.202400007

[15]. Villas-Bôas, S.G., Højer-Pedersen, J., Akesson, M., et al. “Global metabolite analysis of yeast: evaluation of sample preparation methods”, Yeast (Chichester, England) 22(14):1155–1169 (2005). https://doi.org/10.1002/yea.1308

[16]. de Koning, W., van Dam, K. “A method for the determination of changes of glycolytic metabolites in yeast on a subsecond time scale using extraction at neutral pH”, Anal Biochem 204(1):118-123 (1992). https://doi.org/10.1016/0003-2697(92)90149-2

[17]. Ray, L., & Raina, V. “Scale-Up of Engineering Strain for Industrial Applications. In Microbial Engineering for Therapeutics”, Singapore: Springer Nature Singapore. 311-326 (2022). https://doi.org/10.1007/978-981-19-3979-2_14

[18]. Jeppesen, M.J., & Powers, R. “Multiplatform untargeted metabolomics.”, Magnetic Resonance in Chemistry, 61(12), 628-653. (2023). https://doi.org/10.1002/mrc.5350

[19]. Mohd K ,.K ,.Mahamad M ,.Abdul R ,.N ,.et al“ .Bacterial Metabolomics :Sample Preparation Methods .”Biochemistry research international .(2022) 9186536 ,2022 , https://doi.org/10.1155/2022/9186536

[20]. de Jonge, L.P., Douma, R.D., Heijnen, J.J., et al. “Optimization of cold methanol quenching for quantitative metabolomics of Penicillium chrysogenum”. Metabolomics 8:727-735 (2012). https://doi.org/10.1007/s11306-011-0367-3

[21]. Shah, Z. A., Khan, K., Shah, T ,.et al“ .Biological investigations of Aspergillus ficuum via in vivo ,in vitro and in silico analyses .”Scientific reports, 13(1), 17260 .(2023) https://doi.org/10.1038/s41598-023-43819-y

[22]. Pontrelli, S., & Sauer, U“ .Polysaccharide quantification using microbial enzyme cocktails .” Biology methods & protocols, 10(1), bpaf014 .(2025) https://doi.org/10.1093/biomethods/bpaf014

[23]. Lang, J., Ramos, S. E., Smohunova, M ,.et al .“Screening of leaf extraction and storage conditions for eco-metabolomics studies” .Plant Direct ,(4)8 ,e578 .(2024) https://doi.org/10.1002/pld3.578

[24]. McCloskey, D., Utrilla, J., Naviaux, R.K., Palsson, B.O., Feist, A.M. “Fast Swinnex filtration (FSF): a fast and robust sampling and extraction method suitable for metabolomics analysis of cultures grown in complex media”, Metabolomics 11:198-209 (2015). https://doi.org/10.1007/s11306-014-0686-2

[25]. Wordofa, G.G., Kristensen, M., Schrubbers, L., et al. “Quantifying the metabolome of Pseudomonas taiwanensis VLB120: evaluation of hot and cold combined quenching/extraction approaches”, Anal Chem 89(17):8738-8747 (2017). https://doi.org/10.1021/acs.analchem.7b00793

[26]. Pal, P., Singh, A.K., Sarangi, P.K., et al. “Production of gamma-polyglutamic acid microgel by Bacillus species: Industrial applications and future perspectives”, Polymers for Adv Technol 35(9):e6565 (2024). https://doi.org/10.1002/pat.6565

[27]. Pramastya, H., Xue, D., Abdallah, I.I., et al. “High level production of amorphadiene using Bacillus subtilis as an optimized terpenoid cell factory”, New Biotechnol 60:159-167 (2021). https://doi.org/10.1016/j.nbt.2020.10.007

[28]. Yang, Y., Saand, M.A., Huang, L., et al. “Applications of multi-omics technologies for crop improvement”, Front Plant Sci 12:563953 (2021). https://doi.org/10.3389/fpls.2021.563953

[29]. Saleem, M., Rehman, A., Yasmin, R., et al. “Biochemical analysis and investigation on the prospective applications of alkaline protease from a Bacillus cereus strain”, Mol Biol Rep 39:6399-6408 (2012). https://doi.org/10.1007/s11033-011-1033-6

[30]. Sun, B., Zou, K., Zhao, Y., et al. “The fermentation optimization for alkaline protease production by Bacillus subtilis BS-QR-052”, Front Microbiol 14:1301065 (2023). https://doi.org/10.3389/fmicb.2023.1301065

[31]. Goodman, K., Mitchell, M., Evans, A. M ,.et al“ .Assessment of the effects of repeated freeze thawing and extended bench top processing of plasma samples using untargeted metabolomics .” Metabolomics : Official journal of the Metabolomic Society ..(2021) 31 ,(3)17 , https://doi.org/10.1007/s11306-021-01782-7

[32]. Meyer, H., Weidmann, H., Lalk, M. “Methodological approaches to help unravel the intracellular metabolome of Bacillus subtilis”, Microb Cell Fact 12:1-13 (2013). https://doi.org/10.1186/1475-2859-12-69

[33]. Lu, W., Su, X., Klein, M.S., et al. “Metabolite measurement: pitfalls to avoid and practices to follow”, Annu Rev Biochem 86(1):277-304 (2017). https://doi.org/10.1146/annurev-biochem-061516-044952

[34]. Park, C., Yun, S., Lee, S.Y., et al. “Metabolic profiling of Klebsiella oxytoca: evaluation of methods for extraction of intracellular metabolites using UPLC/Q-TOF-MS”, Appl Biochem Biotechnol 167:425-438 (2012). https://doi.org/10.1007/s12010-012-9685-9

[35]. Sedighikamal, H., & Mashayekhan, S“ .Critical assessment of quenching and extraction/sample preparation methods for microorganisms in metabolomics .”Metabolomics : Official journal of the Metabolomic Society .(2025) 40 ,(2)21 ,https://doi.org/10.1007/s11306-025-02228-0

[36]. Lagzian, A., Riseh, R.S., Sarikhan, S., et al. “Genome mining conformance to metabolite profile of Bacillus strains to control potato pathogens”, Sci Rep 13(1):19095 (2023). https://doi.org/10.1038/s41598-023-46672-1

[37]. Park, J.O., Rubin, S.A., Xu, Y.F., et al. “Metabolite concentrations, fluxes and free energies imply efficient enzyme usage”, Nat Chem Biol 12(7):482-489 (2016). https://doi.org/10.1038/nchembio.2077

[38]. Roldán-López, D., Groenewald, M., Pérez-Torrado, R. “Fermentative and metabolic screening of candidate yeast strains hybridisable with Saccharomyces cerevisiae for beer production optimization”, Int J Food Microbiol 426:110899 (2025). https://doi.org/10.1016/j.ijfoodmicro.2024.110899

[39]. Smith, R.L., Soeters, M.R., Wüst, R.C.I., et al. “Metabolic Flexibility as an Adaptation to Energy Resources and Requirements in Health and Disease”, Endocr Rev 39(4):489–517 (2018). https://doi.org/10.1210/er.2017-00211

[40]. Lee, S.J., Trostel, A., Adhya, S. “Metabolite changes signal genetic regulatory mechanisms for robust cell behavior”. mBio 5(1):e00972-13 (2014). https://doi.org/10.1128/mBio.00972-13

[41]. Kocabaş, P. “COMPARATIVE FLUX BALANCE ANALYSES OF SERINE ALKALINE PROTEASE OVERPRODUCTION IN Bacillus subtilis AT GENOME AND SMALL SCALE.”, Eskişehir Teknik Üniversitesi Bilim ve Teknoloji Dergisi-C Yaşam Bilimleri Ve Biyoteknoloji, 12(1), 8-18 (2023). https://doi.org/10.18036/estubtdc.1184733

[42]. Arifoğlu, M. “Effects of bioreactor operation parameters on intracellular reaction rate distribution in beta-lactamase production by bacillus species”, Middle East Technical University (2004). https://hdl.handle.net/11511/14274

[43]. Yuan, K., Song, P., Li, S., et al. “Combining metabolic flux analysis and adaptive evolution to enhance lipase production in Bacillus subtilis”, J Ind Microbiol Biotechnol 46(8):1091–1101 (2019). https://doi.org/10.1007/s10295-019-02205-2

[44]. Çalık, P., Bilir, E., Çalık, G., et al. “Influence of pH conditions on metabolic regulations in serine alkaline protease production by Bacillus licheniformis”, Enzyme Microb Technol 31(5):685-697 (2002). https://doi.org/10.1016/S0141-0229(02)00162-X

Tables Captions:

Table 1. Different quenching-extraction solutions for *B. subtilis* metabolites analysis.

Table 2: The RSM experiment design

Table 3: The ANOVA in Box–Behnken regression model.

Table 1. Different quenching-extraction solutions for *B. subtilis* metabolites analysis.

Quenching-extraction solutions	Percentage in WFI (*v/v*)	Temperature (°C)
CE	75	– 40 ± 1
CM	40	
CMAC	40:40:20	

Table 2: The RSM experiment design

Variables	Experiment levels (mM)		
G6P	0.25	0.50	1.00
F6P	0.04	0.05	0.06
Serine	0.5	1.5	2.5
Glycine	0.05	0.10	0.15
Threonine	0.10	0.30	0.50

Table 3: The ANOVA in Box–Behnken regression model.

Parameter	Quadratic sum	Degree of freedom	Mean square	*F*-value	*p*-value
Models	2.03×10^6	19	1.401×10^5	12.98	< 0.002
G6P	13989.00	1	14291.00	1.16	0.0124
F6P	76008.33	1	76008.33	0.86	0.0179
Serine	4699.97	1	4699.97	7.05	0.0094
Glycine	7401.01	1	7401.01	5.48	0.0189
Threonine	56721.05	1	56721.05	0.51	0.1560
G6P × F6P	35010.31	1	35010.31	3.31	0.0892
G6P × Serine	62008.03	1	62008.03	6.02	0.0312
G6P × Glycine	2.34	1	2.34	2.097×10^{-4}	0.8961
G6P × Threonine	854.69	1	854.69	0.47	0.3214
F6P × Serine	370.00	1	370.00	0.041	0.9012
F6P × Glycine	14998.75	1	14998.75	1.36	0.2604
F6P × Threonine					
Serine × Glycine	9287.19	1	9278.19	0.91	0.3701
Serine × Threonine	1804.78	1	1804.78	1.25	0.5238
$G6P^2$	3.587×10^4	1	3.587×10^4	29.84	0.0014
$F6P^2$	6.942×10^4	1	6.942×10^4	59.73	0.0019
$Serine^2$	7.985×10^4	1	7.985×10^4	68.58	0.0013
$Glycine^2$	7.013×10^4	1	7.013×10^4	66.47	0.0017
$Threonine^2$	2.842×10^4	1	4.620×10^4	46.44	0.1289
Residual	1.391×10^4	19	10603.24		
Lack of fit	91004.02	16	9102.41	0.59	0.761
Pure error	58324.89	3	14455.30		
Cor. total	1.985×10^5	38			
R^2	0.9478				
adj-R^2	0.8870				
coefficient of variation %	7.23				

Figures captions:

Fig. 1: Workflow for sample processing: Quenching metabolism and extracting intracellular metabolites.

Fig. 2: Different quenching- extraction solutions effect on AEC ratio of *B. subtilis.*

Fig. 3: The percentage of RSD of quantified metabolites for different quenching-extraction solutions (sample replicates, n = 5)

Fig. 4: Intracellular metabolites of *B.subtilis* culturing in minimal media with 4.5 g/L of glucose. deoxyadenosine triphosphate (dATP), cytidine triphosphate (dCTP), cytidine triphosphate (CTP), inosine triphosphate (ITP), deoxyinosine triphosphate (dITP), deoxythymidine triphosphate (dTTP), uridine diphosphate (UDP), guanosine diphosphate (GDP), cytidine diphosphate (dCDP), and deoxyadenosine diphosphate (dADP).

Fig. 5: The effect of (A) serine and G6P concentration and (B) F6P and G6P concentration on SAP activity using RSM.

Fig 6. The growth curve and enzyme activity curves of *B. subtilis*, (sample replicates, n = 3).

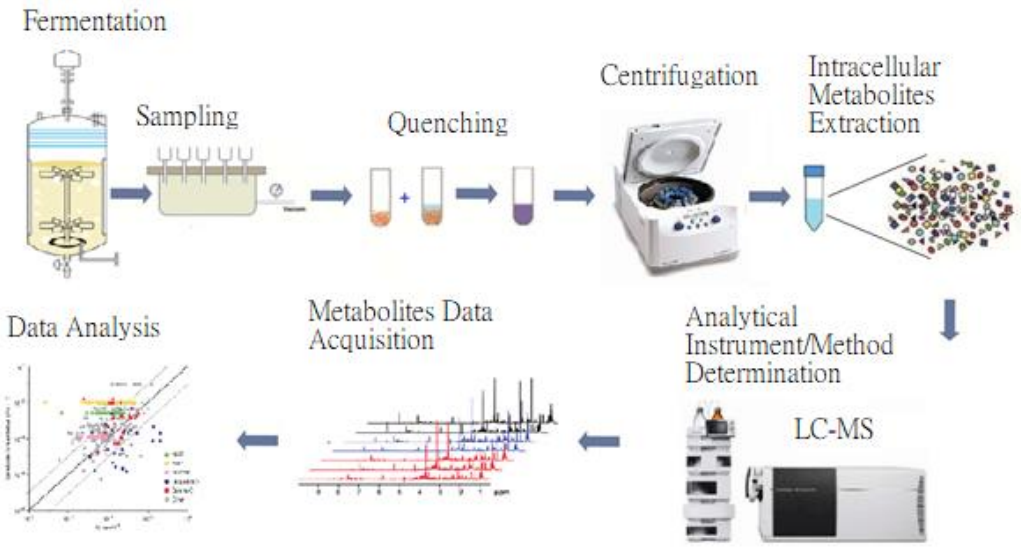

Fig. 1: Workflow for sample processing: Quenching metabolism and extracting intracellular metabolites.

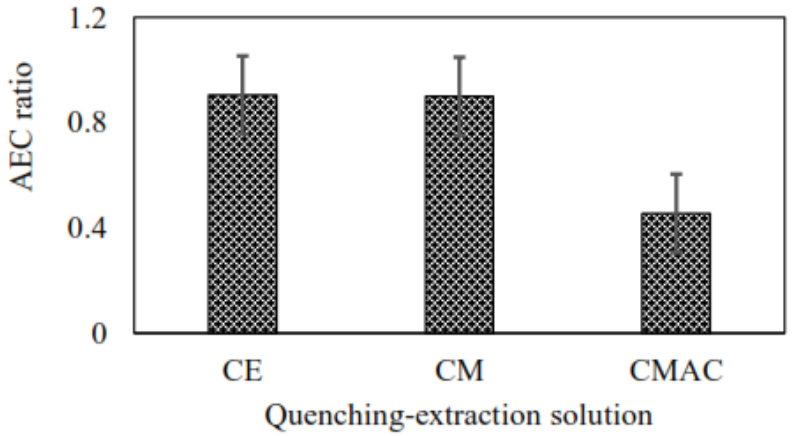

Fig. 2: Different quenching- extraction solutions effect on AEC ratio of *B. subtilis.*

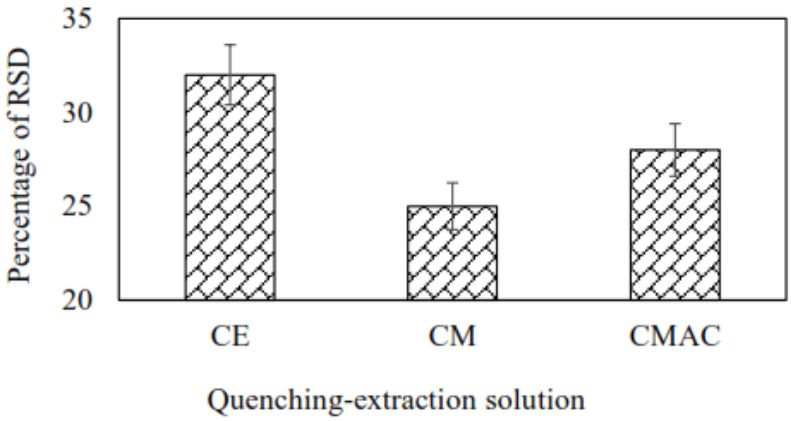

Fig. 3: The percentage of RSD of quantified metabolites for different quenching-extraction solutions (sample replicates, n = 5)

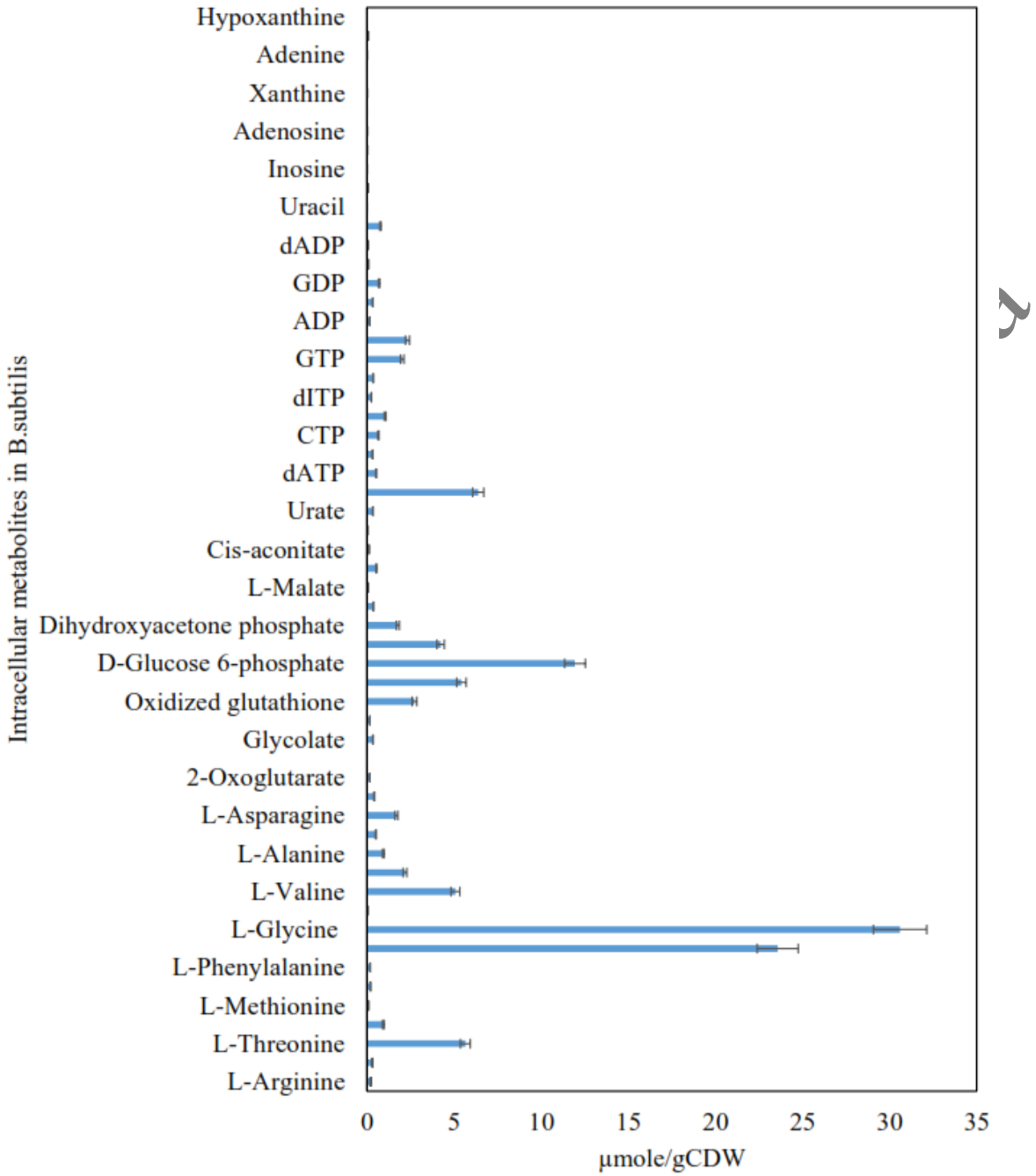

Fig. 4: Intracellular metabolites of *B.subtilis* culturing in minimal media with 4.5 g/L of glucose. deoxyadenosine triphosphate (dATP), cytidine triphosphate (dCTP), cytidine triphosphate (CTP), inosine triphosphate (ITP), deoxyinosine triphosphate (dITP), deoxythymidine triphosphate (dTTP), uridine diphosphate (UDP), guanosine diphosphate (GDP), cytidine diphosphate (dCDP), and deoxyadenosine diphosphate (dADP).

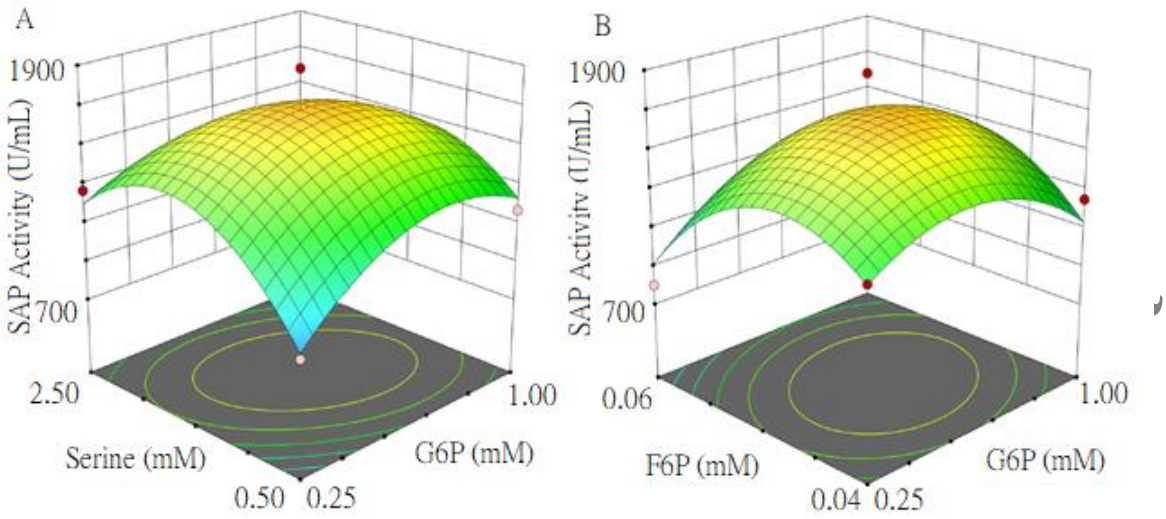

Fig. 5: The effect of (A) serine and G6P concentration and (B) F6P and G6P concentration on SAP activity using RSM.

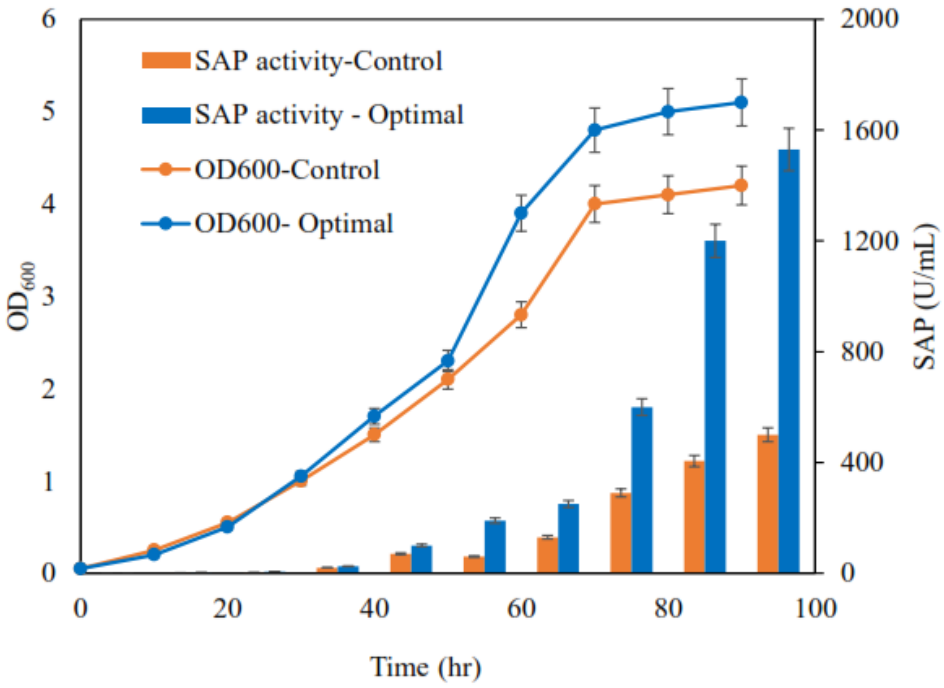

Fig 6. The growth curve and enzyme activity curves of *B. subtilis*, (sample replicates, n = 3).