

Original Research Paper

# Bioconversion of Corn Steep Liquor to Biofertilizer by Symbiotic Fermentation

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**Abstract:** Corn Steep Liquor (CSL) is wastewater from corn starch industry. The huge amount generation made it as a serious environmental problem. But CSL is nutrient-rich with diverse amino acids and other trace elements. In this study, symbiotic biofertilizer was studied using CSL as main substrate. In this study, 9 industrial strains, which were recognized as high performance species in the biofertilizer industry were collected, including four *Bacillus*, four fungus and one *yeast* species. Three dominant strains were selected finally, depending on the ability to dissolve phosphorus, ability for protein hydrolysis and cell growth. They were *Bacillus subtilis* 3301, *Bacillus licheniformis* and *Aspergillus Niger*. The results expressed that the highest growth cell count of *Bacillus subtilis* 3301 reached  $3.1 \times 10^9$  CFU/mL, the highest protein hydrolysis capacity of *Bacillus licheniformis* reached 600 mg/L and the highest phosphorus-dissolving capacity of *Aspergillus Niger* reached 1100 mg/L respectively. Then, symbiotic fermentation of the three strains was carried out both in shake-flask culture and fermentation tank (5 L). Interestingly, series of significant data of symbiotic fermentation were obtained, which were different from individual cultures. Especially, the total growth cell count in the 5 L scale fermenter culture was higher than individual cultures, which proved the mutual promotion effect between different strains. This research would provide a new view to utilize the waste of corn steep liquor as high value-added agricultural products.

**Keywords:** Corn Steep Liquor, Biofertilizer, Symbiotic Fermentation, Microbiome

## Introduction

Corn starch is the main resource for producing glucose and various sugar derivatives. During the industrial process of corn starch production, corn wet milling method is mainly applied with a high energy intensive process and environmentally unfriendly soaking water (Liggett and Koffler, 1948; Zhang and Jia, 2018). Corn Steep Liquor (CSL) is a concentrated liquid derived from the water that is used in the initial stage of the corn wet milling process. As a huge amount of by-product, the yield of CSL is about one billion tons per year globally. However, due to its foul smell, high acid and aflatoxins content, which greatly restricted its application. Currently, the CSL was mainly utilized for feeding in fodder, but the consumed amount was limited. This

phenomenon led to CSL overstocking and discharged as a sewage by factories, resulted in seriously environment problem. In fact, CSL is rich in nutrients such as proteins, amino acids, carbohydrates, vitamins and minerals and it's considered an excellent source of organic nitrogen for microorganisms (Salam and Ishaq, 2019). As previously reported, the CSL is also applicable to use as a component of growth media, fertilizers and soil conditioners when directly added into soil (Filipović *et al.*, 2001; Obayori *et al.*, 2015). Low concentration of corn steep liquor promotes seed germination, plant growth in soybean and promotes lettuce growth via its antifungal and bio-stimulatory effects (Chinta *et al.*, 2014; Zhu *et al.*, 2019).

However, to the best of our knowledge, there have been few reports on the use of CSL for the production of biofertilizer by symbiotic fermentation. This study

investigated the feasibility of using CSL as the main raw material for production of bio-fertilizer as Plant Growth-Promoting Microbes (PGPM). The study developed an innovative view for applying CSL nutrition to cultivate various microorganism for improving the soil matrix and promoting the growth of plants. While reducing CSL overstock, the developed bacteria as biofertilizers can reduce the use of chemical fertilizers and subsequently to reduce the environmental pollution.

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## Materials and Methods

### Raw Materials

CSL were obtained by Luzhou Bio-chem Technology Limited. The CSL with pH  $4.3 \pm 0.1$ , Amino Acid Nitrogen (AAN) content of  $9.71 \pm 0.18$  g/L, moisture content of  $67.14 \pm 3.3\%$ , total sugar content of  $4.52 \pm 0.1\%$ , reducing sugar content of  $40.33 \pm 0.92$  g/L, ash content of  $7.46 \pm 0.3\%$ , soluble phosphorus content of  $4.39 \pm 1.18$  g/L, which were measured in previous studies (Zhao *et al.*, 2020).

Nine strains were collected from industrial production, which including four bacteria (*Bacillus subtilis* 3301, *B. subtilis* A308, *B. subtilis* N52, *B. Licheniformis* F18), one yeast (*Saccharomyces cerevisiae*), four fungi (*Aspergillus Niger*, *A. oryzae*, *Trichoderma harzianum*, *T. longi*). These strains were defined as PGPMs for developing a composite bio-fertilizer.

### Methods

#### Culture

The corn pulp liquor stock was diluted 5 times with water 20% (w/v) and was adjusted to pH 6.5-7.0 with NaOH (1M). Then the CSL medium was sterilized at  $121^\circ\text{C}$ , for 20 min. The bacteria were cultivated in nutrient broth LB (10 g/L tryptone, 5 g/L yeast extract, 5g/L NaCl); while fungi were cultivated in a modified Martin medium with peptone 5.0 g/L, dipotassium hydrogen phosphate 1.0 g/L, magnesium sulfate 0.5 g/L, yeast extract powder 2.0 g/L, glucose 20.0 g/L for seed culture. All fermentation processes were performed by transferring 10% (w/v) of selected strain inoculums. A 50 mL CSL medium was added in 250 mL flask and incubated at  $30^\circ\text{C}$  with shaking speed 160 rpm for 72 h for biomass production.

### CSL Microbial Diversity Investigation

For deep understanding microbial preference in CSL, the original microbial diversity was analyzed by high-throughput sequencing technology. Genomic DNA was extracted using the Mag-Bind Soil DNA Isolation Kit (Omega Bio-tek, Norcross, GA, U.S.) according to the manufacturer's instructions. The extraction procedures of CSL were conducted in triplicate. Each sample was tested for DNA integrity by agarose gel electrophoresis. Qubit2.0 DNA detection kit was used to accurately quantify genomic DNA. The primers 27 F and 1541 R were used for the amplification of bacterial 16S rDNA genes. High-throughput sequencing was performed using an Illumina Miseq™ platform. The Ribosomal Database Project (RDP) classifier was applied for the alignment analysis of each sample sequence and the species information of the original flora in CSL was expressed at the genus level.

### Phosphorus-Dissolving Characteristics Determination

The ability of the tested strains to dissolve different insoluble Phosphates (P) in liquid medium were investigated in Pikovskaya (PVK) medium containing 5.0 g  $\text{Ca}_3(\text{PO}_4)_2$  (Ogbo, 2010). To determine the dissolving ability of organophosphorus,  $\text{Ca}_3(\text{PO}_4)_2$  was substituted by phosphatidylcholine in the PVK medium. Inoculate 10% (v/v) of the tested strains individually into 50 mL inorganic/organic PVK mediums and cultured them at  $30^\circ\text{C}$ , 160 rpm, for 12 h. All the experiments were carried out in triplicates. The soluble phosphorus content and pH of each culture were determined after 72 h.  $\Delta\text{P} = \text{C}_\text{P} - \text{C}_{\text{P}0}$  ( $\text{C}_\text{P}$  the content of soluble phosphorus in fermentation broth.  $\text{C}_{\text{P}0}$  represented the initial content in CSL medium without microbial inoculation).

### Protein-Dissolving Characteristics Determination

In order to study the decomposition ability of protein to Amino Acid Nitrogen (AAN) by different strains,  $\Delta\text{AAN}$  was determined for different strains cultured at 72 h in CSL medium.  $\Delta\text{AAN} = \text{C}_\text{N} - \text{C}_{\text{N}0}$  ( $\text{C}_\text{N}$  the content of Amino Acid Nitrogen (AAN) in fermentation broth;  $\text{C}_{\text{N}0}$  represented the initial AAN content in CSL medium without microbial inoculation).

### The Effect of Different Neutralizer on PGPMs' Biomass, Phosphorus and Protein Dissolving Ability in CSL Culture

The initial pH of corn pulp is very low (~2) and a large amount of pH regulator need to be added to adjust the CSL medium to neutral. However, because different pH

regulator contains different kinds of salt ions and the addition of these salt ions may have different effects on the growth of PGMs as well as on the ability of phosphorus and nitrogen hydrolysis. Therefore, we did a comparative study, to find out the best pH regulator for promoting microbial proliferation in corn pulp.

In this test, the CSL medium 20% (w/v) was adjusted to pH 6.5 with different pH neutralizers, which including NaOH (2M), KOH (2M), Ca (OH)<sub>2</sub> (2M) and NH<sub>3</sub>·H<sub>2</sub>O (2M), respectively. Then three selected dominant strains (*Bacillus subtilis* 3301, *Bacillus licheniformis* and *Aspergillus Niger*) were cultured in CSL mediums and the fermentation processes were sampled to determine the indicators.

### *Symbiotic Fermentation*

Spore suspension of *A. Niger* was inoculated into a 5 L fermenter with 5% inoculum volume. The initial aeration rate was 2 NL/min; the agitation speed was 300 rpm and the pH was controlled to 6.5 by automatic flow of 2 mol/L KOH and H<sub>2</sub>SO<sub>4</sub>, respectively; the initial culture temperature was 30°C. The seed solution of *B. subtilis* and *B. licheniformis* was inoculated into a 5 L fermenter with 5% inoculum after 24 h, respectively; then the pH was adjusted to 7.0 by 2 mol/L KOH and H<sub>2</sub>SO<sub>4</sub>, respectively; the temperature was adjusted to 37°C; the aeration volume was controlled to 4 NL/min. During the fermentation process, the glucose was fed with 40% at a concentration of 10 mL/L flow rate for 24 h.

### *Analysis Methods*

Soluble phosphorus in culture supernatant was determined by molybdate blue colorimetric method (Zhu *et al.*, 2012). Reducing sugars were analyzed applying 3,5-Dinitrosalicylic acid (DNS) method (Deng and Tabatabai, 1994). Total sugars were assayed with sulphuric acid-phenol method (Lu *et al.*, 2008). The microbiological analysis was carried out using plate count method (Namfon *et al.*, 2017). Reference analysis of amino acid nitrogen (AAN) content was in accordance with the Formaldehyde Colorimetry (Zhang *et al.*, 2018). All the experiments were conducted in triplicates.

## **Results and Analysis**

### *Original Colonized Microbial Diversity Investigation in CSL*

For deep understanding microbial preference in CSL, the original microbial diversity was analyzed. Results showed that the diversity of bacteria was

relatively rich than fungi in CSL as show in Fig. 1. The results showed the ratio of each species as *Clostridium sensu stricto* (68.76%), *Bacillus* (3.98%), *Brevundimonas* (2.53%), *Pseudomonas* (1.6%), *Lactobacillus* (1.45%), *Paenibacillus* (1.42%), *Lysinibacillus* (1.41%), respectively. So, the *C. sensu stricto* is the dominant genus of the original bacteria group in CSL. It is a strictly anaerobic bacilli, which generally produces acetic acid and butyric acids and other small organic molecules, however, it is not efficient as compared with Plant Growth-Promoting Microbes (PGPM) (Hu *et al.*, 2016). Results shows *Bacillus* species take account of 3.98% in the bacterial species and it is the second dominant species in the CSL, which indicate it is compatible with the CSL environment. Meanwhile, *Bacillus* species are robust producing bio-fertilizers, so this study selected *Bacillus* spp. for CSL culture performance.

### *Comparison of Biomass in CSL with Different Potential Strains*

Nine strains represent the robust producing bio-fertilizers in various application fields were tested in this part. They are *B. subtilis* 3301; *B. subtilis* A308; *B. subtilis* N52; *B. licheniformis*; *S. cerevisiae*; *A. Niger*; *A. oryzae*; *T. harzianum*; *T. longi*. The growth of different strains in CSL medium for 72 h was shown in Fig. 2. Obviously, the number of *Bacillus* spp. was significantly higher than that of other strains after 72 h, which expressed obvious performance growth in CSL medium. The cells count of *B. Subtilis* 3301 was up to 3.1×10<sup>9</sup> CFU/mL in CSL medium. While, *T. longi* grew well in the CSL medium as well, which produced a large number of hyphae during liquid culture. The spore number of *T. longi* was up to 7.6×10<sup>6</sup> CFU/mL in CSL medium after cultivation for 72 h and expressed the highest living fungal count according to the cultivation result on petri dish. However, *A. niger* and *A. oryzae* showed lower growth levels (cell concentration of 3.5×10<sup>6</sup> CFU/mL and 3.2×10<sup>6</sup> CFU/mL respectively) in CSL medium culture.

And the cell number of yeast *S. cerevisiae* reached up to 5.5×10<sup>8</sup> CFU/mL in CSL medium after 72 h culture. However, the growth condition is not comparable with *S. cerevisiae* in other researches. Therefore, *B. subtilis* strain 3301 and *T. longi* performed better growth in CSL fermentation medium.

### *Comparison of Phosphorus-Dissolving Ability- A. Niger Expressed Excellent Phosphorus-Dissolving Ability*

Figure 3, all strains expressed certain capacity to higher than that of other strains which dissolving organic and

inorganic phosphorus and reached at highest 1100 mg/L inorganic phosphorus. On the other hand, the ability of dissolve organophosphorus for all other organisms were higher than that of *Bacillus* (the lowest is *B. subtilis* 3301,

which shows 28 mg/L) in this study and *A. Niger* and *T. harzianum* have a stronger dissolving capacity for organophosphorus than other strains with about 140 mg/L.

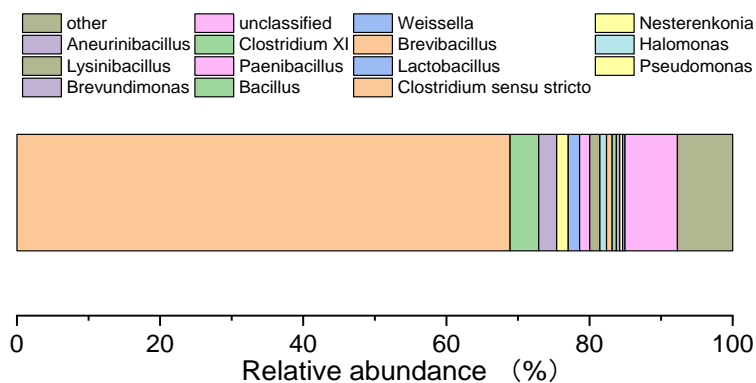


Fig. 1: Microbiome abundance of CSL sample at the level of genus

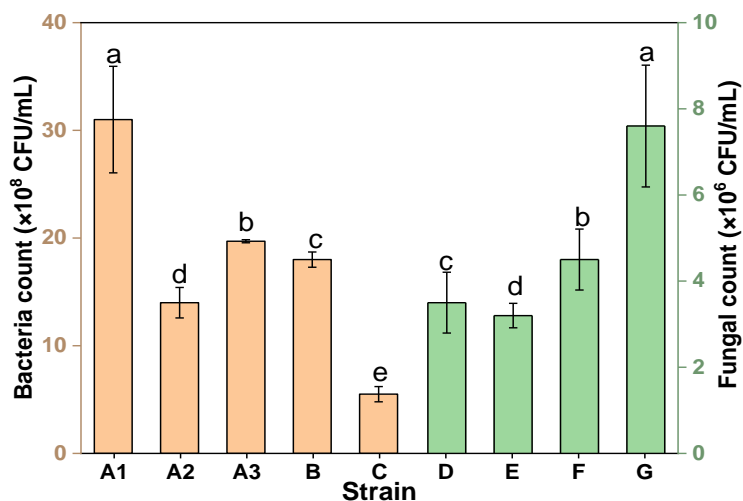
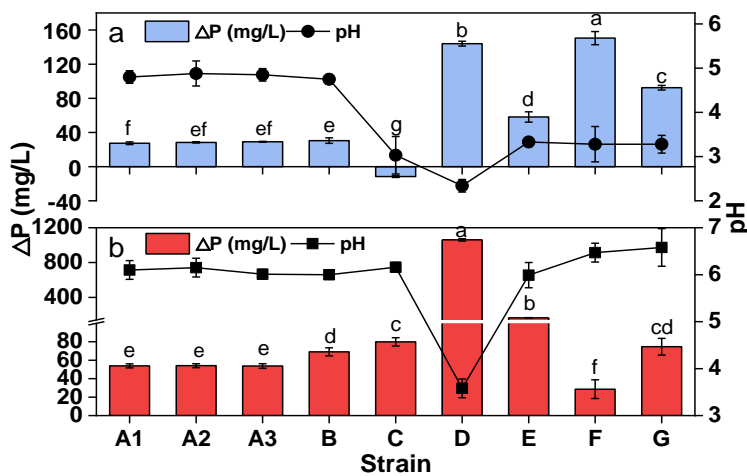
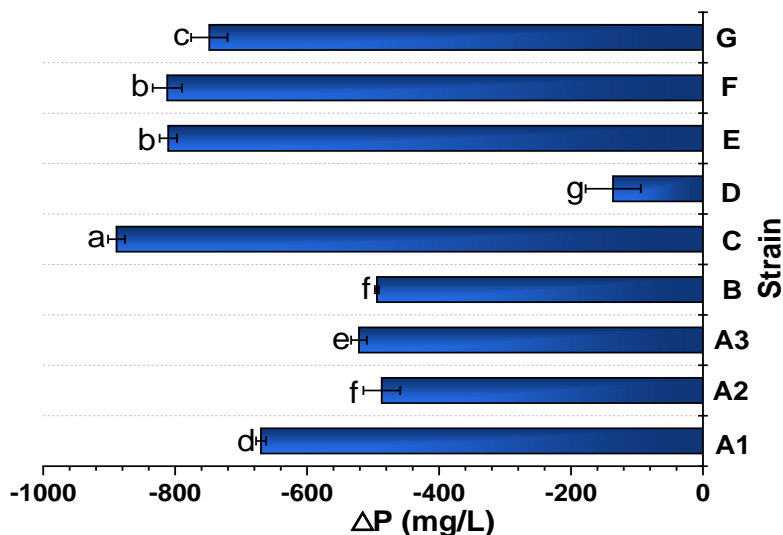


Fig. 2: Microbial count of tested stains cultivated in CSL medium for 72 h. A1: *B. subtilis* 3301; A2: *B. subtilis* A308; A3: *B. subtilis* N52; B: *B. licheniformis*; C: *S. cerevisiae*; D: *A. Niger*; E: *A. oryzae*; F: *T. harzianum*; G: *T. longi*. Error bars represent standard deviations ( $n = 3$ ), Lowercase letters indicate significant differences ( $P < 0.05$ )





**Fig. 3:** The  $\Delta P$  and pH of different PGPMs cultivated in PVK medium for 72 h. (a)  $\Delta P$  and pH in medium with organic P; (b)  $\Delta P$  and pH in medium with inorganic P. A1: *B. subtilis* 3301; A2: *B. subtilis* A308; A3: *B. subtilis* N52; B: *B. licheniformis*; C: *S. cerevisiae*; D: *A. Niger*; E: *A. oryzae*; F: *T. harzianum*; G: *T. longi*. Error bars represent standard deviations ( $n = 3$ ). Lowercase letters indicate significant differences ( $P < 0.05$ ). The  $\Delta P$  of different PGPMs cultivated in CSL medium for 72 h. A1: *B. subtilis* 3301; A2: *B. subtilis* A308; A3: *B. subtilis* N52; B: *B. licheniformis*; C: *S. cerevisiae*; D: *A. Niger*; E: *A. oryzae*; F: *T. harzianum*; G: *T. longi*. Error bars represent standard deviations ( $n = 3$ ). Lowercase letters indicate significant differences ( $P < 0.05$ )

Meanwhile, the *A. Niger* expressed a lowest pH (2.3 in organic PVK medium and 3.5 in inorganic PVK medium respectively) and all strains showed that a trend with that the low pH value accompanied high soluble phosphorus content. These results suggests that the low pH significant related to the robust conversion ability from insoluble phosphorus to soluble phosphorus. Also, our findings demonstrated that *A. Niger* has a stronger ability to dissolve both insoluble organic phosphorus and inorganic phosphorus than other strains, which suggested *A. Niger* a promising candidate for servicing a functional microorganism as bio-fertilizer.

CSL is rich in phosphorus, partially in the form of calcium and magnesium phytate. The phosphorus-dissolving experiment was also tried in the CSL. The net added value ( $\Delta P$ ) of phosphorus was investigated and found soluble phosphorus of only *A. Niger* was significantly higher than that of other tested strains, which expressed same comparing with the results in PVK medium. The  $\Delta P$  was negative in the fermentation supernatant of all the strains, which indicated that, all the strains consumed the soluble phosphorus in the CSL for microbial growth. However, when added 4% glucose in CSL, the  $\Delta P$  value in *A. Niger* CSL culture changed from negative to positive, which indicates adding glucose as carbon source improved the ability of *A. Niger* to dissolve phosphorus. The results showed that soluble phosphorus could effectively promote the growth of all strains and *A. Niger* could better convert insoluble calcium magnesium phosphorus into soluble phosphorus.

#### Amino Acid Nitrogen (AAN) Generation of PGPMs in CSL

CSL is rich in soluble protein which supports excellent resource of organic nitrogen for various cells growth. AAN decomposed from protein could be directly absorbed and utilized by plant roots and soil microorganisms, a great candidate for bio-fertilizer (Zhang *et al.*, 2016). The  $\Delta AAN$  value indicated the dynamic changes of amino acid nitrogen generated from protein hydrolysis in CSL by different strains. Results showed that *B. licheniformis* obtained the highest  $\Delta AAN$  value of 600 mg/L, which means this strain has high ability for protein hydrolysis. In addition, the  $\Delta AAN$  in yeast was significantly lower than that in other strains, which expressed low protein hydrolysis ability.

#### Symbiotic Fermentation Studies

To improve the efficiency of CSL bio-fertilizers, three strains *B. subtilis* 3301, *B. licheniformis* and *A. niger* have been selected for mixed culture in different combinations in CSL medium for 72 h. The cell count results were showed in Fig. 5a. It can be seen that when the three strains were mixed together (A1+B+D as shown in Fig. 5a), the total microbial count was the highest. The bacterial count could reach  $3.8 \times 10^8$  CFU·mL<sup>-1</sup> and the fungal count could reach  $0.9 \times 10^6$  CFU·mL<sup>-1</sup>. While when *B. licheniformis* and *Aniger* were mixed (B+D as shown in Fig. 5a), the total cell count was the lowest, but the fungal count of *A. niger* was the highest, reached up to  $5.1 \times 10^6$  CFU·mL<sup>-1</sup>. As the pH could be significantly reduced by *A. niger* in the previous result.

Our findings also indicated that the pH decrease affect the growth of *Bacillus* when *Bacillus* was mixed with *A. Niger*. Therefore, it is necessary that pH is controlled at an appropriate level during the symbiotic fermentation process.

The fermentation property of soluble phosphorus and amino acid nitrogen in various mixed culture in CSL medium for 72 h were shown in Fig. 5b. The  $\Delta P$  was positive when mixed with *Bacillus* and *A. Niger* (A1+D and B + D as shown in Fig. 5b). The results indicated that insoluble phosphorus was converted into soluble phosphorus in the mixed culture during fermentation process. The content of AAN in mixed culture of *B. licheniformis* and *A. Niger* (B+D as shown in Fig. 5b) was higher than other combinations, but the difference was not clear. However, during the mixed culture of *B. subtilis* and *B. licheniformis* (A1+B) and the mixed culture of the three strains (A1+B+D), soluble phosphorus and amino acid nitrogen in the culture medium was used for self-growth and metabolism, resulting in the decrease of  $\Delta P$  and  $\Delta AAN$ .

#### The Effect of Different Neutralizer on three Selected Strains' Biomass, Phosphorus and Protein Dissolving Ability in CSL Culture

Multiple neutralizers, NaOH, KOH, Ca (OH)<sub>2</sub> and NH<sub>3</sub>·H<sub>2</sub>O were applied to control the pH in the CSL medium and the mentioned indicators were investigated following fermentation time. The results showed that, Ca(OH)<sub>2</sub> and KOH could increase slightly the cells count than other pH neutralizer. The  $\Delta AAN$  of each strain with NH<sub>3</sub>·H<sub>2</sub>O as pH neutralizer was significantly lower than that of other pH neutralizers. While for soluble phosphorus generation, we found that Ca(OH)<sub>2</sub> could reduce  $\Delta P$  in the fermentation broth. This may because the Ca<sup>2+</sup> in Ca(OH)<sub>2</sub> could chelate with phosphate radical into insoluble Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> and reduce the content of soluble phosphorus in fermentation broth. The results showed that KOH had no adverse effect on fermentation if compared with other pH, meanwhile, it could increase the K<sup>+</sup> content of fermentation broth which suitable for plant utilization. Therefore, KOH was defined as the appropriate pH neutralizer for bio-fertilizer of CSL fermentation in this study.

To evaluate the growth characteristics of three strains in CSL medium with KOH as a pH regulation, the changing of tested strains of *B. subtilis* 3301, *B. licheniformis* and *A. Niger* were carried out and results shown in (Fig. 7). Firstly, the three strains entered stable period after 48 h and living cells count seems to be stable. Secondly, the AAN of the two *Bacillus* species reached the highest at 48 h and then decreased. But *A. Niger* showed increasing AAN from 24 h, because it could produce protease to decompose the protein into free amino acids after cells growth. On the contrary, phosphorus of two *Bacillus* species started to decrease after 24 h when *A. Niger* increase. *Bacillus* had low phosphorolysis capacity but cells growth consumed huge soluble phosphorus during the growth process. However,  $\Delta P$  first decreased and then increased in the fermentation

supernatant of *A. Niger*, so the content of soluble phosphorus first decreased and then increased in the growth process. *A. Niger* has strong phosphorus hydrolysis ability and may used soluble phosphorus for its cell synthesis and at the same time, it metabolized organic acid production and phosphatase to convert insoluble phosphorus into soluble P.

#### Symbiotic Fermentation in 5L Scale Fermenter

It can be seen that the logarithmic growth phases of the three strains were different during the symbiotic fermentation process in CSL. While, *B. subtilis* entered the logarithmic growth phase and grew rapidly to the highest cell number after 8 h of inoculation and the maximum cell number of *B. subtilis* reached 3.0×10<sup>9</sup> CFU·mL<sup>-1</sup> at 32 h. Also, *A. Niger* entered the logarithmic phase and the maximum cell number reached 1.1×10<sup>6</sup> CFU·mL<sup>-1</sup> at 45 h. Finally, *B. licheniformis* entered the logarithmic growth phase, with the highest cell number reaching 3.2 ×10<sup>9</sup> CFU·mL<sup>-1</sup> at 52 h. *B. subtilis* and *A. Niger* are aerobic microorganisms. The Dissolved Oxygen (DO) decreased rapidly from 0 to 15 h in the fermentation system. *A. Niger* quickly capture DO in the fermentation substrate for growth and metabolism. After 24 h inoculation with *B. subtilis* and *B. licheniformis*, the ventilation rate was adjusted from 2 NL/min to 4 NL/min. Competed with *A. Niger* for dissolved oxygen, *B. subtilis* quickly utilized DO in the culture medium and grew rapidly. In the late growth stage, the DO was insufficient and the number of *B. subtilis* cells slowly decreased at 32 h. Meanwhile, *A. Niger* fungus start to grow till the highest number at 45 h. *B. licheniformis*, as a facultative anaerobe, began to grow rapidly at 36 h. In the late stage of growth, the nutrient substances in the medium are lacking and the numbers of *B. licheniformis* and *A. Niger* decreased. Therefore, it is necessary to extend the fixed period of *B. subtilis* by increasing the DO and feeding in the fermentor Fig. 4.

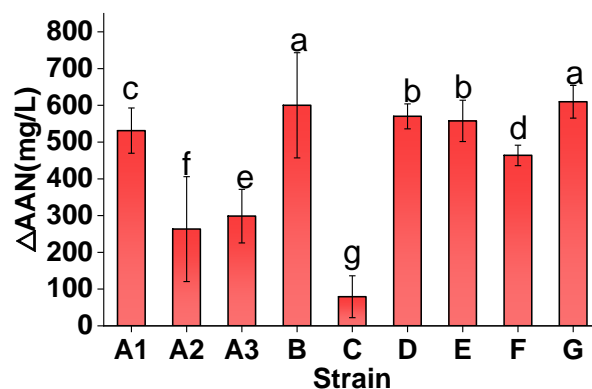
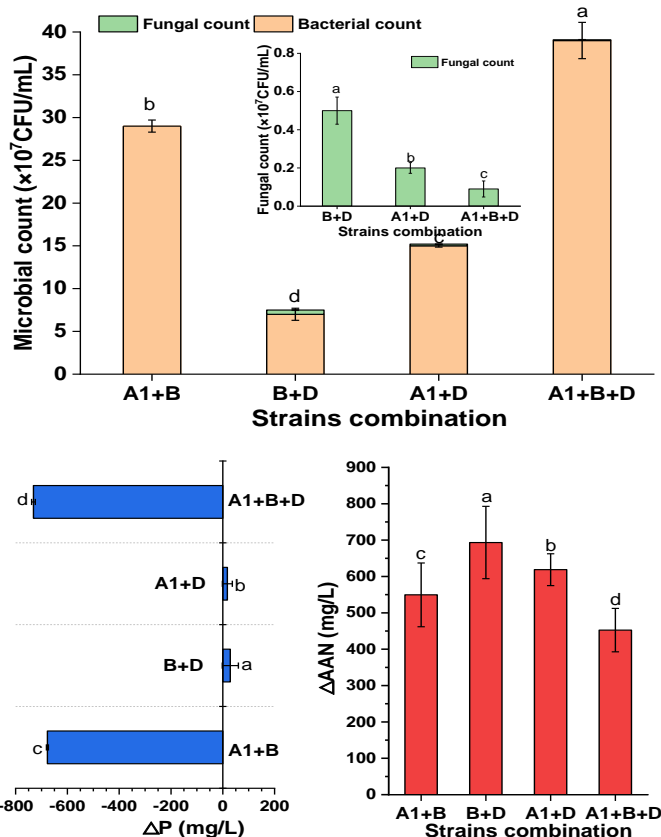
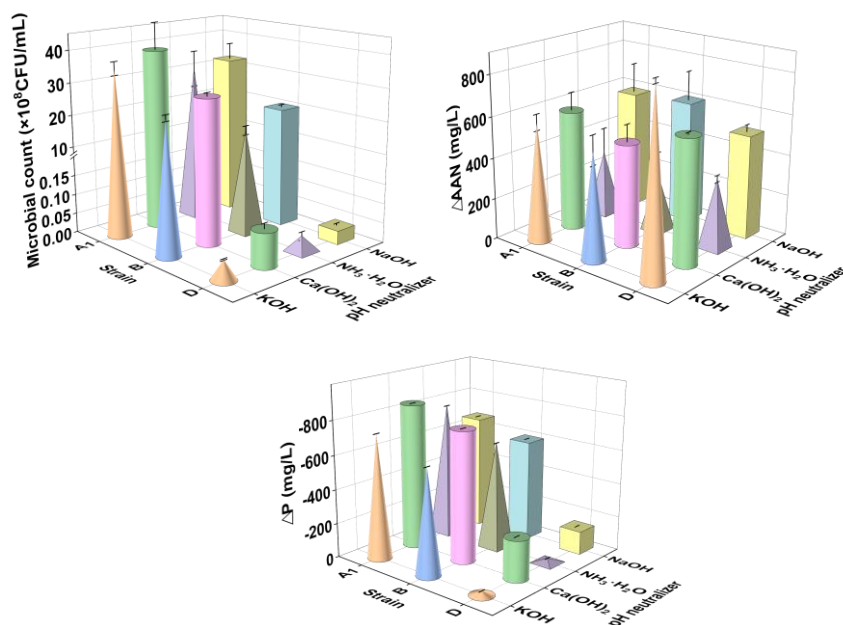


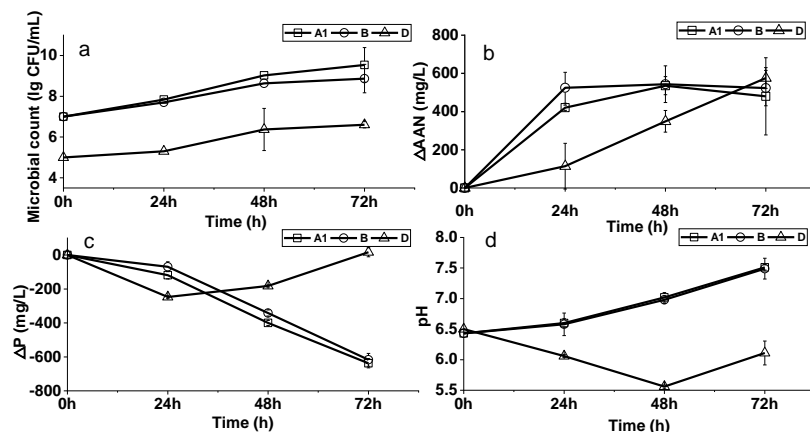
Fig. 4: The  $\Delta AAN$  of different PGPMs cultivated in CSL medium for 72 h. A1: *B. subtilis* 3301; A2: *B. subtilis* A308; A3: *B. subtilis* N52; B: *B. licheniformis*; C: *S. cerevisiae*; D: *A. niger*; E: *A. oryzae*; F: *T. harzianum*; G: *T. longi*. Error bars represent standard deviations (n = 3). Lowercase letters indicate significant differences (P<0.05)



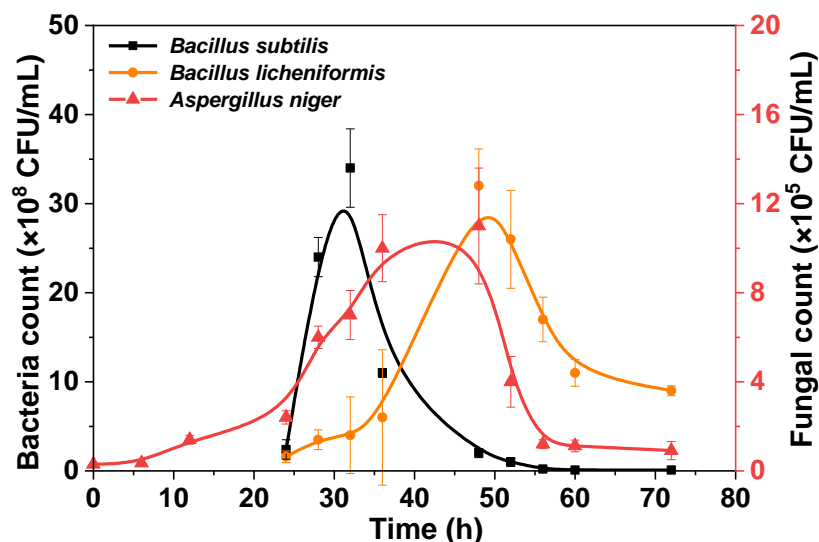
**Fig. 5:** Microbial count of selected strains mixed cultivated in CSL medium for 72 h. A1: *B. subtilis* 3301; B: *B. licheniformis*; D: *A. Niger*. Error bars represent standard deviations (n = 3). Lowercase letters indicate significant differences (P < 0.05). The  $\Delta P$  and  $\Delta AAN$  of selected strains mixed cultivated in CSL medium for 72 h. A1: *B. subtilis* 3301; B: *B. licheniformis*; D: *A. Niger*. Error bars represent standard deviations (n = 3). Lowercase letters indicate significant differences (P < 0.05).



**Fig. 6:** Microbial count,  $\Delta AAN$  and  $\Delta P$  of selected strains cultivated in CSL medium with different pH neutralizer (NaOH, KOH, Ca(OH)<sub>2</sub>, NH<sub>3</sub>·H<sub>2</sub>O) for 72 h. A1: *B. subtilis* 3301; B: *B. licheniformis*; D: *A. Niger*. Error bars represent standard deviations (n = 3).



**Fig. 7:** Change in microbial count,  $\Delta$ AAN,  $\Delta$ P and pH during cultivating in CSL medium. A1: *B. subtilis* 3301; B: *B. licheniformis*; D: *A. Niger*. Error bars represent standard deviations (n = 3)



**Fig. 8:** Time course of microbial count during the symbiotic fermentation process in a 5L fermentor. Error bars represent standard deviations (n = 3)

Time data of the  $\Delta$ P and  $\Delta$ AAN during the symbiotic fermentation process was shown in Fig. 8.  $\Delta$ AAN showed the opposite trend to  $\Delta$ P in fermentation broth when mixed culture.  $\Delta$ AAN is negative at the beginning of fermentation and then  $\Delta$ AAN increased fastly between 24 h to 52 h, which expressed a high increasing speed about  $30 \text{ mg}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$ . *Bacillus* and *A. Niger* produces more proteases to break down the protein into amino acids during fermentation in CSL.  $\Delta$ AAN increased to the peak at 52 h, then start to decrease, because the *A. Niger* and *B. licheniformis*, both reached the max biomass ( $10.4 \times 10^5$  CFU $\cdot$ mL $^{-1}$  and  $30.0 \times 10^8$  CFU $\cdot$ mL $^{-1}$  respectively) at about 52 h, which use the amino acids in CSL for growth and metabolism at the beginning of fermentation. So, the

nutrient substances were deficient in CSL after 52 h, the protein was basically decomposed and used and the microorganisms began to use amino acids for their cell growth, resulting in  $\Delta$ AAN reduction.

$\Delta$ P is positive at the beginning of fermentation and then  $\Delta$ P decreased fast between 15 h to 35 h, which expressed a high decreasing speed about  $35 \text{ mg}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$ . *A. Niger* could convert part of insoluble phosphorus into soluble phosphorus during this period in CSL.  $\Delta$ P decreased to the minimum value at 52 h, because the *A. Niger* and *Bacillus* reached the max biomass at about 52 h. They converted phytic acid in CSL into free phosphate for own cell growth. Therefore, the soluble phosphorus in CSL can effectively promote the growth of functional microbe.



## Discussion

As a beneficial soil supplements, bio-fertilizers have made important contributions to ecological agriculture (Bhardwaj *et al.*, 2014). In particularly, there is great potential to develop bio-fertilizers with agricultural waste as fermentation substrate (Du *et al.*, 2018). For example, Xu *et al.* (2014) successfully transformed the waste water from the production of sweet potato starch into a bio-fertilizer for tea tree polymyxomycetes (Xu *et al.*, 2014); Ogbo and Namfon converted cassava waste and monosodium glutamate by-product into bio-fertilizer respectively (Namfon *et al.*, 2017; Ogbo, 2010). Many reports showed that the low concentration of CSL could promote the germination of soybean seeds and the growth of plants (Zhu *et al.*, 2019) and it can also promote the root of lettuce and repair the soil (Chinta *et al.*, 2014; Salam and Ishaq, 2019).

In this study, the microbial diversity of original CSL environment was analyzed by high-throughput sequencing technology, which provided a theoretical basis for understanding CSL microbial preference. Results showed that the original microbiome is in an anaerobic environment and 68.76% strains were *Clostridium sensu stricto* in CSL, which was the dominant group. At the same time, various *Bacillus* genera also take part in high abundance. Therefore, in the further study, facultative anaerobe bacteria were tried for bio-fertilizers fermentation by CSL. Finally, nine strains were collected that had been used in the bio-fertilizers industry. These strains have been shown to be effective in promoting plant growth and play an important role in agricultural production (Al-Taweil *et al.*, 2010; García-Fraile *et al.*, 2015; Karajeh, 2013; Kayasth *et al.*, 2013; Ogbo, 2010; Sun *et al.*, 2018).

An effective way to improve the sustainability of plant phosphorus nutrition is to find microorganisms with excellent phosphorus-increasing properties to produce bio-fertilizers. In this study, the ability of 9 strains to dissolve phosphorus were investigated. All strains have a certain ability to dissolve insoluble P, especially *A. niger* species. This is basically consistent with previous studies (Kapri and Tewari, 2010; Ogbo, 2010; Paul and Rao., 1971; Rojas *et al.*, 2001). Many researchers have studied the growth of phosphorus-dissolving bacteria in insoluble phosphorus and studied the mechanism of phosphorus-dissolving microbes (Wang *et al.*, 2015; Whitelaw, 1999). In this study, pH was negatively correlated with the content of soluble P. This perhaps that during the fermentation process, phosphorylating microorganisms produced malic and citric acids and other organic acids that reduced the pH value of the fermentation broth (Zhu *et al.*, 2012). Organic acids dissociate insoluble phosphoric acid and hydrochloric acid, chelate and exchange reactions to dissociate phosphoric acid (Illmer and Schinner, 1995). In addition, phosphatase produced by phosphorus-dissolving microorganisms also

has a role in digestion and degradation of insoluble P (Achal *et al.*, 2007; Raghothama, 1999).

The key fact affecting the quality of bio-fertilizer is the number of functional microorganisms. In this study, the number of viable bacillus could reach  $10^9$  CFU/mL and the number of fungi can reach  $10^6$  CFU/mL after 72 h of fermentation in CSL medium. Xu *et al.*, 2014 optimized the conditions of fermentation sweet potato starch production wastewater and the maximum biomass of *P. polymyxa* was  $9.7 \times 10^9$  CFU/mL at 16 h (Xu *et al.*, 2014). they indicated the bioconverted agricultural waste into compost and the fungal biomass in the compost inoculated with *Aspergillus fumigatus* reached a maximum of  $1.7 \times 10^6$  CFU/g (Chang and Yang, 2009). However, CSL can achieve high fermentation density without adding other nutrients, which indicates that CSL has great advantages in the development of bio-fertilizers. Moreover, the content of soluble phosphorus of each strain in the fermentation broth increased after adding 4% glucose, indicating that the addition of glucose could increase the content of soluble phosphorus in the fermentation broth of each strain. This may be due to the increase of C/N and organic acid content after the addition of glucose, which improves the chelation of insoluble phosphorus and other reactions and may also increase the activity of phosphatase (Relwani *et al.*, 2008). Reported that mannitol and glucose were the best carbon sources for phosphorus dissolution in *A. niger* (Seshadri *et al.*, 2004).

Based on the number of viable cell count, protein and phosphorus-dissolving capacity of 9 strains in CSL, *B. subtilis*, *B. licheniformis* and *A. niger* were selected as target strains. Three strains had a certain ability to dissolve phosphorus and notably *B. subtilis* grew rapidly in CSL with the highest number of viable bacteria and *B. licheniformis* had strong protease activity with the highest content of AAN in CSL. Kayasth *et al.* (2013) identified *B. licheniformis* as an ideal strain for the development of multifunctional bio-fertilizer, which can produce Indoleacetic Acid (IAA) and iron-chelating agent, dissolved phosphate and has the characteristics promoting growth plant microbe (Kayasth *et al.*, 2013). Multiple studies have been confirmed that *B. subtilis* bacteria promotes plant growth in IAA production, biological control, soil restoration and other aspects (Idris *et al.*, 2007; Mikutta *et al.*, 2012; Ye *et al.*, 2012). In addition, the phosphorus dissolution capacity of *A. niger* was significantly higher than that of other strains and was also better than that of *A. niger* which Frank C. Ogbo had reported.

CSL has a high acidity with pH value of about 4.3. Adjust the pH with conventional NaOH will introduce a large amount of  $\text{Na}^+$ , which will cause soil salinization.  $\text{Ca}(\text{OH})_2$  is low in alkalinity which widely used as a pH neutralizing agent.  $\text{NH}_3 \cdot \text{H}_2\text{O}$  as a pH neutralizing agent will make the C/N ratio lower, which will have an adverse effect on the fermentation process and metabolites (Wang *et al.*, 2000).

In this study, the effects of different pH on the fermentation of CSL bio-fertilizer were studied. The effect of KOH and pH on the fermentation of CSL was not significantly different and the content of K<sup>+</sup> in bio-fertilizers was increased. As results demonstrated in Fig. 6, the growth of the three strains in CSL medium with KOH neutralizer increased. The results showed that the content of AAN and soluble phosphorus in the fermentation broth of two *Bacillus* spp. and *A. Niger* was complementary.

Symbiotic fermentation is widespread in nature and sometimes the stable co-existence of microorganisms is more effective for multiple functions than single culture (Bader *et al.*, 2010). Marcel Gutierrez-Correa *et al.* (1999) studied mixed fermentation of *A. Niger* and *Trichoderma* spp. on sugarcane bagasse (Gutierrez-Correa *et al.*, 1999). Zuroff *et al.* (2013) mediated cellulose into ethanol by co-culture of symbiotic *Clostridium* bacteria with yeast. Although studies on symbiotic fermentation have been extensively reported, different microorganisms in the co-culture system may compete for the substrate and inhibit the metabolites, so co-culture has an effect on the growth of microorganisms compared to a single culture (Wu *et al.*, 2016). In this study, *B. subtilis* and *B. licheniformis* co-cultured with *A. Niger* respectively and the viable count was lower than that of single culture. The microbial count co-cultured by *B. licheniformis* and *A. Niger* was superior to that of *B. subtilis* and *A. Niger*. After co-culture with *A. Niger*, the content of soluble phosphorus in the culture solution increased, the pH decreased and the content of AAN increased, which also solved the adverse effect of excessive pH after *Bacillus* fermentation. The symbiotic fermentation was implemented through the fermentor and the interaction between the three strains was further tracked and evaluated in CSL. The growth of *Aspergillus Niger* was slow and it was inoculated at first to inhibition of inter-species competition led to the low number of microorganisms after co-culture. *B. subtilis* grows fast and has a high oxygen demand and it is inoculated after feeding at the later stage of fermentation and the decline of inter-microorganisms due to the lack of nutrients in the later stage. *B. licheniformis* is a facultative anaerobic bacterium. It should be inoculated in the middle of fermentation to increase the fermentation pH and decompose to produce more amino acids for later use by *B. subtilis* in CSL. The establishment of a symbiotic system between *Bacillus* and *A. Niger* and the increase in the number of *A. Niger* in the symbiotic system will be discussed further in future studies.

## Conclusion

In this study, the original microbial community of CSL was determined. 9 selected strains were studied as bio-fertilizer PGPMs depending their capacity of dissolving phosphorus and protein, as well as biomass

growth in CSL medium. Moreover, 3 strains *B. subtilis*, *B. licheniformis* and *A. Niger* were then selected for symbiotic fermentation. Based on the results, it is undeniable that this study is a systematic study on the development of bio-fertilizer for symbiotic fermentation of CSL and provides the importance and potential applications for the production of bio-fertilizer of CSL.

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## Author's Contributions

**Xiaojie Ren and Zhuangzhuang He:** Participated in the whole experiment process and also contributed to the interpretation of the results and manuscript preparation.

**Huihui Peng, Jing Zhang, Sihua Wang, Xiaoyu Li:** Participated in part of the experimental design and manuscript preparation.

**Yubin Zhao and Yuanda Song:** Ameliorated the manuscript.

**Xinhe Zhao and Chengye Ma:** Contributed to the guidance of experimental design and ameliorated the manuscript.

## Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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