# An alternative peptone preparation using *Hermetia illucens* (Black soldier fly) hydrolysis: process optimization and performance evaluation (#91199)



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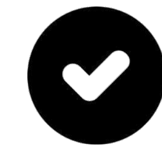

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# An alternative peptone preparation using *Hermetia illucens* (Black soldier fly) hydrolysis: process optimization and performance evaluation

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**Background.** *Hermetia illucens* (HI), commonly known as black soldier fly, recognized for their prowess in resource utilization and environmental protection, have the ability to transform organic waste into animal feed for livestock, poultry, and seafood. However, their high protein content's potential for value-added applications has been largely unexplored. **Methods.** Our research innovatively explores the potential of *Hermetia illucens* larvae (HIL) protein as a peptone substitute for microbial culture media. Four commercial proteases (alkaline protease, trypsin, pancreatin, and papain) were explored to hydrolyze the defatted HIL, the experimental conditions were optimized via response surface methodology experimental design. The hydrolysate of the defatted HIL was subsequently vacuum freeze-dried and deployed as a growth medium for two bacterial strains (*Staphylococcus aureus* and *Bacillus subtilis*) to determine the growth kinetics between the HIL peptone and commercial tryptone. **Results.** The optimal conditions were 1.70% (m/m) complex enzyme (alkaline protease: pancreatin at 1:1 ratio) at pH 7.0 and 54.00 °C for a duration of 4 hours. Under these conditions, the hydrolysis of defatted HIL yielded 19.25% ± 0.49%. Growth kinetic analysis showed no significant difference in growth parameters (μ, X and λ) between the HIL hydrolysate-based medium and a conventional tryptone medium, demonstrating that the HIL hydrolysate could serve as an effective, low-cost alternative. Our study introduces an innovative approach to HIL protein resource utilization, broadening its application beyond its current use in animal feed.

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24 ## Abstract:

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40 based medium and a conventional tryptone medium, demonstrating that the HIL hydrolysate
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43 **Key words:** *Hermetia illucens* larvae; Peptone; Bacterial culture; Growth kinetics

## 44 1. Introduction

45 *Hermetia illucens* (HI), or black soldier fly (BSF), is increasingly gaining recognition as a remarkable
46 bio-converter and decomposer. This insect is adept at consuming a plethora of organic materials, such as

47 kitchen waste, spoiled fruits and vegetables, animal manure, and food processing waste, and converting them

48 into a rich b

49 iomass containing proteins, lipids, amino acids, and peptides(Amrul et al., 2022; Mangindaan, Kaburuan

50 & Meindrawan, 2022). Intriguingly, the dry biomass of *Hermetia illucens* larvae (HIL) boasts crude protein

51 content ranging from 32 to 60% (w/w)(Al-Qazzaz et al., 2016; Ruhnke et al., 2018). Moreover, HIL acts as a

52 bulwark against harmful microbials like *Escherichia coli* and *Salmonella*. During the 10 – 20day breeding

53 phase, the larvae synthesize a high concentration of antibacterial peptides, creating an inhospitable

54 environment for bacterial and fungal growth(Secci et al., 2018; Cullere et al., 2019). Even more, HIL

55 encouraging is the absence of typical mycotoxins such as deoxynivalenol, fumonisin 1 and 2, and zearalenone

56 in HIL biomass, regardless of the nature of the substrate on which they are reared(Leni et al., 2019). The

57 legislative landscape is also shifting in favor of HIL, with the European Union, the United States, Australia,

58 and Canada having enacted legislation to approve its use in animal feed(Alagappan et al., 2022). Currently, the

59 breeding industry predominantly uses HIL as a protein-rich alternative to traditional feeds like soybean meal

60 and fishmeal, attributing this preference to its high protein content, feed safety, and cost-effectiveness(Petrova

61 et al., 2021). However, other than the use for animal protein feed, few studies have been investigated for the

62 HIL protein application.

63 Commercially available tryptones, which are indispensable nitrogen sources in microbial culture media,

64 owe their widespread use to their proven efficiency and stability(Chapman, Mariano & Macreadie, 2015;

65 Fallah, Bahram & Javadian, 2015). These tryptones are typically derived from hydrolyzed sources such as fish,

66 casein, meat, or soybean. However, there are shortages to use these sources, including high costs and the

67 potential risk of bovine spongiform encephalopathy (BSE) virus contamination during beef hydrolysis(Zhang

68 et al., 2023). These limitations have ignited the pursuit for feasible alternatives.

69 Intriguingly, a growing body of research has begun to explore unconventional sources for peptone
70 derivation. Materials such as wool, tuna heads, and salmon skeletons have been used with different proteases
71 to yield peptones that demonstrate comparable bacterial growth performance to their commercially available
72 counterparts(Broli et al., 2021; Tuysuz et al., 2021; Vázquez et al., 2022). However, despite the significant
73 potential of HIL, in nutrient recycling and as a protein-rich feed alternative, very few studies have delved into
74 the preparation of peptones using HIL. This research gap represents an opportunity for exploration, especially
75 considering the impressive credentials of HIL as a sustainable and efficient nutrient source.

76 In the quest for efficient, cost-effective, and safe solutions to the high costs and biosafety concerns of
77 conventional peptone sources, in this context, we propose the utilization of HIL as a promising source for
78 alternative peptone preparation. Recognizing HIL as a prospective alternative peptone source, we have
79 developed a method to hydrolyze HIL proteins using a variety of protease combinations. The outcome is a
80 protein hydrolysate that has shown potential in serving as a microbial medium that can stand toe-to-toe with
81 traditional tryptone. Our study is geared towards optimizing the HIL peptone preparation process, which
82 includes examining the physicochemical properties of the resultant HIL peptone and conducting a performance
83 evaluation. To assess performance, we focused on the growth kinetics of *Bacillus subtilis* and *Staphylococcus*
84 *aureus* in HIL-based media.

## 85 2 Materials & Methods

86 2.1 Raw materials and enzymes

87 The HIL were purchased from Gansu Guorui Environmental Protection Biotechnology Co. Ltd (Lanzhou,
88 China). General chemical reagents were obtained from Tianjin Baishi Chemical Industry Co. Ltd (Tianjin,

89 China). Lastly, biochemical-grade yeast extract and agar tryptone was purchased from Beijing Solarbio
90 Science & Technology Co. Ltd (Beijing, China). Trypsin, trypsin and papain were purchased from Nanning
91 Donghenghuadao Biotechnology Co. Ltd (Nanning, China). Alkaline protease was purchased from Shandong
92 Longke Bio-Products Co. Ltd (Linyi, China). *Bacillus subtilis* ATCC 6051, purchased from Beijing Bai'ou
93 Bowei Biotechnology Co. Ltd (Beijing, China). *Staphylococcus aureus* ATCC6583, preserved by Northwest
94 Microbial Strain Preservation Center (Gansu, China).

95 2.2 Instruments and Equipment

96 The Automatic Kjeldahl Nitrogen Analyzer (Kjeltec 8200) used in this study was manufactured by FOSS
97 Co. Ltd. in Denmark. Additionally, the Hitachi Amino Acid Analyzer (L-8900) was purchased from Shanghai
98 Baiga Instrument Technology Co. Ltd. The Vacuum Freeze Dryer (LGJ-20F) employed in the study was
99 purchased from Beijing Songyuan Huaxing Technology Development Co. Ltd.

100 2.3 Microbial culture medium

101 The Luria-Bertani (LB) medium contained 10.0 g tryptone, 5.0 g yeast extract, 10.0 g NaCl, and 15.0 g
102 agar dissolved in distilled water to a volume of 950 mL. The pH was then adjusted to 7.2~7.4 and topped up to
103 a final volume of 1000 mL with distilled water at 120 °C. The medium was sterilized for 20 minutes.

104 The HIL peptone medium, which contained the same amount of nitrogen (11.2 g), replaced tryptone with
105 HIL peptone. The same procedure was applied to prepare this medium, which was called HIL peptone
106 medium.

107 2.4 Response Surface Methodology (RSM) Experimental design

108 Data detailing the impact of enzyme complexes (specific enzymes and their proportions), pH, and
109 hydrolysis time on HIL hydrolysis will be presented in the Supplementary File. According to our preliminary

110 factorial experimental test result the variables of enzyme addition (A) and temperature (B) on proteolysis of
111 HIL are the most significant variables, thus they were selected to investigated to optimize by response surface
112 methodology. Polynomial equations, relating the effect of independent variables on maximum hydrolysis
113 (Hm), were obtained after applying the orthogonal least-squares method (Eq. 1)(Vázquez et al., 2022).

114 $$Y = m_0 + \sum_{i=1}^{n} m_i X_i + \sum_{\substack{i=1 \\ k>i}}^{n-1} \sum_{k=2}^{n} m_{ik} X_i X_k + \sum_{i=1}^{n} m_{ii} X_i^2 \ldots (1)$$

115 Where, $Y$ is the response evaluated, $m_0$ is the constant coefficient, $m_i$ is the coefficient of linear effect, $m_{ik}$ is
116 the coefficient of combined effect, $m_{ii}$ is the coefficient of quadratic effect, $n$ is the number of variables, and
117 $X_i$ and $X_k$ are the independent variables studied in each case. We tested five combinations of temperature and
118 enzyme addition, based on previous data from our group and provided by the enzyme marketers, to establish
119 the most suitable optimal working conditions. The experimental conditions studied were (pH=7, 4 h) for
120 enzyme complex (alkaline protease: trypsin = 1:1, g: g). In all cases after proteolysis, each liquid hydrolysate
121 was heated at 95 ◦C for 15 min to inactivate the enzyme. Following the hydrolysis, both the hydrolysate and
122 sediment were subjected to centrifugation. The resultant liquid hydrolysate was then dried using a vacuum
123 freeze dryer (LGJ-20F). This material was distributed evenly on the instrument's rack, under a vacuum
124 pressure of 20pa. The process involved a linear temperature increase, from -45 °C to 25 °C, over the course of
125 a 40-hour vacuum freeze-drying cycle. The dried samples were subsequently collected, pulverized, and
126 preserved through vacuum packaging in sterile bags. To optimize the hydrolysis process of the HIL, the
127 Design-expert 8.0.6 software was used. The Center-Composition Design experimental setup is shown in Table
128 1.

129 2.4.2 Measurement of main indicators

130 The physicochemical properties of HIL hydrolysate were obtained by measuring: The determination of

131 the total nitrogen content was performed using the Kjeldahl method. The content of amino-nitrogen (a-amino)
132 was estimated using the formol titration method according to as described by Kosasih(Kosasih et al., 2018).
133 HIL hydrolysate (2 ml) was dissolved in deionized water (5 mL), then and pH was adjusted to 8.2 using NaOH
134 (0.1 mol/L). 5 mL of previously neutralized formaldehyde (pH= 8.2) was added to the above mixture and
135 subsequently titrated with NaOH (0.1 mol/L) to attain pH 9.2. The total volume of NaOH in the titration was
136 used to calculate the amino-nitrogen as shown in equation 1. The protein hydrolysis DH (%) was determined
137 by separately determining the amount of total nitrogen and amino-state nitrogen in the protein solution and
138 calculating the result using equation (2). Where, v is volume of NaOH used in titration, w stands for weight of
139 sample (g), and N(NaOH) indicates NaOH normality. The degree of hydrolysis (DH) was estimated by the
140 ratio of amino nitrogen to total nitrogen in each sample (Eq. 3)(Kosasih et al., 2018). Where, A was Amino
141 acid nitrogen in enzymatic digest; T was Total nitrogen in the sample.

142 $$Amino\ nitrogen\ (\%) = \left(\frac{v}{w \times 10}\right) \times M_{(NaOH)} \times 14.008 \ldots (2)$$

143 $$\mathrm{DH}\ (\%) = \frac{\mathrm{A}\,(\mathrm{g}/100\mathrm{g})}{\mathrm{T}\,(\mathrm{g}/100\mathrm{g})} \times 100 \ldots\ldots (3)$$

144 2.4.3 Amino acid analysis

145 The sample processing involved dissolving the lyophilized powder in pure water, centrifuging it at 1200
146 r/min for 15 minutes to remove the protein, and then treating the supernatant with trichloroethylene. After
147 dilution with TCA, the filtrate was filtered through a 0.22 μm filter, and the amino acid species and content in
148 the samples were determined through an amino acid analyzer(NHC&CFDA, 2016).

149 2.4.5 HIL Peptone and tryptone chemical property test

150 The fat protein, content, and ash moisture of the samples were determined according to methods 955.04,
151 934.01, 2003.06 and 942.05 in AOAC. The ash content of hydrolysates was evaluated with reference to AOAC

152 International methods 942.05(Patrica, 1997). The protein solubility index (WSI), were determined with
153 reference to Hou Lixiao's method(Lixiao, 2017).
154 2.4.7 Effect of HIL peptone and tryptone on the growth effect of bacteria
155 The experiment was conducted in four separate sections, each serving a distinct objective. Firstly, the
156 solubility of peptone was assessed. A precise quantity of 1.0 g of peptone was dissolved in 50 mL of distilled
157 water. The solution was immediately shaken and completely processed within 30 minutes. In the second
158 sections, the clarity and precipitation of the peptone solution were evaluated under alkaline conditions. A
159 solution was prepared by dissolving 2.0 g of peptone in 100 mL of distilled water. The pH was adjusted to 8-9
160 using 0.1 mol/L NaOH. Subsequently, the solution was autoclaved at 121 °C for 30 minutes, followed by
161 cooling to room temperature for observation. The third sections involved examining the interaction between
162 peptone and phosphate. A solution was prepared by dissolving 2.0 g of peptone and 0.5 g of $KH_2PO_4$ in 100
163 mL of distilled water. The pH was adjusted to 7.4-7.6 using 0.1 mol/L NaOH. Similar to the second stage, the
164 solution was autoclaved and cooled before evaluating its clarity and precipitation. In the fourth sections, the
165 coagulability of peptone was tested. A 5% aqueous peptone solution was filtered and boiled, and any observed
166 precipitation was noted.
167 The microorganisms employed in this study included Bacillus subtilis, a commonly used industrial
168 bacterium, and Staphylococcus aureus, a pathogenic bacterium. This choice allowed us to assess the efficacy
169 of HIL peptones in comparison to commercial peptones for cultivating microorganisms, taking into account
170 their distinct genera and natures. To determine the ideal bacterial inoculum concentrations, Bacillus subtilis
171 (cultivated at 30.0 °C) and Staphylococcus aureus (cultivated at 37.0 °C) were separately inoculated into both
172 Luria-Bertani (LB) and HIL peptone liquid media. Inoculation concentrations ranging from 1.0% to 5.0% (v/v)

173 were utilized. Following a 12-hour shaking incubation at a consistent agitation speed of 180 rpm, we measured
174 the absorbance at 600 nm to identify the optimal inoculum concentration for each bacterium. Subsequently,
175 Bacillus subtilis and Staphylococcus aureus were inoculated at their respective optimal concentrations into LB
176 and HIL peptone liquid media. These cultures were incubated at 30.0 °C and 37.0 °C, and samples were
177 collected every 2.0 hours for bacterial count determination. The viable bacterial counts (cfu/mL) and
178 corresponding time intervals were utilized to construct the growth curves.

179 Finally, the growth kinetics of the bacterial strains in both media types were evaluated using the Verhulst
180 logistic model (equation formula 4(Ding et al., 2016)). The growth curves provided the necessary data to
181 determine the maximum biomass concentration ($X_{max}$), maximum specific growth rate ($\boldsymbol{\mu_{max}}$), and lag phase (
182 λ). These parameters were calculated to assess the effect of HIL and commercial peptone on bacterial growth.

183 $$X_t = \frac{X_{max}}{1 + e^{2 + \mu_{max}(\lambda - t)}} \ldots\ldots \quad (4)$$

184 Where, the biomass concentration $X_t$ (cfu /mL) during the time course, $X_{max}$ (cfu /mL) as the maximum
185 biomass concentration, $\boldsymbol{\mu_{max}}$ ($h^{-1}$) as the maximum specific growth rate, and λ (h) as the lag phase were
186 calculated using OriginPro 2021 software.

187 2.4.8 Data Analysis Software

188 The raw data of this experiment were organized in Microsoft Excel and statistically analyzed using IBM
189 SPSS Statistics 22.0. The experimental results were expressed as mean values ± standard deviations. Response
190 surface optimization was carried out using Design-Expert.V8.0.6.1. Growth curves and fitting curves were
191 plotted using Origin Pro 2021 (9.8.0.200).

## 192 3 Results and Discussion

193 3.1 RSM experimental results

194 3.1.1 Variance Analysis and Model Validation

195 The regression equation obtained is as follows: $Y = 19.56 + 1.52A + 0.74B + 0.56AB – 4.00A^2 – 3.53B^2$

196 The regression analysis demonstrated that A, B, $A^2$ and $B^2$ were highly significant, as evidenced by P-

197 values less than 0.01. Furthermore, the model itself was found to be highly significant ($P < 0.01$). The lack-of-

198 fit term was not significant ($P > 0.05$), indicating that the model provides a good fit to the experimental data

199 and has good stability. The determination coefficient ($R^2$) was 0.9752, suggesting that 97.52% of the

200 variability in the response could be explained by the model. The adjusted $R^2$ was 0.9574, further confirming

201 the model's reliability in predicting experimental outcomes. The model can effectively fit the real response

202 surface, thereby elucidating the interrelationship among enzyme concentration, temperature, and hydrolysis.

203 As such, it is deemed suitable for analyzing and forecasting the results of the defatted HIL hydrolysis process.

204 3.1.2 Response Surface Analysis

205 Our study revealed a notable trend: the hydrolysis degree, at a constant enzyme concentration, initially

206 increased and later decreased as the temperature varied between 45°C and 60°C (Fig.1). The hydrolysis degree

207 peaked at 55°C. The underlying mechanism can be attributed to the progressive depletion of the substrate

208 concentration as it is hydrolyzed by the increasing enzyme quantity until fully hydrolyzed. Consequently, the

209 concentration of free amino nitrogen and the hydrolysis degree reach a plateau. Moreover, the higher slope for

210 temperature in comparison to enzyme dosage in the response surface analysis indicates a more pronounced

211 impact of temperature on the enzyme-catalyzed reaction rate.

212 3.1.3 Optimization and validation

213 The predicted optimal conditions using the regression equation for hydrolysis were determined to be an

214 enzyme dosage of 1.7% (w/w) at 54.0°C, with a theoretical hydrolysis degree of 19.75% ± 0.995%. Three

215 validation experiments under these conditions yielded a hydrolysis degree of 19.25% ± 0.49% for the defatted
216 HIL. The relative error between the observed and theoretical values was 2.60%, and the difference was
217 statistically non-significant ($P > 0.05$). These results provide compelling evidence of the model's practicality
218 and reliability for experimental optimization and identifying the best hydrolysis conditions. Therefore, model
219 obtained can be used for experimental optimization to obtain the best hydrolysis conditions.
220 3.2 General characterization of HIL peptone and tryptone.
221 As delineated in Table 4, the HIL peptone exhibited a darker color in comparison to tryptone. This
222 variation is likely attributed to the higher sugar content in the raw material, instigating Maillard reactions and
223 caramelization under elevated temperature conditions. These reactions subsequently intensify the color of the
224 final product. Nevertheless, the product's appearance aligns with the quality standard of peptone and does not
225 adversely influence the growth or reproduction of microorganisms. Optimal peptone coloration should steer
226 clear of extreme darkness, as it could interfere with morphological observation and the growth and
227 reproduction assays of microorganisms.[25] In assessments concerning clarity, sedimentation, and solubility,
228 both HIL peptone and tryptone demonstrated comparable results, displaying clarity, transparency, and full
229 dissolution within 30 minutes. Clarity and transparency are fundamental properties for peptones utilized as
230 biochemical reagents in microbial culture, owing to the necessity to observe microbial morphologies during
231 cultivation. A clouded peptone solution may compromise the accuracy of observational results.
232 The pH value of HIL protein hydrolysate was 8.83, while the pH value of pancreatic protein hydrolysate
233 was 7.10. The higher pH value of HIL protein hydrolysate may be attributed to the substantial feeding
234 requirement of the larvae stage of HIL, aiming to fulfill their later survival needs. Consequently, the HIL
235 protein hydrolysate contains a diverse range of digestive enzymes (including cellulases, lipases, α-amylases,

236 proteases, and pancreatic proteases). In the process of preparing HIL protein hydrolysate, the pH value of the
237 reaction system was not adjusted, leading to alkaline precipitation. Both alkaline and phosphate precipitates
238 were observed in the HIL protein hydrolysate, while pancreatic protein hydrolysate did not exhibit such
239 precipitation. This phenomenon may be attributed to the presence of a considerable amount of chitin, which
240 cannot be removed, in the larvae stage of HIL, resulting in the formation of slight precipitates in the protein
241 hydrolysate solution. Nevertheless, both HIL peptone and tryptone met the growth requirements of
242 microorganisms in terms of coagulable peptones.

243 3.3 Chemical property of HIL peptone and tryptone

244 Table 5 shows that the moisture, ash, total nitrogen, amino nitrogen, phosphorus, and chloride content of
245 HIL peptone and tryptone met the quality standards for peptone. This demonstrates that HIL peptone possesses
246 the requisites to replace tryptone as a nitrogen source in culture media for microbial growth and reproduction.

247 3.4 Water solubility index (WSI) of HIL peptone and tryptone

248 In Figure 2, the water solubility index (WSI) of HIL protein hydrolysate and tryptone is illustrated at
249 various pH levels. Both HIL protein hydrolysate and tryptone exhibited an initial increase in water solubility,
250 followed by a subsequent decrease. At pH 9, the HIL protein hydrolysate achieved its highest water solubility
251 index of 5.20%, while tryptone reached a peak value of 6.46% at pH 7. These comparable values and similar
252 trends suggest functional parallels between the hydrolysates. Remarkably, the lowest solubility for both
253 hydrolysates was observed at pH 1. At this pH value, the absence of repulsive electrostatic forces between
254 protein molecules leads to their aggregation or precipitation, indicating the isoelectric point of the proteins.
255 This phenomenon implies that under non-extreme pH conditions, the properties of HIL peptones remain stable.

256 3.5 Analysis of amino acids in the HIL hydrolysate

257 Figure 3 delineates the respective amino acid compositions of HIL peptone, commercial peptone, and
258 bovine blood peptone. Prominently, the HIL peptone harbors a higher percentage of tyrosine (12.92%),
259 arginine (11.59%), leucine (11.23%), and phenylalanine (9.38%) in comparison to the other two peptones. On
260 the other hand, the commercial peptone was found to be significantly abundant in Glutamic acid (17.53%),
261 Glycine (16.79%), Aspartic acid (12.22%), and Alanine (10.1%). Lastly, the bovine blood peptone, as
262 researched by Nasim Rezaee et al., primarily contains Lysine (18.54%), Glutamic acid (12.83%), Aspartic acid
263 (10.95%), and Alanine (8.706%). The amino acids present in these peptones, integral to microbial growth,
264 perform critical roles such as aiding microbial protein synthesis, catalyzing enzymatic reactions, and providing
265 antioxidative properties. Rini Triani's(Triani et al., 2021), research indicated that enzymatic hydrolysis can
266 effectively elevate the amino acid content in black soldier fly prepupae, the protein hydrolysate is not only
267 abundant in amino acids, but also contains short chain peptides, which are more readily assimilable by
268 microorganisms.
269 As shown in Figure 4, principal component analysis (PCA) revealed amino acid differences between HIL
270 peptone, bovine blood peptone, and commercial peptone based on 22 different amino acids. The direction and
271 length of the arrows in the figure indicate the direction of the principal components and the contribution of
272 each other's differences. Particularly, the HIL peptone exhibits distinct characteristics from the bovine blood
273 peptone and commercial peptones, implying a low correlation between them. The primary differences amongst
274 the three peptones are attributable to the content of tyrosine, arginine, and leucine. Furthermore, bovine blood
275 and commercial peptones share a high correlation, as indicated by the close proximity of their respective
276 arrows. Glycine, Glutamic acid, and Lysine are highlighted as the principal amino acids responsible for their
277 dissimilarity. Despite substantial differences in amino acid composition, the HIL peptone and commercial

278 peptone did not significantly impact microbial growth. This fact enhances the appeal of HIL peptone as an
279 economically viable and environmentally sustainable substitute to conventional peptones. It is particularly
280 beneficial for microbial cultures that demand swift nutrient absorption and energy conservation.
281 In conclusion, the amino acid composition variations among the peptones could exert unique effects on
282 the growth and metabolism of microorganisms. This is particularly relevant in the field of industrial
283 biotechnology, where modifying the nutrient composition of the growth medium could optimize specific
284 microbial processes. Future research could exploit this potential by investigating the impacts of these peptones
285 on a range of industrially relevant microorganisms.
286 3.6 Effect of HIL peptone and tryptone on the growth effect of the strain
287 Figure 5 showed the optimal inoculum levels for *Bacillus subtilis* and *Staphylococcus aureus* were
288 ascertained to be 2.0% (v/v) in HIL peptone medium and 3.0% (v/v) in tryptone medium. The seed solutions of
289 *Bacillus subtilis* and *Staphylococcus aureus* were inoculated into HIL peptone liquid medium and LB liquid
290 medium at their respective optimal inoculation levels. Growth curves were measured every 2 hours, revealing
291 analogous growth trends in HIL protein hydrolysate and pancreatic protein hydrolysate for both *Bacillus*
292 *subtilis* and *Staphylococcus aureus*, as shown in Figure 5. The growth phases included a lag phase, logarithmic
293 growth phase, and stationary phase.
294 The use of peptone in media tends to enhance growth, with its key role being to serve as an organic
295 nitrogen source that fulfills bacterial cellular requirements for amino acids and peptide. In this study, we
296 undertook a comparative analysis of the logistic kinetic parameters for bacterial strains grown in both tryptone
297 and HIL peptone mediums. This comparison, outlined in table 6, takes into account the maximum population
298 size ($X_m$), maximum specific growth rate ($\mu$), and lag phase ($\lambda$) of *Staphylococcus aureus* and *Bacillus subtilis*

299 cultivated in both mediums. An intriguing finding was the higher biomass yield ($X_m$=4.29 × $10^9$cfu) of

300 *Staphylococcus aureus* cultured in HIL peptone, compared to that in tryptone (4.13 × $10^9$cfu). While other

301 growth parameters did not show significant differences s ($p > 0.05$), the maximum specific growth rate of

302 *Bacillus subtilis* in HIL peptone ($\mu_{max}$=0.42$h^{-1}$) was slightly higher than that in tryptone ($\mu_{max}$=0.32$h^{-1}$),

303 suggesting potential advantages of HIL peptone for fast-growing cultures. The growth curves in Figure 6

304 illustrate the variation in growth patterns of different bacterial strains under different culture conditions. The

305 fitting of the logistic model with the growth data of all bacterial strains examined substantiates its suitability

306 for this study. Furthermore, a shorter lag phase was observed for both *Staphylococcus aureus* ($\lambda$=3.97h) and

307 *Bacillus subtilis* ($\lambda$=14.67h) when cultured in HIL peptone, in comparison with the tryptone medium. This

308 expedited lag phase and the accelerated increase in bacterial concentration during the logarithmic growth phase

309 underscore the potential of HIL peptone for microbial cultivation.

310 Given that bacteria cultured in either HIL peptone or tryptone yielded similar results in terms of growth

311 profile and maximum bacterial growth, it is reasonable to conclude that HIL peptone could serve as an

312 effective substitute for meat-derived tryptone. This conclusion aligns with the proposition of Vazquez et al.,

313 who suggested the potential of aquaculture peptones as alternatives to meat-derived tryptone. In similar

314 studies, Vazquez et al(Vázquez et al., 2020). successfully used thermal or enzymatic techniques to derive

315 peptone from fish byproducts for the cultivation of Lactobacillus bacteria. Nasim Rezaee et al. also

316 demonstrated that bovine blood protein hydrolysate, obtained via integrated heat and enzymatic treatment,

317 shows a marked physico-chemical similarity to commercial Merck peptone, a meat-derived product.

318 However, fish protein products are often marred by the rancidity and unpleasant odor of fish oil, as

319 pointed out by Bridson and Brecker(Bridson & Brecker, 1970). Consequently, HIL peptone may present a

320 superior option, although further research should be conducted on a broader range of bacteria and their
321 metabolites in fermentation. This will allow for a more comprehensive assessment of the potential of HIL
322 peptone as an alternative to meat-derived tryptone.

## 323 4 Conclusions

324 Our work provides a key insight into the feasibility of HIL-derived peptones for microbial culture,
325 positioning this novel raw material as a valuable asset for a multitude of scientific and industrial applications.
326 Despite these promising results, it is important to note that the analysis did not show a significant difference
327 between the use of HIL protein hydrolysate and traditional tryptone for both *Bacillus subtilis* and *Staphylococcus*
328 *aureus* cultivation. This suggests that the HIL medium is at least as efficient as conventional media. However,
329 further investigation is needed to confirm these initial findings and fully understand the implications of using
330 HIL as a peptone source. Therefore, this study aims to delve deeper into these aspects, including further
331 optimization of the HIL peptone preparation process and its comprehensive performance evaluation. These
332 steps are essential to establish the viability of HIL as a sustainable and alternative peptone source.

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401

## Table 1(on next page)

Factors and Levels of Central Composition Design

1 Table 1 Factors and levels of Center-Composition Design

| Level | Factors | |
|---|---|---|
| | A:Enzyme addition（%） | B: Temperature（°C） |
| -1.414 | 0.79 | 40.86 |
| -1 | 1.0 | 45 |
| 0 | 1.5 | 55 |
| 1 | 2.0 | 65 |
| 1.414 | 2.21 | 69.14 |

2

## Table 2 (on next page)

Center-Composition Design test results

1 Table 2 Center-Composition Design test results

| Serial number | Factors | | Hydrolysis predictions（%） | Hydrolysis degree actual value （%） |
|---|---|---|---|---|
| | A（%） | B（°C） | | |
| 1 | 0 | 0 | 19.56 | 19.42±0.27 |
| 2 | 1.414 | 0 | 13.71 | 14.13±0.31 |
| 3 | 0 | 1.414 | 20.61 | 19.07±0.27 |
| 4 | -1 | -1 | 13.86 | 14.97±0.27 |
| 5 | 0 | 0 | 19.56 | 18.97±0.31 |
| 6 | -1 | 1 | 14.22 | 15.20±0.41 |
| 7 | 1 | -1 | 15.78 | 17.33±0.27 |
| 8 | 1 | 1 | 18.38 | 20.40±0.27 |
| 9 | 0 | 0 | 19.56 | 18.62±0.15 |
| 10 | 0 | 0 | 19.56 | 18.91±0.13 |
| 11 | 0 | -1.414 | 18.51 | 17.33±0.15 |
| 12 | -1.414 | 0 | 9.41 | 10.63±0.15 |
| 13 | 0 | 0 | 19.56 | 18.20±0.27 |

2

## Table 3(on next page)

variance analysis results of response surface quadratic regression equation model

1 Table 3 variance analysis results of response surface quadratic regression equation model

| Source | Sum of squares | Degree of freedom | Mean Square | F Value | P-Value |
|---|---|---|---|---|---|
| Models | 46.77 | 5 | 9.35 | 54.99 | < 0.0001** |
| A- Enzyme addition | 4.13 | 1 | 4.13 | 24.25 | 0.0017** |
| B-Temperature | 2.19 | 1 | 2.19 | 12.90 | 0.0088** |
| AB | 0.20 | 1 | 0.20 | 1.17 | 0.3159 |
| $A^2$ | 7.04 | 1 | 7.04 | 41.41 | 0.0004** |
| $B^2$ | 35.40 | 1 | 35.40 | 208.07 | < 0.0001** |
| Residuals | 1.19 | 7 | 0.17 | | |
| Misfit term | 0.97 | 3 | 0.32 | 6.02 | 0.0578 |
| Pure error | 0.22 | 4 | 0.05 | | |
| Total difference | 47.96 | 12 | $R^2$=0.9752; $R^2_{adj}$ = 0.9574 | | |

2 ** means extremely significant difference (P<0.01).

3

## Table 4(on next page)

Test results of general indexes of HIL peptone and tryptone

Table 4 Test results of general indexes of HIL peptone and tryptone

| Item | HIL peptone | Tryptone |
|---|---|---|
| Color | Light brown | Light yellow |
| Clarity | Clarity and transparency | Clarity and transparency |
| Precipitation | Small amount of precipitation | No precipitation |
| Dissolution time | ＜30 min | ＜30 min |
| pH | 8.83±0.03 | 7.10±0.01 |
| Alkaline precipitation | A little precipitation | No precipitation |
| Phosphate precipitation | little precipitation | no precipitation |
| Coagulable peptone | No precipitation | No precipitation |

## Table 5 (on next page)

Results of chemical properties of HIL peptone and tryptone

Table 5 Results of chemical properties of HIL peptone and tryptone

| Item | HIL peptone | Tryptone | Peptone for biochemical reagent |
|---|---|---|---|
| Moisture（%） | $4.34\pm0.01^{b}$ | $4.89\pm0.02^{a}$ | ＜5.0 |
| Ash content（%） | $12.77\pm0.23^{b}$ | $13.08\pm0.08^{a}$ | ＜15.0 |
| Total nitrogen（%） | $12.38\pm0.12^{b}$ | $12.70\pm0.12^{a}$ | ＞12.0 |
| Amino nitrogen（%） | $2.53\pm0.02^{b}$ | $3.70\pm0.03^{a}$ | ＞2.5 |
| Phosphorus content（%） | $0.53\pm0.03^{b}$ | $0.75\pm0.03^{a}$ | - |
| Chloride content (as chlorine)（%） | $1.43\pm0.03^{a}$ | $0.40\pm0.12^{b}$ | ≤2.0 |

Data for HIL peptone and tryptone were derived from this study, while data for biochemical reagent peptone were sourced from literature(Xueyan et al., 2018). Different letters in the shoulder labels of data in the same row in the table indicate significant differences ($P<0.05$), and the same letters indicate no significant differences ($P>0.05$).

## Table 6 (on next page)

Growth kinetic parameters generated from the Logistic model

1 Table 6 Growth kinetic parameters generated from the Logistic model

| | | $\mu_{max}(h^{-1})$ | $X_{max}$(cfu/mL) | λ(h) | $R_{adj}^2$ |
|---|---|---|---|---|---|
| *Bacillus* | Tryptone | 0.35 ± 0.04[a] | 8.37 ± 0.32[a] | 15.28 ± 0.54[a] | 0.9880 |
| *subtilis* | HIL peptone | 0.42 ± 0.05[a] | 7.98 ± 0.22[b] | 14.67 ± 0.50[a] | 0.9888 |
| *Staphylococcu* | Tryptone | 0.54 ± 0.04[a] | 4.13 ± 0.07[a] | 4.48 ± 0.24[a] | 0.9967 |
| *s aureus* | HIL peptone | 0.47 ± 0.03[a] | 4.29 ± 0.08[a] | 3.97 ± 0.26[a] | 0.9964 |

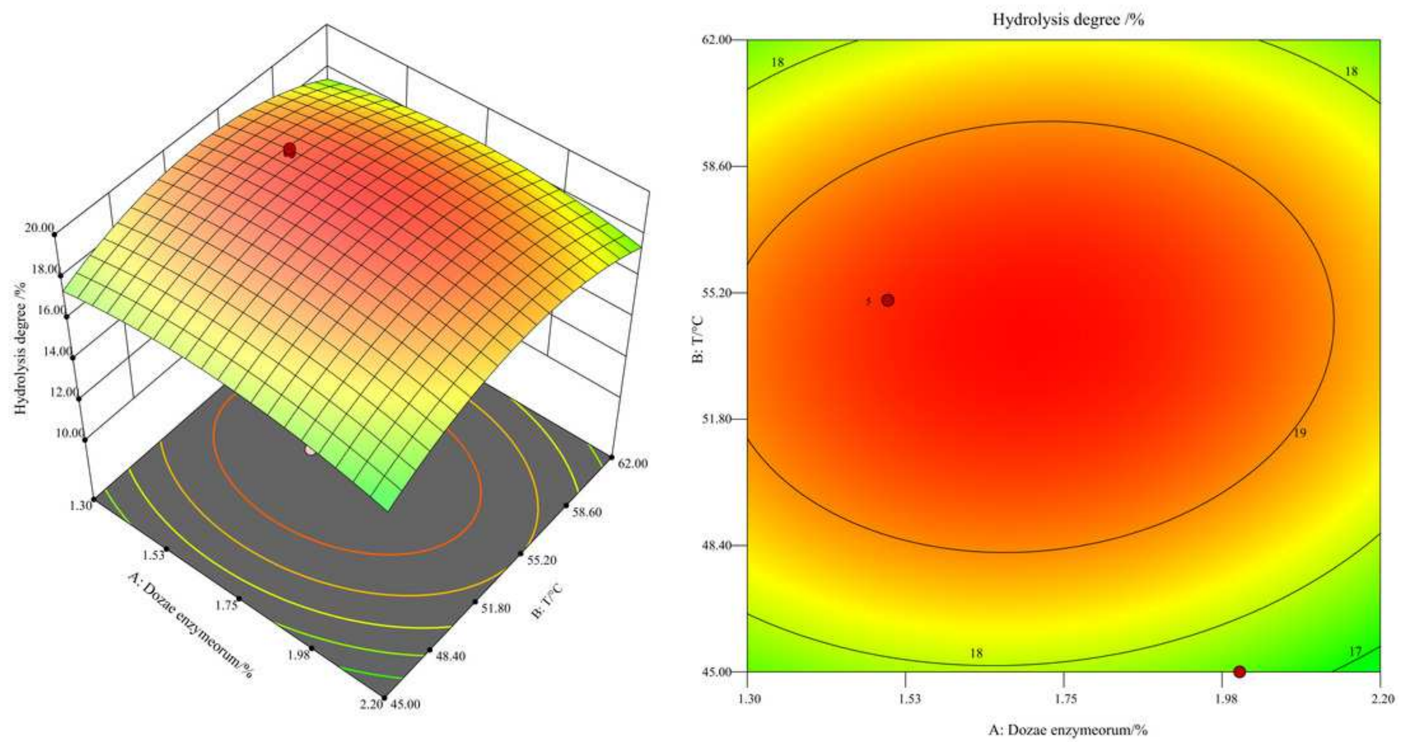
2 In the table, different letters on the shoulder labels of the same strain and the same column data indicate significant differences

3 ($P<0.05$), and the same letters indicate no significant differences ($P>0.05$)

4

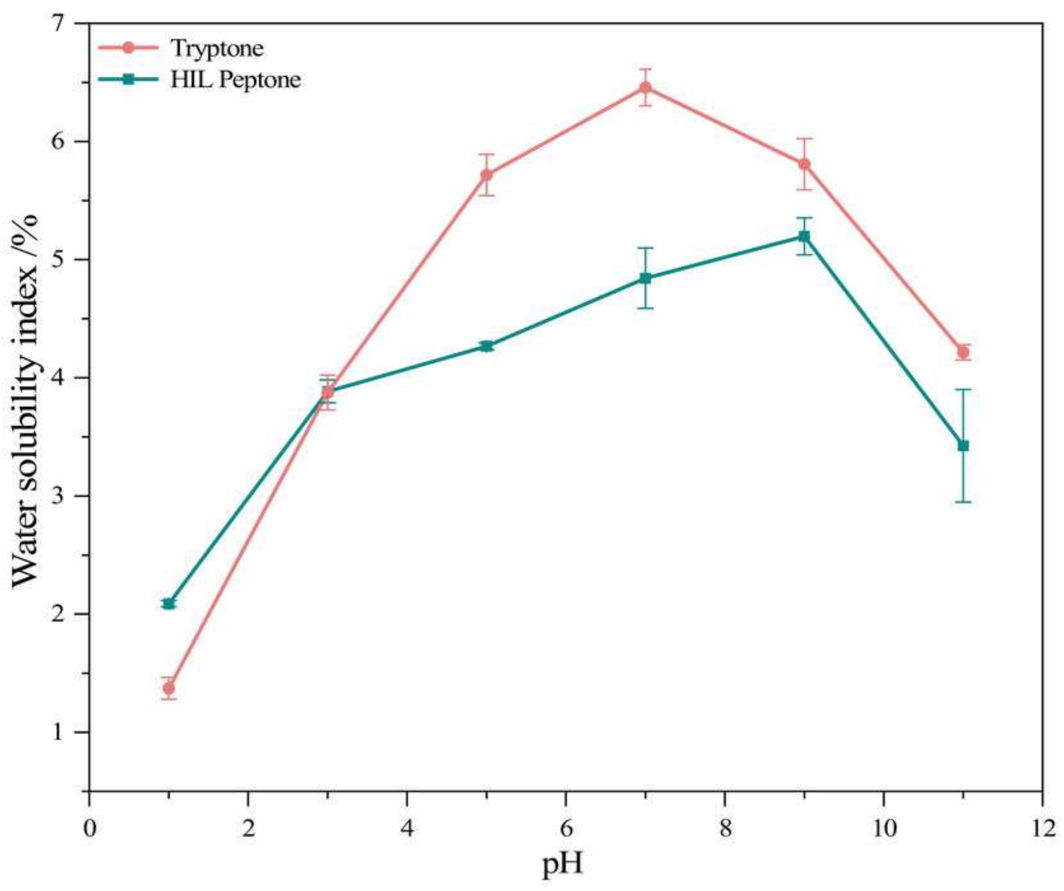
# Figure 1

Response surface and contours of the effect of enzyme addition and temperature on the degree of hydrolysis



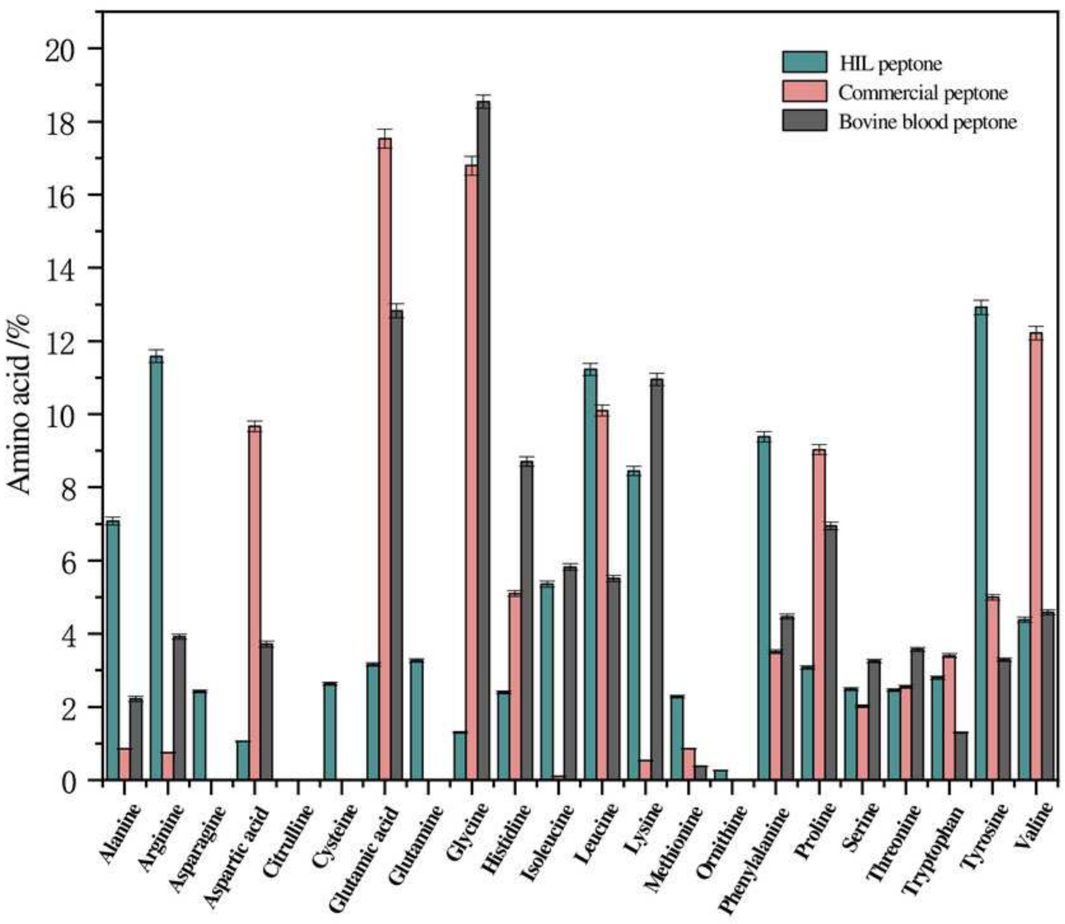
# Figure 2

Water solubility index of two peptones at different pH values



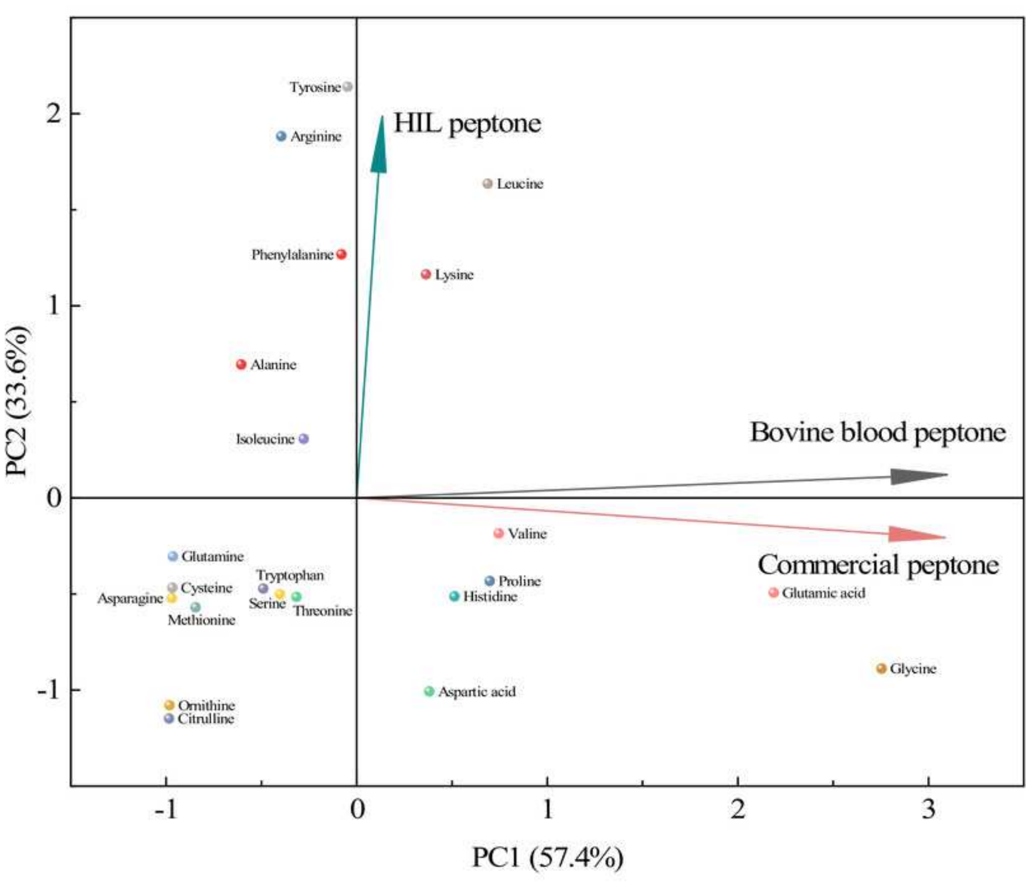
# Figure 3

Percentage contribution of each amino acid to the total amino acids in peptone sample (HIL peptone, commercial peptone and bovine blood peptone)



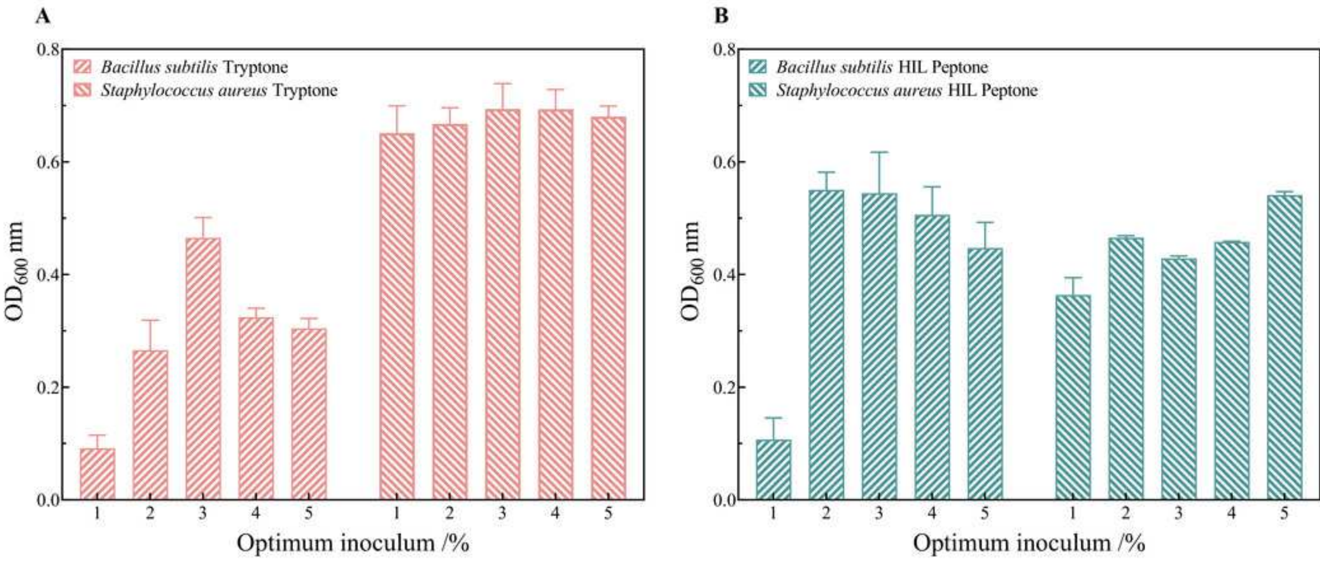
# Figure 4

Principal component analysis for assessing similarities among of HIL peptone, commercial peptone and bovine blood peptone based on amino acid compositions



# Figure 5

Determination of the optimum inoculum volume of Bacillus subtilis and Staphylococcus aureus in tryptone (A) and HIL peptone (B)



# Figure 6

Growth curve and fitting curve