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Amino acids production using pineapple plant stem by optimised one-step fermentation

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Abstract

Background The surge in global pineapple production has led to an excess of waste, demanding a sustainable approach for bioconversion. Despite its substantial volume, pineapple plant stems remain largely neglected, often discarded as on-farm waste. These stems, composed of intricate structures, necessitate a multi-step process for effective bioconversion. A promising alternative involves a single-step approach using microorganisms to combine hydrolysis and fermentation processes, yielding significant amino acid production from pineapple plant stems. This is aligned with Sustainable Development Goals 13 in reducing carbon dioxide and greenhouse gas emissions from traditional waste disposal methods.

Results The utilisation of *Bacillus subtilis* ATCC 6051 for amino acid production demonstrated success, yielding 1.28 mg/mL of total free amino acids with a remarkable 67.13 mg/g yield. This represents a 13% increase in concentration and a 12% boost in yield compared to commercial starch. The study underscores the pivotal role of medium composition, highlighting the significance of pineapple plant stems as a substrate and other key components to enhance amino acid production.

Conclusion Notably, the study achieved a substantial improvement in total amino acids production, reaching 9.57 mg/mL with a yield of 423.97 mg/g—an impressive 6.32-fold increment. This emphasises the enhanced potential of pineapple plant stems as a valuable resource for amino acid production, shedding light on the importance of optimising medium composition for maximum yield.

Keywords Pineapple plant stem, One-step fermentation process, Amino acids production, *Bacillus subtilis*, Optimisation

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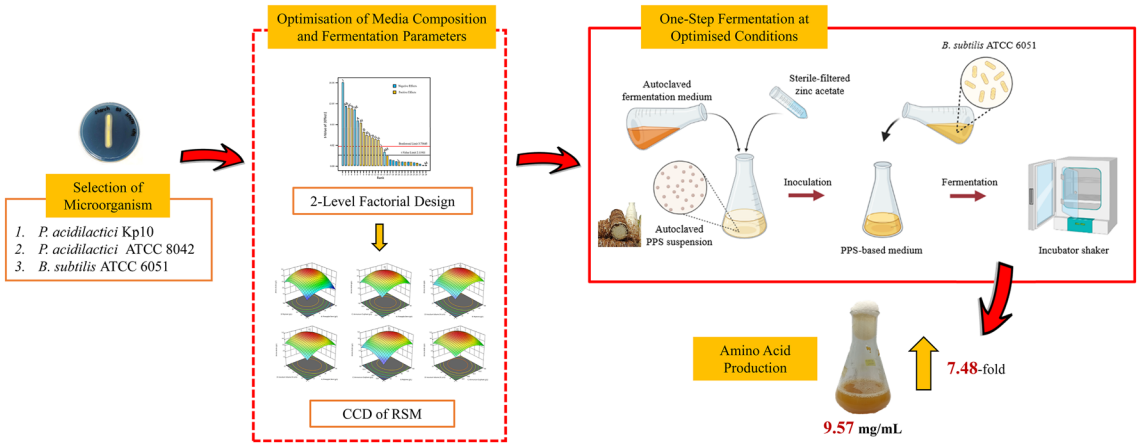
Graphical Abstract



Background

Due to the continuous increase in the production of pineapples worldwide, particularly in Asian countries, significant agricultural biomass was disposed of as waste. As much as 80% of the pineapple production was discarded [1]. This issue demands urgent attention and innovative solutions, especially in light of environmental concerns. Rising carbon dioxide emissions from biomass combustion contribute to deteriorating air quality, causing more respiratory diseases, increased healthcare demand, and, consequently, increased government health sector expenditure [2]. In 2021, the global pineapples production has reached 28 million tonnes. Malaysia alone produced 323 thousand tonnes of pineapples, yielding 258 thousand tonnes of waste. Inadequate disposal practices like dumping and open burning pose environmental risks. Concerning this, the valorisation of biomass should be explored. Despite its significant content, pineapple plant stem (PPS) comprising 78% (w/w) starch, 12% (w/w) protein, 8% (w/w) crude fibre, and 2% (w/w) nitrogen on a dry basis, remains underutilised [3]. The fermentable sugars in PPS make it a promising feedstock for producing valuable products like amino acids.

The one-step fermentation of amino acids from pineapple plant stem (PPS) offers an environmentally friendly and economically viable solution, aligning with Sustainable Development Goals (SDGs). Repurposing PPS for amino acid production addresses SDG 13 (Climate Action) by reducing greenhouse gas emissions from traditional waste disposal methods. Globally, this approach mitigates combustion of approximately 5.8 million tonnes of pineapple plant stem annually, cutting carbon dioxide and greenhouse gas emissions. Additionally, this leads to reduction of health expenditure which costs 4.3% Gross Domestic Product (GDP) in Malaysia, equivalent to RM 64.3 billion [4]. This innovative waste valorisation strategy serves as a sustainable alternative to open burning and effectively curbing carbon dioxide emissions. This effort can also aid Malaysia fulfilling its commitment to the Paris Agreement's to achieve a 45% reduction in GHG emissions by 2030 [5].

The vast applications of amino acids in various industries, such as animal feed, cosmetics, food, agriculture, and polymer materials, have increased their demand as well as production [6]. While glucose is the primary carbon source for amino acid production, attention is shifting to low-cost substrates like starch and lignocellulose to reduce manufacturing costs [7]. Current fermentation processes from biomass involve gelatinisation, enzymatic hydrolysis of starch, and subsequent fermentation, but are time-consuming and costly due to the need for expensive commercial enzymes [8].

Selecting an efficient amino acid producer is crucial for biomass utilisation and production. The common microorganisms for industrial amino acid application are *Corynebacterium glutamicum* [7] and *Escherichia coli* [9]. Tateno et al. [10] showed wild-type *C. glutamicum* unable to grow with soluble starch as sole carbon source. Integrating enzyme production, hydrolysis, and fermentation into one step is gaining interest for cost reduction and time efficiency. *B. subtilis* is a promising candidate, widely characterised, generally regarded as

safe, adaptable to wide range of temperatures, fermenting various carbohydrates, having low-nutrient requirements, and efficiently utilising starch [11]. It produces essential enzymes like alpha-amylase [12], cellulase, protease [13], and pullulanase [14]. Notably, *B. subtilis* also serve as valuable feed additive, benefiting the growth of broiler chickens [15] and piglets [16]. To date, limited research exists on one-step fermentation for amino acid production from agricultural biomass, and comprehensive research on amino acid production by *Bacillus subtilis* ATCC 6051 is notably absent.

Agricultural waste can be a cost-effective and sustainable feedstock for bioproduction, replacing processed sugar. Enzyme production, hydrolysis and fermentation may take place at different optimum conditions [17]. Challenges in one-step fermentation from PPS include low hydrolytic efficiency and amino acid yield and addressing these require optimising media selection and fermentation parameters. In this context, the application of response surface methodology (RSM) emerges as a highly promising tool. This study seeks to make a meaningful contribution to planetary health and the advancement of sustainable agriculture with a specific focus on the underexplored potential of pineapple plant stems in the optimised one-step fermentation of amino acids. Therefore, this study is aimed to use pineapple plant stem for one-step amino acids fermentation, offering a dual benefit of waste valorisation and environmental impact reduction. Apart from that, the determination of media composition conducted in this study aimed to establish the significant relationship in the production of amino acids from PPS.

Materials and methods

Sample collection and preparation

Pineapple plant stem (PPS) was collected from Ladang Nanas Dengkil, Selangor, Malaysia. The leaves were removed completely and the stem was chopped into smaller sizes. It was then rinsed and dried in an oven (Memmert GmbH + Co. KG, Germany) at 60 °C for 48 h. Next, the dried PPS was ground into powder form using a grinder (Philips, China). The powdered PPS was further sieved using a sifter and stored at room temperature.

Inoculum preparation

Three wild-type bacteria strains were selected for the screening of amino acid production through CBP, namely *Pediococcus acidilactici* Kp10, *P. acidilactici* ATCC 8042, and *B. subtilis* ATCC 6051. *P. acidilactici* Kp10 and *P. acidilactici* ATCC 8042 employed in this study were obtained from the culture collection of Professor Dr Arbakariya Ariff [18] and culture lab, respectively, from the Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia. *B. subtilis* ATCC 6051 was purchased from American Type Culture Collection (ATCC). For the routine growth of *P. acidilactici*, de Man Rogosa Sharpe (MRS) medium at pH 7.0 was used. Subculturing was done using 1% (v/v) of the culture, incubated in an incubator shaker (Mecasys Co., Ltd, Korea) at 37 °C, 150 rpm for 24 h. Nutrient broth was used for the routine growth of *B. subtilis* ATCC 6051, which consisted of peptone (0.5% w/v) and meat extract (0.3% w/v) with pH of the medium adjusted to pH 7.0. Subculturing was done using 1% (v/v) of the culture, incubated in an incubator shaker at 30 °C, 150 rpm for 24 h.

Primary screening of bacteria strain

Preliminary screening on amino acids producers were done based on several factors, including the ability to produce amylase as well as amino acids. Three selected microorganisms were subjected to these preliminary screening factors.

Amylase-producing bacteria

Starch hydrolysis assay was conducted as described by Abbasiliasi et al. [19] with modification using growth medium supplemented with 1% (w/v) soluble starch. After incubation, the clear halo zone formation around the colony upon flooding with Gram's iodine, consisting of 0.2% (w/v) I_2 and 2.0% (w/v) KI, indicated the ability of the bacteria strain to produce extracellular amylase. Starch hydrolysis ratio (SHR) was calculated as shown in Eq. 1:

$$\mathrm{SHR} = \frac{\text{Clear halo zone diameter (mm)}}{\text{Colony growth diameter (mm)}}. \quad (1)$$

Amino acids-producing bacteria

The bacteria strains were allowed to grow on a growth medium supplemented with 20.00 g/L glucose and incubated in an incubator shaker (Mecasys Co., Ltd, Korea) at 37 °C and 150 rpm for 24 h. After incubation, ninhydrin analysis was conducted using the cell-free supernatant for the determination of amino acids produced by the bacteria strains. The bacteria selected was then used for further study.

Batch production of amino acid in shake flask

B. subtilis ATCC 6051 was selected for further study. Preculture of the bacteria was conducted at 30 °C and 150 rpm for 24 h. A 10% (v/v) of the precultured strain was transferred into production medium in a 250-mL shake flask with 100 mL working volume consisting of nutrient broth supplemented with 20.00 g/L PPS.

Incubation was conducted at 30 °C for 72 h at 150 rpm using an incubator shaker (Mecasys Co., Ltd, Korea). Sampling was done at 4-h intervals in triplicates, followed by centrifugation at 10,000 rpm for 5 min using a microcentrifuge (Sorvall Legend Micro 17, Thermo Fisher Scientific, Germany) to obtain the cell-free supernatant for reducing sugars, residual starch, and amino acids analysis.

Figure 1 illustrates the production of amino acid in PPS-based medium using one-step fermentation. Excess distilled water was added into the flask containing PPS before subjected to autoclave. Both PPS suspension and medium were autoclaved separately and subjected to sterilisation at 121 °C for 20 min at 15 psi. The autoclave medium was then added aseptically into the PPS suspension, followed by inoculation and fermentation.

Experimental design and parameters optimisation

The influences of various media compositions and process parameters were studied to optimise the production of amino acids from PPS by *B. subtilis* ATCC 6051 through one-step fermentation. Screening and optimisation of the variables were conducted using a 2^{14-9} factorial design of RSM. The results were further analysed using statistical analysis to obtain the prediction model for the study of interactions between the factors and optimum independent parameters for maximum amino acid production.

2-Level factorial design

Fourteen factors were selected as the variable components to determine the influence of media compositions and various process parameters on amino acids production from PPS as shown in Table 1. For thiamine hydrochloride, calcium chloride, and zinc acetate, filter sterilisation was conducted on a separate solution before it was added to the autoclaved basal medium.

Table 1 Range of variables at different coded levels for the 2^{14-9} factorial design on amino acids production

Code (X_i)	Variables	Levels of variables		
		−1	0	+1
Coded levels of the independent variables for medium components				
X_1	PPS (g/L)	1.0	20.5	40.0
X_2	Peptone (g/L)	1.0	5.5	10.0
X_3	Yeast extract (g/L)	1.0	5.5	10.0
X_4	Ammonium sulphate (g/L)	0.0	20.0	40.0
X_5	Ammonium chloride (g/L)	0.0	20.0	40.0
X_6	Magnesium sulphate heptahydrate (g/L)	0.0	2.5	5.0
X_7	Calcium carbonate (g/L)	0.0	5.0	10.0
X_8	Manganese (II) sulphate monohydrate (g/L)	0.0	1.0	2.0
X_9	Thiamine hydrochloride (mg/L)	0.0	5.0	10.0
X_{10}	Calcium chloride (mg/L)	0.0	5.0	10.0
X_{11}	Zinc acetate (mg/L)	0.0	5.0	10.0
Coded levels of the independent variables for process parameters				
X_{12}	Temperature (°C)	30.0	40.0	50.0
X_{13}	Rotational speed (rpm)	50.0	115.0	180.0
X_{14}	Inoculum volume (% v/v)	1.0	8.0	15.0

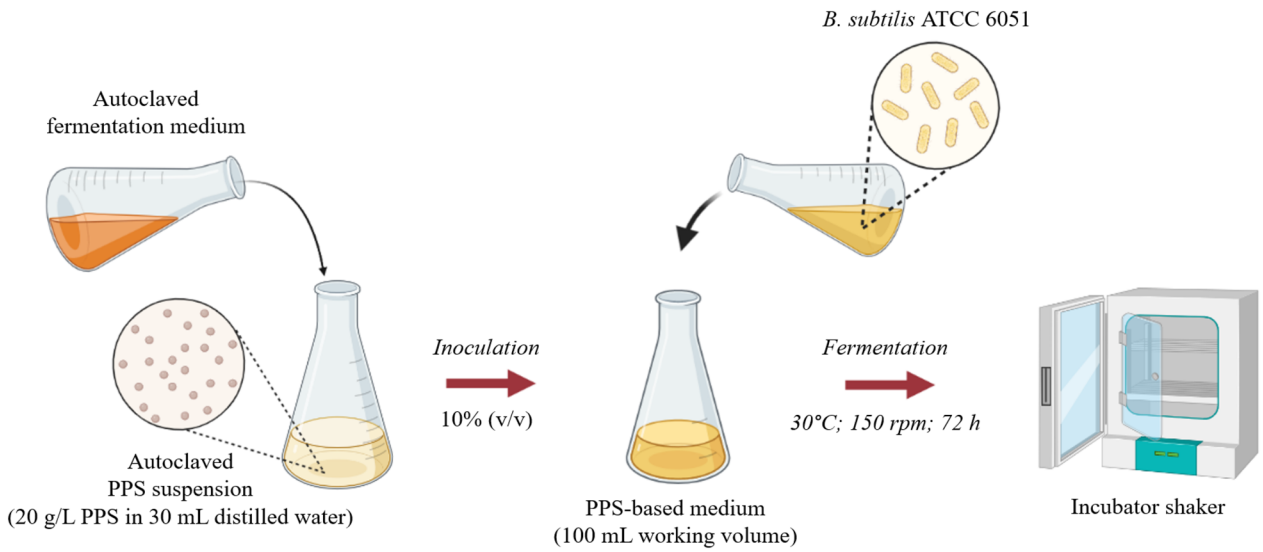


Fig. 1 Fermentation of amino acid from pineapple plant stem using one-step fermentation

By the experimental matrix generated by the software, 34 experiments were performed in total including 4 centre points. The samples were then analysed for amino acid production after 48 h of incubation using the ninhydrin method and the results are generated by the software for the significant effects. The significant parameters with greater effects on the amino acids production through CBP were then further optimised using CCD for optimum production.

Central composite design (CCD)

A face-centred central composite design (CCD) was employed to determine the influences of media compositions and various process parameters on the one-step fermentation of amino acids from PPS. The important factors screened by 2^{14-9} factorial design were given as input to CCD in the optimisation of amino acid production. Fermentation was conducted at its optimised condition based on the optimisation result using 2-level factorial design. The effects of four selected parameters, namely A: PPS (X_1), B: peptone (X_2), C: ammonium sulphate (X_3), and D: inoculum volume (X_4) on amino acids production were investigated using a 2^3 face-centred design, coded as $-1, 0, +1$, in which the number of α is 1.000. The minimum and maximum level for PPS, peptone, ammonium sulphate, and inoculum volume were set at 0.50–40.00 g/L, 1.00–160.00 g/L, 1.00–160.00 g/L, and 5.00–20.00% (v/v), respectively.

Amino acid production was selected as the response variable (Y). A total of 30 experiments in triplicates were performed based on the experimental design matrix generated from CCD, with four independent variables at three levels for each variable, including 6 centre points (for estimation of pure error sum of squares), and 24 factorial points. The run order of the experiments was randomised to avoid systematic errors. The samples were then analysed for amino acid production using the ninhydrin analysis. The results were then fitted to the regression model equation and analysed using the software to obtain ANOVA, multivariant analysis, and contour plot with the 3D surface.

Amino acid production from pineapple plant stem at optimised condition

The optimised conditions based on optimisation of amino acid production using CCD was selected for method validation. The production medium as determined based on the optimised conditions consisted of 22.57 g/L PPS, 108.70 g/L peptone, 95.23 g/L ammonium sulphate, yeast extract (1.31 g/L), $MgSO_4.7H_2O$ (4.83 g/L), $MnSO_4.4H_2O$ (2.00 g/L), and zinc acetate (8.72 mg/L). Inoculum volume was set at 14.04% (v/v) and fermentation was conducted at 30 °C and 180 rpm for 48 h using an incubator shaker (Mecasys Co., Ltd, Korea).

Analytical methods

Ninhydrin solution was prepared by dissolving 0.35% (w/v) ninhydrin in 95% (v/v) ethanol and the mixture was stored in an amber Schott bottle. A ninhydrin test was conducted for routine determination of total amino acids as described by Marathe et al. [20] with modifications by heating at 95 °C for 15 min and absorbance was read at 570 nm after incubation. Reducing sugar concentration was determined using the dinitrosalicylic acid (DNS) method as described by Miller [21].

The fermented samples were centrifuged at 10,000 rpm for 5 min using a microcentrifuge (Sorvall Legend Micro 17, Thermo Fisher Scientific, Germany). The sample pellet was used for cell concentration analysis through an optical density method. A 1 mL distilled water was introduced to the cell pellet and subjected to vortexing. Following this, the cells underwent a washing step with distilled water before being transferred to a plastic cuvette. The determination of cell density was conducted by measuring absorbance at 600 nm using a spectrophotometer (GENESYS 20, Thermo Scientific, US), employing distilled water as the reference blank.

The supernatant was filtered through a 0.22 µm nylon membrane filter into a sterile Eppendorf tube. The filtered sample was kept at −20 °C prior to HPLC analysis. The amino acids profile of the cell-free supernatant was performed at an accredited laboratory (UNIPEQ, UKM-MTDC). After acid hydrolysis, samples were analysed for amino acid content with ACCQ Tag Waters Method using HPLC equipped with a fluorescence detector. Amino acid yield and productivity were calculated as shown in Eqs. 2, 3, respectively:

$$\text{Amino acid yield (mg/g)} = \frac{\text{Concentration of amino acid produced (mg/mL)}}{\text{Amount of substrate (g/L)}} \times 1000, \tag{2}$$

$$\text{Amino acid productivity (mg/L/h)} = \frac{\text{Concentration of amino acid produced (mg/mL)}}{\text{Incubation time (h)}} \times 1000. \tag{3}$$

Statistical analysis

Statistical analysis of 2-level factorial design was analysed using the Design-Expert® version 13.0.5 Stat-Ease Inc., (Minneapolis, USA) software. SPSS Statistics software Version 28.0.0.0 and Microsoft Excel 2010 were used for the statistical analysis of the data, with the average three independent replicates presented as mean value ± standard deviation. One-way analysis of variance (ANOVA), Tukey HSD and Duncan's post hoc test were used for the determination of significant differences ($p < 0.05$) between different samples.

Results and discussion

Batch production of amino acids

Based on the primary screening for amylase-producing microorganisms, both *P. acidilactici* Kp10 and *B. subtilis* ATCC 6051 exhibited positive effects on the extracellular amylase activity. *B. subtilis* ATCC 6051 is determined to be a superior producer of amylase, with high SHR (27.33 ± 2.31). *P. acidilactici* Kp10 also showed a positive result, although lower SHR (1.00 ± 0.00) was obtained. Generally, the size of SHR is directly proportional to the amylase production by the microorganism. This suggests that the bacterial strain can produce extracellular amylase, thus allowing it to hydrolyse starch. Further screening of microorganisms was conducted to determine the strains with amino acid producing ability. When tested with ninhydrin assay, only *P. acidilactici* Kp10 and *B. subtilis* ATCC 6051 showed positive result for the production of amino acids after fermentation. In term of concentration, *B. subtilis* ATCC 6051 (280.83 ± 12.35 mg/L) produced higher amount of amino acid compared to *P. acidilactici* Kp10 (35.86 ± 5.52 mg/L). Based on the results obtained in the primary screening phase, *B. subtilis* ATCC 6051 was the most promising strain to further employed in the one-step fermentation of amino acid. This is due to its ability to hydrolyse starch and the ability to produce amino acids.

Production of amino acids from pineapple plant stem

To gain insight into the feasibility of one-step fermentation in amino acids production by *B. subtilis* ATCC 6051, changes in the concentration of starch and reducing sugar were studied as illustrated in Fig. 2. Soluble starch was used as the control in this study. The term one-step fermentation in this study is referring to consolidated bioprocessing approach in which the enzyme production, enzymatic hydrolysis and saccharification, and amino acid fermentation is carried out in a one-step process using *B. subtilis* ATCC 6051. The enhanced one-step fermentation refers to the optimised medium composition and fermentation parameters of the amino acid production.

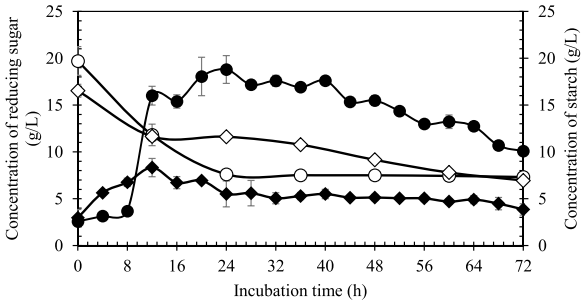


Fig. 2 One-step fermentation of amino acids using PPS (*diamond*) and soluble starch (*circle*) as carbon sources. Changes in the concentration of reducing sugar (*closed symbols*) and concentration of starch (*open symbols*). Error bars indicate the mean value ± standard deviation of triplicates of the sample

Figure 2 shows higher starch hydrolysis in soluble starch-based (63%-consumed) compared to PPS-based (58%-consumed) fermentation. The results were slightly higher than Tanimura et al. [22], where 40–50% of the starch remained unutilised. After 12 h of fermentation, a sheer drop was observed in residual starch concentration, whereas the reducing sugars increased abruptly in both cases. This indicates starch hydrolysis by the amylase produced by *B. subtilis* ATCC 6051. Soluble starch-based fermentation produced more reducing sugar at 12 h, while PPS-based fermentation yielded less reducing sugars due to its complex structure with cellulose, hemicellulose, protein, and other components hindering enzymatic hydrolysis. Starch hydrolysis plateaued after 60 h of fermentation with both fermentations having similar residual starch after 72 h (7.31 ± 0.48 g/L for soluble starch-based and 6.94 ± 0.22 g/L for PPS-based).

To determine the behaviour of reducing sugar released from starch and other components of the PPS, the glucose concentration was also monitored. In soluble starch-based fermentation, reducing sugar concentration was 10.47 g/L higher than in PPS-based fermentation. Soluble starch fermentation consumed 16.23 g/L reducing sugar, higher than the 5.35 g/L consumed in PPS-based fermentation. This suggests that reducing sugar, mainly glucose, was consumed by *B. subtilis* ATCC 6051 for fermentation while being simultaneously produced from enzymatic hydrolysis. However, in the PPS-based fermentation, the reducing sugars produced may be the result of enzymatic hydrolysis from starch, cellulose, and hemicellulose components of the PPS. The results were in agreement with previous studies [12, 23] reporting *B. subtilis* producing multiple extracellular enzymes, including amylase, cellulase and pullulanase.

The total amino acid produced was quantified with ninhydrin solution for preliminary determination and the result revealed that 0.15 g/L of amino acid was produced

Table 2 The maximum amino acids produced from PPS and starch by *B. subtilis* ATCC 6051 through one-step fermentation

Amino acid	Substrate						
	PPS				Soluble starch		
	Amount (mg/mL)	Time (h)	Yield (mg/g)	Productivity (mg/L/h)	Amount (mg/mL)	Yield (mg/g)	Productivity (mg/L/h)
Threonine	0.02^{ab}	24	1.00^{c}	0.83^{e}	0.00^{a}	0.00^{a}	0.00^{a}
Valine	0.06^{cd}	72	3.01^{f}	0.84^{e}	0.00^{a}	0.00^{a}	0.00^{a}
Methionine	0.02^{ab}	72	1.00^{c}	0.28^{c}	0.03^{a}	1.50^{ab}	0.63^{b}
Lysine	0.01^{a}	48	0.50^{b}	0.21^{b}	0.53^{c}	26.55^{b}	11.06^{d}
Isoleucine	0.10^{e}	72	5.01^{h}	1.39^{g}	0.00^{a}	0.00^{a}	0.00^{a}
Leucine	0.05^{bcd}	24	2.51^{e}	2.09^{j}	0.00^{a}	0.00^{a}	0.00^{a}
Phenylalanine	0.03^{abc}	48	1.50^{d}	0.63^{d}	0.00^{a}	0.00^{a}	0.00^{a}
Aspartic acid	0.08^{de}	48	4.01^{g}	1.67^{h}	0.00^{a}	0.00^{a}	0.00^{a}
Serine	0.14^{f}	24	7.01^{i}	5.85^{j}	0.00^{a}	0.00^{a}	0.00^{a}
Glutamic Acid	0.38^{h}	48	19.04^{k}	7.93^{l}	0.00^{a}	0.00^{a}	0.00^{a}
Glycine	0.02^{ab}	24	1.00^{c}	0.84^{e}	0.00^{a}	0.00^{a}	0.00^{a}
Alanine	0.38^{h}	48	19.04^{k}	7.93^{l}	0.03^{a}	1.50^{a}	0.63^{b}
Proline	0.18^{g}	24	9.02^{j}	7.52^{k}	0.00^{a}	0.00^{a}	0.00^{a}
Tyrosine	0.06^{cd}	48	3.01^{f}	1.25^{f}	0.10^{b}	5.01^{ab}	2.09^{c}

Superscripts with different letters are significantly different from each other within the same column at $p < 0.05$ using Tukey HSD post hoc test

in the PPS-based fermentation. Table 2 summarises the amino acid profiling for the PPS-based and soluble starch-based fermentation. The total amount of free amino acids produced from PPS-based fermentation was double that of soluble starch-based fermentation.

Soluble starch-based fermentation yielded higher production of essential amino acids, notably 0.53 mg/mL lysine and 0.03 mg/mL methionine. In contrast, PPS-based fermentation produced higher amounts of non-essential amino acids, particularly 0.38 mg/mL each of glutamic acid and alanine. Overall, it was noteworthy that fermentation using PPS as the substrate led to various essential and non-essential amino acids being produced, in which 14 out of 17 free amino acids were detected, with a total of maximum 1.28 mg/mL free amino acids produced after 24 h of fermentation. On the flip side, soluble starch-based fermentation only produced 4 out of 17 types of free amino acids, resulting in the total maximum amount of 0.69 mg/mL free amino acids being produced.

The results obtained in this section have illustrated the feasibility of *B. subtilis* ATCC 6051 in one-step fermentation of amino acids production. The higher total free amino acids, in which 85.5% in concentration and 94.2% in yield, was obtained from PPS as compared to commercial starch. This situation might be due to the complex nutrient component of the PPS, since it also contained cellulosic and protein components that contributed to the higher amino acid production. The ability of *B. subtilis* ATCC 6051 to produce amylase, cellulase, and protease may help in utilising these components of the PPS for amino acids production.

Determination of media composition effect on the production of amino acids from pineapple plant stem

A two-level factorial design (2^{14-9}) was used to screen for the significant effects of 14 variables in 34 runs and the Pareto chart is illustrated in Fig. 3 for the main effects on amino acids production from PPS by *B. subtilis* ATCC 6051. A total of 14 factors were studied, with 11 factors on medium components and 3 for fermentation conditions (temperature, rotational speed, and inoculum volume). Screening of the media components included 1 substrate (PPS); 2 organic nitrogen sources (peptone and yeast extract); 2 inorganic nitrogen sources (ammonium sulphate, ammonium chloride); 5 mineral sources (magnesium sulphate heptahydrate, calcium carbonate, manganese (II) sulphate tetrahydrate, calcium chloride, zinc acetate) and 1 vitamin (thiamine hydrochloride). The selection of the medium components was conducted by referring to the published reports on the common components used in amino acid production.

The significant variables were determined from the Pareto chart based on the Bonferroni limit (3.756) and t-value limit (2.119). The effects of factors that extend beyond the Bonferroni indicate that they are extremely significant factors, and the effects with values in between the Bonferroni limit and the t-value limit

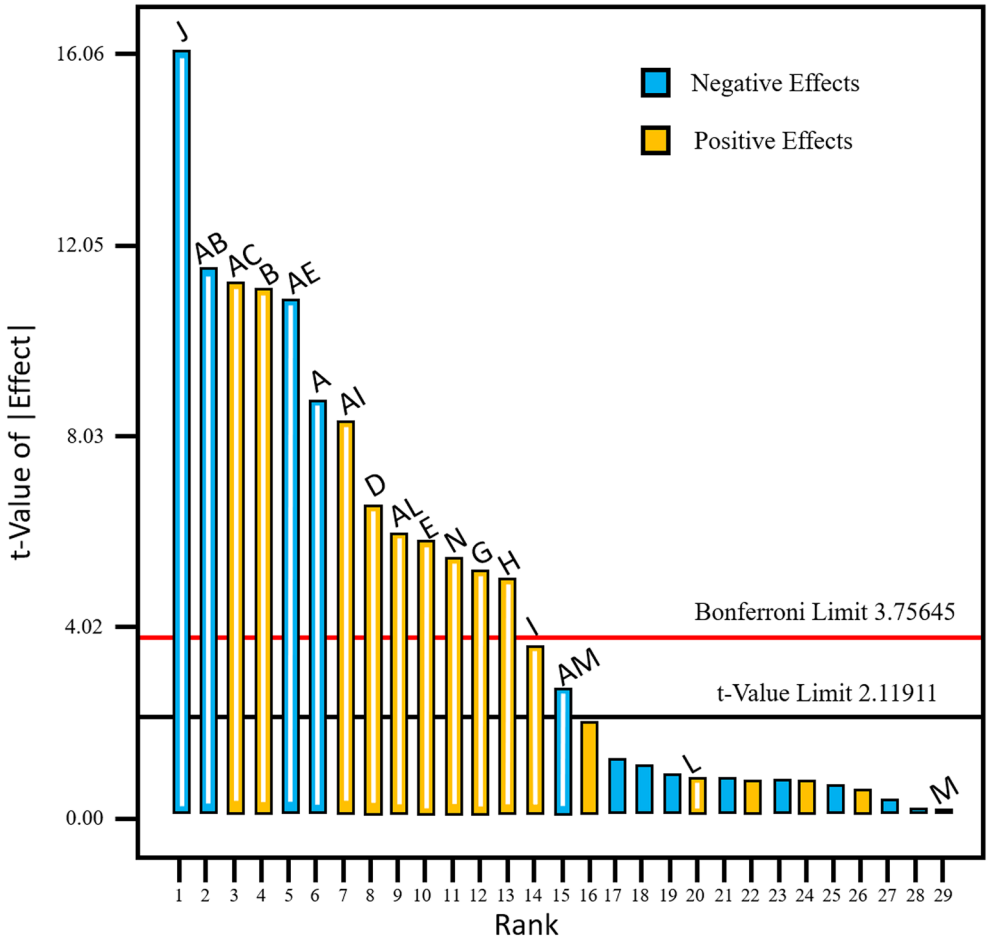


Fig. 3 Pareto charts of main effects for 2^{14-9} experimental design on amino acids production. A: PPS; B: peptone; C: yeast extract; D: $(NH_4)_2SO_4$; E: NH_4Cl; F: $MgSO_4.7H_2O$; G: $CaCO_3$; H: $MnSO_4.4H_2O$; I: thiamine HCl; J: $CaC1_2$; K: $Zn(CH_3CO_2)_2$; L: temperature; M: rotational speed; N: inoculum volume

are significant and possibly important for the model. In contrast, the factors with effects lower than the t-value limit imply that the effect was not significant and can only be selected to support the model hierarchy [24]. The significance of the variables was determined as a *p*-value less than 0.05. Backward elimination analysis was selected to reduce the insignificant terms, thus resulting in the overall significant but reduced quadratic model. Verification of the statistical model was then conducted using ANOVA as summarised in Table 3.

The Fisher variation (F-value) of the model was 60.87, which implies that the model was significant and there was only a 0.01% chance that the F-value could occur on account of noise. *p*-values less than 0.0500 suggested that the model terms are significant. In this case, A, B, D, E, G, H, I, J, N, AB, AE, AG, AI, AL, and AM were significant model terms. The coefficient of determination (R^2) obtained from this study was 0.9848, which demonstrated that it was a very good fit for the model estimation. The predicted R^2 (0.9131) was in reasonable agreement with the adjusted R^2 (0.9686), in which the difference was less than 0.2. Adequate precision of 36.806 suggested that the model can be used to navigate the design space as it measures the signal-to-noise ratio. This value of adequate precision stipulated an adequate signal as a ratio more than 4 is desirable. The model showed a non-significant lack-of-fit with a *p*-value of 0.1800, indicating their insignificance relative to pure error and there was only 18.00% chance this could occur due to noise.

The variables were further optimised by selecting the four most significant variables obtained from the screening analysis. Based on the Pareto chart, four variables, namely PPS, peptone, ammonium sulphate, and inoculum volume were selected for further optimisation. Although J has the most significant result, however, it exerted negative effects on amino acid production. Therefore, it was not selected for further analysis. In the one-step fermentation of amino acids, PPS not only acts as the carbon source, but also organic nitrogen source as it

Table 3 Analysis of variance (ANOVA) of the two-level factorial design for the prediction of significant variables for amino acid production

Source	Sum of squares	Degree of freedom (*F*)	Mean square	*F*-value	*p*-value Prob > *F*	Comment
Model	37.42	17	2.20	60.87	< 0.0001	Significant
A-PPS	2.82	1	2.82	78.06	< 0.0001	Significant
B-Peptone	4.61	1	4.61	127.40	< 0.0001	Significant
D-$(NH_4)_2SO_4$	1.56	1	1.56	43.01	< 0.0001	Significant
E-NH_4Cl	1.25	1	1.25	34.46	< 0.0001	Significant
G-$CaCO_3$	0.9880	1	0.9880	27.32	< 0.0001	Significant
H-$MnSO_4.4H_2O$	0.9210	1	0.9210	25.47	0.0001	Significant
I-Thiamine HCl	0.4636	1	0.4636	12.82	0.0025	Significant
J-$CaC1_2$	9.58	1	9.58	264.84	< 0.0001	Significant
L-Temperature	0.0205	1	0.0205	0.5676	0.4622	
M-Rotational Speed	0.0003	1	0.0003	0.0095	0.9236	
N-Inoculum volume	1.10	1	1.10	30.29	< 0.0001	Significant
AB	4.91	1	4.91	135.69	< 0.0001	Significant
AE	4.35	1	4.35	120.40	< 0.0001	Significant
AG	4.66	1	4.66	128.88	< 0.0001	Significant
AI	2.55	1	2.55	70.56	< 0.0001	Significant
AL	1.31	1	1.31	36.31	< 0.0001	Significant
AM	0.2632	1	0.2632	7.28	0.0158	Significant
Residual	0.5786	16	0.0362			
Lack of fit	0.5404	13	0.0416	3.26	0.1800	Not significant
Pure error	0.0383	3	0.0128			
Cor total	38.00	33				

Std. deviation: 0.1902; mean: 0.8677; coefficient of variation %: 21.92; R^2: 0.9848; adjusted R^2: 0.9686; predicted R^2: 0.9131; adequate precision: 36.8061

consisted of 1.85% (w/w) of total nitrogen on a dry weight basis [3]. Apart from that, PPS also consists of 11.56% (w/w) protein, thus proteolysis may also take place during the fermentation, resulting in amino acid production. Furthermore, peptone as the organic nitrogen source also plays a significant role in amino acid production. In terms of inorganic nitrogen sources, both ammonium sulphate and ammonium chloride exerted significant effects on amino acid production. With respect to this, only the most significant factor was selected, whereby ammonium sulphate has a higher effect on the production. A face-centred CCD matrix with actual values along with observed amino acids concentration experimentally obtained from 30 statistically designed experiments is shown in Table 4.

The statistical analysis for the significance of all four factors conducted in this section was summarised by ANOVA as shown in Table 5. The model *F*-value obtained revealed that the model was significant, in which there was only a 0.01% chance that the *F*-value this large could occur due to noise. The study also revealed that the model terms were significant with a *p*-value less than 0.05 ($p < 0.0001$). In this aspect, all the linear coefficients (A, B, C, D) and quadratic coefficients (A^2, B^2, C^2, and D^2) were proved to be significant. Apart from that, the model terms AB, BD, and CD were also significant.

In order to illustrate the main and interactive effects of the variables, 3D quadratic response surface plots were drawn visually as depicted in Fig. 4. The graph was plotted with two factors at a time and the other factors were kept constant at their centre points. The 3D surface plots in this study shows a typical bell curve with a concave down graph, with its peak at the middle. This indicates that the maximum point of the amino acid concentration was located inside the experimental region.

The interaction between all the factors showed almost symmetrical bell-curve graph. In amino acids production, PPS not only acts as the carbon source, but also organic nitrogen source as it consisted of 1.85% (w/w) of total nitrogen on a dry weight basis [3]. Nitrogen plays a vital role in sustaining the physiological and biochemical functions of bacteria. Apart from that, PPS also consists of 11.56% (w/w) protein, thus proteolysis may also take place during the fermentation, contribute to higher amino acid production. Furthermore, peptone as the additional organic nitrogen source also plays a

Table 4 Design matrix and corresponding responses of CCD on amino acids production

Run order	*A* (g/L)	*B* (g/L)	*C* (g/L)	*D* (% (v/v))	Amino acids concentration (g/L)	
					Experimental	Predicted
1	20.25	160.00	80.50	12.50	3.64 ± 0.71^{ghijk}	3.68
2	20.25	80.50	80.50	12.50	1.61 ± 0.23^{abcde}	1.67
3	40.00	160.00	160.00	5.00	2.65 ± 0.63^{defghi}	2.55
4	40.00	80.50	80.50	12.50	1.90 ± 0.92^{abcdef}	1.97
5	20.25	80.50	80.50	5.00	1.49 ± 0.31^{abcde}	1.54
6	40.00	160.00	1.00	20.00	1.08 ± 0.27^{abcd}	0.99
7	20.25	80.50	160.00	12.50	2.54 ± 1.88^{cdefgh}	2.47
8	20.25	80.50	1.00	12.50	3.79 ± 1.56^{ghijk}	3.91
9	40.00	160.00	1.00	5.00	2.55 ± 1.81^{cdefgh}	2.62
10	0.50	160.00	160.00	20.00	4.14 ± 1.05^{hijk}	4.28
11	0.50	80.50	80.50	12.50	2.41 ± 0.27^{bcdefg}	2.45
12	0.50	160.00	1.00	5.00	0.76 ± 0.16^{a}	0.85
13	0.50	1.00	1.00	5.00	0.75 ± 0.01^{a}	0.65
14	20.25	80.50	80.50	12.50	1.39 ± 0.50^{abcde}	1.48
15	40.00	160.00	160.00	20.00	0.93 ± 0.08^{abc}	0.86
16	0.50	160.00	1.00	20.00	3.02 ± 0.07^{efghij}	3.09
17	0.50	160.00	160.00	5.00	0.81 ± 0.09^{ab}	0.88
18	40.00	1.00	1.00	20.00	4.24 ± 1.33^{ijk}	4.33
19	20.25	80.50	80.50	12.50	3.38 ± 0.34^{fghijk}	3.28
20	40.00	1.00	160.00	5.00	4.51 ± 0.07^{jk}	4.56
21	40.00	1.00	1.00	5.00	4.74 ± 0.85^{k}	4.56
22	0.50	1.00	160.00	5.00	3.83 ± 0.81^{ghijk}	3.86
23	20.25	80.50	80.50	12.50	4.61 ± 0.49^{jk}	4.56
24	40.00	1.00	160.00	20.00	4.68 ± 0.77^{jk}	4.56
25	20.25	80.50	80.50	20.00	3.49 ± 1.74^{fghijk}	3.53
26	0.50	1.00	1.00	20.00	4.63 ± 0.58^{jk}	4.56
27	20.25	1.00	80.50	12.50	1.18 ± 0.25^{abcd}	1.1
28	20.25	80.50	80.50	12.50	3.95 ± 0.14^{ghijk}	4.08
29	0.50	1.00	160.00	20.00	1.67 ± 0.78^{abcde}	1.58
30	20.25	80.50	80.50	12.50	4.73 ± 0.96^{k}	4.56

A: PPS; *B*: peptone; *C*: ammonium sulphate: *D*: inoculum volume

Superscripts with different letters are significantly different from each other within the same column at $p < 0.05$ using Duncan's post hoc test

significant role in amino acid production. This is supported by Lim et al. [25], whose study revealed that the addition of peptone at its optimum condition exerted a positive effect on threonine production. Inorganic nitrogen sources, specifically ammonium sulphate and ammonium chloride, significantly impact amino acid production. Ammonium sulphate, identified as the most significant factor, aligns with Anakwenze et al. [26] study on maximum methionine production by *Bacillus thuringiensis*. The aforementioned author also revealed that optimum inoculum led to increased methionine production. In addition, since methionine contains sulphur, elemental sulphur provided by ammonium sulphate in the medium was anticipated to enhance synthesis.

Yeast extract, known for increasing methionine concentration, also positively influenced amylase and protease production, aiding enzymatic hydrolysis. Zhou et al. [27] have also reported both yeast extract and $MgSO_4.7H_2O$ have significant effects in increasing methionine production. Furthermore, the significant effect of $MgSO_4.7H_2O$ on enhanced methionine production was in agreement with the study conducted by Venkata et al. [28]. Furthermore, Lim et al. [25] identified $MnSO_4$ as a key component positively affecting threonine production. Additionally, Zn^{2+} was reported to enhance lysine and methionine production in studies by Ekwealor and Obeta [29] and Anakwenze et al. [26], respectively.

Table 5 Analysis of variance (ANOVA) of the response surface quadratic model for amino acids concentration

Source	Sum of squares	Degree of freedom (*F*)	Mean square	*F*-value	*p*-value Prob > *F*	Comment
Model	57.9200	14	4.1400	247.2700	< 0.0001	Significant
A-PPS	0.6502	1	0.6502	38.8600	< 0.0001	Significant
B-Peptone	6.8600	1	6.8600	410.0200	< 0.0001	Significant
C-Ammonium sulphate	0.7101	1	0.7101	42.4400	< 0.0001	Significant
D-Inoculum volume	0.7766	1	0.7766	46.4100	< 0.0001	Significant
AB	0.7311	1	0.7311	43.6900	< 0.0001	Significant
AC	0.0069	1	0.0069	0.4115	0.5309	
AD	0.0591	1	0.0591	3.5300	0.0798	
BC	0.0554	1	0.0554	3.3100	0.0888	
BD	0.1464	1	0.1464	8.7500	0.0098	Significant
CD	0.4600	1	0.4600	27.4900	< 0.0001	Significant
A^2	1.8600	1	1.8600	111.0900	< 0.0001	Significant
B^2	1.8900	1	1.8900	112.9600	< 0.0001	Significant
C^2	1.2100	1	1.2100	72.6000	< 0.0001	Significant
D^2	0.6340	1	0.6340	37.8900	< 0.0001	Significant
Residual	0.2510	15	0.0167			Significant
Lack of fit	0.2128	10	0.0213	2.78	0.1349	Not significant
Pure error	0.0382	5	0.0076			
Cor total	58.1700	29				

$p < 0.05$ is considered as significant. Std. deviation: 0.1294; mean: 2.84; coefficient of variance %: 4.56; R^2: 0.9957; adjusted R^2: 0.9917; predicted R^2: 0.9785; adequate precision: 42.8363

Validation of the experimental design

The optimum condition for amino acids production from PPS by one-step fermentation was predicted according to Eq. 4.

$$\begin{aligned}\text{Amino acids} =& 4.56495 + 0.190056\,\text{A} + 0.617363\,\text{B} + 0.198623\,\text{C} + 0.20771\,\text{D} + 0.213756\,\text{AB} \\ &+ 0.0207446\,\text{AC} - 0.0607686\,\text{AD} - 0.0588524\,\text{BC} + 0.0956576\,\text{BD} + 0.16956\,\text{CD} \\ &- 0.847018\,\text{A}^2 - 0.854113\,\text{B}^2 - 0.684703\,\text{C}^2 - 0.494673\,\text{D}^2,\end{aligned} \quad (4)$$

where *A* is PPS, *B* is peptone, *C* is ammonium sulphate and *D* is inoculum volume.

The validation experiment was conducted with the optimised parameters to ensure the accuracy of the developed empirical model. The optimum condition as predicted by the model for maximum amino acid concentration was 22.57 g/L PPS, 108.70 g/L peptone, 95.23 g/L ammonium sulphate, and 14.04% (v/v) inoculum volume. The maximum amino acid production as predicted by the model was 4.74 g/L. A confirmation run was conducted at the optimised parameters and it was observed that a maximum of 4.57 (± 0.51) g/L with 0.20 g/g yield amino acid was being produced at the said parameters. This is in accordance with the predicted value, with a 3.54% difference between the actual and predicted values within a 95% confidence interval. Therefore, the model is acceptable since the error falls below 5.00%, indicating that the error might be caused by a random error [30]. The result showed a 30.48-fold increase in the total free amino acids production when tested with ninhydrin assay after optimisation of media composition and fermentation parameters.

Based on the results obtained, other significant factors in the amino acids production from PPS by *B. subtilis* ATCC 6051 are yeast extract (1.31 g/L), $MgSO_4.7H_2O$ (4.83 g/L), $MnSO_4.4H_2O$ (2.00 g/L), and zinc acetate (8.72 mg/L). Apart from that, fermentation parameters including temperature (30 °C) and rotational speed (180 rpm) were also set at their optimum condition based on the screening results. Under the given conditions, profiling of the amino acid composition was conducted, and the finding is revealed in Table 6. The highest amino acids production was hydroxyproline at 3.61 mg/mL.

The amino acid profiling indicates a substantial increase in the concentration of almost all amino acids, resulting in a 7.48-fold increment. In terms of yield and

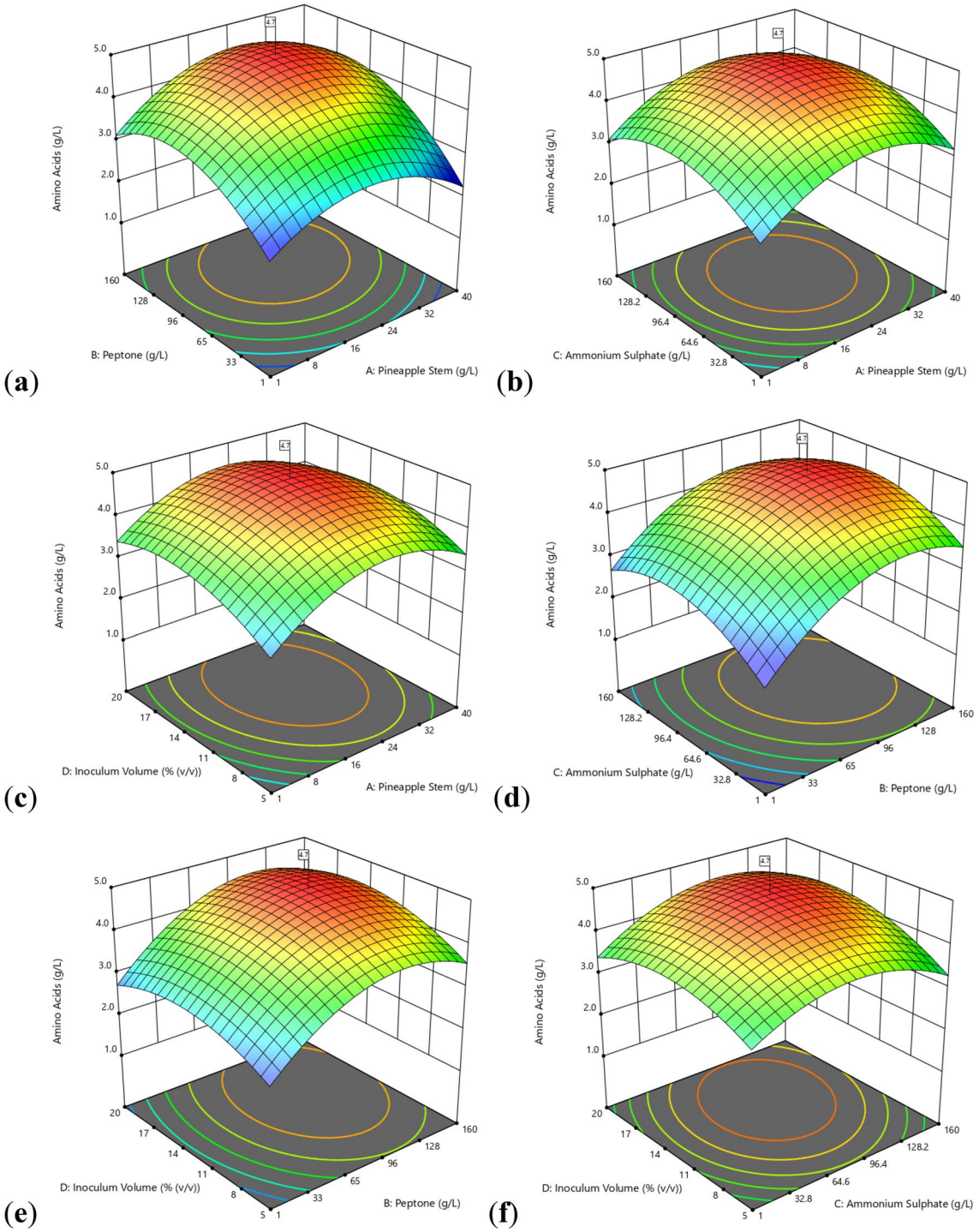


Fig. 4 Response surface plots for the effects of **a** PPS and peptone; **b** PPS and ammonium sulphate; **c** PPS and inoculum volume; **d** peptone and ammonium sulphate; **e** peptone and inoculum volume; **f** ammonium sulphate and inoculum volume on the amino acids production

productivity, it has led to a 6.32 and 3.56-fold increment, respectively. The highest increment of amino acid concentration was threonine (70%), followed by methionine (50%), aspartic acid (21%), and tyrosine (13%). The diverse amino acids produced have applications in various industries, including animal feed (methionine and threonine), food (glutamic acid), and pharmaceutical, nutrition, and cosmetics (hydroxyproline). The amino acid production

Table 6 Amino acids profiling using optimum production media and PPS as feedstock

Amino acid	Concentration (mg/mL)	Yield (mg/g)	Productivity (mg/L/h)
Histidine	1.50^{h}	66.59^{j}	31.31^{i}
Threonine	1.41^{g}	62.60^{i}	117.74^{l}
Methionine	1.02^{e}	45.28^{g}	85.17^{k}
Isoleucine	0.06^{a}	2.66^{b}	5.01^{c}
Phenylalanine	0.06^{a}	2.66^{b}	1.25^{a}
Hydroxyproline	3.61^{j}	159.82^{l}	75.15^{j}
Aspartic acid	1.72^{i}	76.36^{k}	143.62^{m}
Serine	1.16^{f}	51.50^{h}	24.22^{h}
Glutamic acid	0.52^{c}	23.09^{d}	21.71^{f}
Glycine	0.04^{a}	1.78^{a}	1.67^{b}
Arginine	0.17^{b}	7.55^{c}	7.10^{d}
Proline	0.53^{c}	23.53^{e}	22.13^{g}
Tyrosine	0.81^{d}	35.96^{f}	16.91^{e}

Superscripts with different letters are significantly different from each other withinthe same column at $p < 0.05$ using Tukey HSD post hoc test

in this study surpassed that reported by Lim et al. [31], where methionine concentrations ranged from 8.28 mg/L to 49.14 mg/L using different lactic acid bacteria strains of *P. pentosaceus* and *Lactobacillus plantarum* RS5. The variations could be attributed to differences in fermentation routes, biomass, and microorganisms used.

In our previous study [32], there was as much as 23.53 mg/mL total amino acid produced through separate enzymatic hydrolysis and fermentation. This method utilised a simple medium composition with basic nutrient broth and PPS hydrolysate. However, in the current study, a more sophisticated approach was adopted with an optimised production medium containing specific nutrients tailored for enhanced amino acid production. By utilising production medium containing precise concentrations of PPS, peptone, ammonium sulphate, and additional nutrients, one-step fermentation provides optimal conditions for microbial growth and amino acid production. Specific nutrients such as yeast extract, magnesium sulphate, manganese sulphate, and zinc acetate further enhanced microbial metabolism and amino acid synthesis, leading to improved productivity and yield. This highlights the significance of medium composition optimisation in maximising the potential of microbial fermentation for amino acid production from pineapple stem, offering a sustainable and efficient approach for value-added product generation. Apart from medium composition, separate enzymatic hydrolysis and fermentation required specific enzyme (Dextrozyme DX 1.5) and specific conditions for gelatinisation and enzymatic hydrolysis before it can be applied in the amino acid fermentation, which further increased the cost, time, energy, and steps for amino acid production.

In this study, one-step fermentation, employing a streamlined process, demonstrates superior performance in terms of amino acid concentration, yield, and productivity compared to our previous study using separate hydrolysis and fermentation. Despite higher total amino acid concentration, especially lysine, was produced in the previous study, a diverse range of amino acids with higher concentrations was produced through one-step fermentation. In separate hydrolysis and fermentation, lysine production stood out prominently, with a maximum production of 20.81 mg/mL, indicating the efficacy of the process in synthesising this essential amino acid from pineapple stem hydrolysate. However, while lysine production was significant in separate hydrolysis and fermentation, other amino acids demonstrated varying levels of production. On the other hand, amino acids such as histidine, threonine, methionine, and aspartic acid showed notable improvements in concentration and productivity in one-step fermentation. This suggests that more efficient production of a wider range of amino acids from pineapple stem can be facilitated by one-step fermentation, combined with optimised medium composition and control parameters.

All in all, the optimised condition of media composition and fermentation parameters proved that this study can be a torchbearer and throws a light on the potential valorisation of PPS as agricultural biomass in the amino acid industry. This can be a basis to further explore the application of one-step fermentation in utilising other starch-based and lignocellulosic-based agricultural by-products, not only in amino acid industry, but also in the production of other value-added products. Future research can also be conducted on the application of co-culture system utilising the same approach for higher concentration of amino acid production.

Additionally, traditional method of burning PPS will release carbon dioxide (CO_2) due to the combustion process. The quantity of CO_2 emitted per tonne of PPS combusted varies based on factors like combustion efficiency, moisture content, and carbon content. Dumping PPS as waste also contributes to CO_2 emissions indirectly. As the organic matter decomposes, it undergoes microbial respiration, releasing CO_2 into the atmosphere. Furthermore, if the waste is left to decompose anaerobically in landfills, it can produce methane, another potent greenhouse gas. Fermenting PPS for amino acid production offers a sustainable alternative, reducing CO_2 emissions compared to traditional disposal methods. While fermentation does produce CO_2, it can be carbon-neutral or negative if sourced sustainably. The amino acids

produced can replace carbon-intensive production methods, aligning with UN Sustainable Development Goals, especially SDG 13 (Climate Action). Thus, a comprehensive life cycle analysis can be conducted as future study to accurately quantify emissions savings and ensure alignment with sustainability goals.

Conclusions

This study demonstrates the importance of fermentation media in enhanced one-step fermentation of amino acid production from pineapple plant stem. This approach offers a sustainable alternative to the conventional separate enzymatic hydrolysis and fermentation by utilising agricultural biomass for value-added product without the need of expensive commercial enzymes. The amino acids production using PPS as a substrate by *Bacillus subtilis* ATCC 6051 has successfully produced 1.28 mg/mL of total free amino acids with the yield of 67.13 mg/g. The increment was 13% in concentration and 12% in yield of total free amino acids production as compared to commercial starch. The determination of medium composition has significantly improved the production of amino acids, with the total amino acids production of 9.57 mg/mL with the yield of 423.97 mg/g. The yield has successfully been improved by 6.32-fold of increment. Overall, this study highlights the importance of media composition and presents a promising alternative approach for amino acid production from pineapple plant stem through enhanced one-step fermentation for a more sustainable bioeconomy as aligned with the Sustainable Development Goals.

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Author contributions
CPH: methodology, software, validation, formal analysis, investigation, data curation, writing—original draft, writing—review and editing, visualisation. MAJ: validation, data curation, writing—original draft, writing—review and editing, visualisation. PLY: conceptualisation, validation, supervision. MFI: conceptualisation, validation, supervision. PP: conceptualisation, validation, funding acquisition. SH: conceptualisation, validation, funding acquisition. SAA: conceptualisation, resources, writing—original draft, writing—review and editing, supervision, project administration, funding acquisition.

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Declarations

Ethical approval and consent to participate
The authors assert that ethical approval is not required.

Competing interests
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Availability of data and materials
The data are presented in the article, further inquiries can be directed to the corresponding author upon reasonable request.

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