Conversion of Food Waste and Feldspar into Biofertilizer Using a Stress-Tolerant Keldspar-Solubilizing *Bacillus Subtilis* Xue-113168

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Abbreviations

CMF	Compound Microbial Fertilizer
PGPR	Plant growth-promoting rhizosphere
SSF	Solid-state fermentation
SMF	Submerged fermentation
SMS	spent mushroom substrate
KSM	Potassium dissolving microorganism
LiCl	Lithium chloride
KCl	Kalium chloratum

1 Introduction

With the exhaustion of non-renewable resources like coal and peat, the advancing of technology also prompt the development of new sustainable sources of humic products (e.g. organic wastes) [1]. Biofertilizer without chemical fertilizer has a slow effect on crops, Compound Microbial Fertilizer (CMF) with stress-tolerant microorganisms combine the advantages of both biofertilizer and chemical fertilizer.

Microbial fertilizers promote biological fertility of soil, maintain and slow the release of chemical fertilizer, reduce the amount of nitrate nitrogen, heavy metals,

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and pesticides in crops, and reduce the occurrence of crop diseases [2]. Biofertilizers could mitigate crisis, e.g., the energy crisis, scarcity of resources, and environmental pollution [3]. Plant growth-promoting rhizosphere (PGPR) raises the resistance to pathogen by producing antibiotics and System resistance, etc. The CMF can palliate Jujube and Selenium-enriched Jujube from rust and anthracnose. Solid state fermentation using waste is a popular way of biofertilizer manufacturing. Low water activity in solid state fermentation (SSF) medium facilitates spore forming on the solid substrate. Unlike in submerged fermentation (SMF) processes, humidity is an important parameter of SSF. Microbial releases of potassium from K-bearing minerals have two steps, fermentation and bioleach [4]. The solubilization of potassium-bearing rock powder by Aspergillus niger in submerged fermentation lasted for 35-day period [5]. This study achieves it in one process for 7 days, saving production cycle and enhancing robustness. The imbalance of soil nutrients in China is the consequence of available potassium deficiency in the soil and shortages of potassium fertilizer, although K-bearing minerals in soil and mine, such as K-feldspar is rich, the amount of potassium dissolved by bacteria in soil is limited.

Keldspar can be dissolved by silicate bacteria such as *B. mucilaginosus, B. edaphicus, B. circulans*, *B. subtilis* which are part of the PGPR [6, 7]. Humus and Humic acids, which exist in spent mushroom substrates and biofertilizer, can complex with the potassium released from K-feldspar to improve its dissolving efficiency. Conversion of food waste and feldspar into biofertilizer using a stress-tolerant keldspar-solubilizing *Bacillus subtilis* Xue-113168 make the best use of agricultural and mineral resources, enhance crop yields and play an important role in the sustainable development.

2 Materials and Methods

2.1 Food Waste and Feldspar for SSF

In an SSF process, the solid substrate not only supplies anchorage for the cells, but also nutrients and air channel. A number of cheaply available raw substrates have been screened for the SSF. Shrimp shell, spent mushroom substrate (SMS) and corn bran of 0.1 mm, are all support material for ventilation in SSF. Chitin in shrimp shell and SMS can induce chitinase. K-feldspar containing 10.0% total potassium and insoluble potassium, was purchased from Lingshou County, Hebei, P.R. China. The shrimp shell was from Whiteleg. SMS of Pleurotus ostreatus, constitute of 4.45% organic matter, 1.58% total N, C/N ratio 28/1, water absorption rate 78–80%, humic acid content 20–30%, obtained from a local market. Corn bran constitute of 11.8% protein, 32% glucose, 1% cellulose, purchased from the North China Pharmaceutic Corporation, Hebei, P.R. China.

2.2 Potassium Dissolving Microorganism (KSM)

KSM are isolated from medium containing feldspar as the only potassium source (Hutchens et al. 2003). Medium's ingredients are as follows (g/L): starch, 5.0; yeast extract, 1.0; $MgSO_4 \cdot 7H_2O$, 0.5; $CaCO_3$, 0.1; $FeCl_3 6H_2O$, 5 mg, feldspar 10, pH 7.5. After 1-day incubation at 30 °C, the transparent colonies, which resemble half round glass bead, were picked and purified by growth of K-feldspar medium.

Stress-tolerance induction was carried out by semi-continuous culture in 50-ml shaking flasks at 30 °C on a reciprocal shaker (150 rpm). The cells were transferred to the induction solution which contains KCl, monosodium glutamate 4.27 mg%. Concentration of k is increasing at a rate of about 2.5% per circulation, from 2.5 to 15%.

This isolates were originally named *Bacillus circulans*, later identified as *Bacillus subtilis* by culturing characteristics and morphological observation, physiological and biochemical characteristics and identification of genetic characterization by Institute of Microbiology, Chinese Academy of Sciences in December 2016. The potassium dissolving rate was determined as = (St–Sc)/It*100%, where "St" and "Sc" are the water-soluble potassium in the treatment and matrix, respectively, and "It" = the total potassium in the treatment.

For UV's convenience and efficacy, breeding a high potassium dissolving rate *B. subtilis* strain had been performed using UV mutagenesis, screen in a fermentation broth using the potassium tetraphenylborate spectrophotometric method. After a series of mutation steps (Fig. 1), *B. subtilis* Xue-113168 has been obtained, which have chitinase and dissolving potassium function, preserved as patent strain in the Chinese General Microbiological Culture Collection Center (CGMCC) with the accession number 5155 [8].

2.3 CMF Preparation by Solid-State Fermentation (SSF)

The biofertilizer was made from SMS, shrimp shell, corn bran and feldspars using a simultaneously dissolving K-feldspar and SSF. Besides solid material, flour and feldspar were used as substrates at 10 and 2% (w/v), respectively, medium are sterilized in 121 °C, 0.1 MPa, for 30 min. An initial moisture content is 60–65%, and culture temperature is 30 °C. The SSF was stirred every 48 h for 7 days. The pH was nature during the fermentation of the SSF with *B. subtilis* Xue-113168. After 168 h of fermentation, the spore number and dissolving K rate are tested.

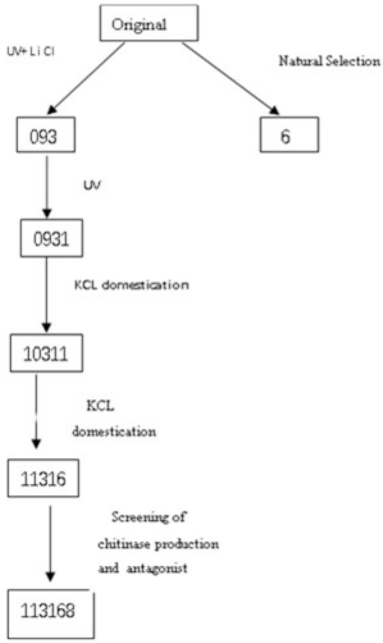

Fig. 1 Breeding pedigree of *B. subtilis* Xue-113168

2.4 Testing the Quality Index of the CMF

The amount of viable *B. subtilis* Xue-113168 in the CMF was calculated by counting colonies of the medium: (g/L) sucrose (5.0), $MgSO_4$ (1.0), $FeCl_3$ (0.2), yeast extract (0.2), $(NH_4)_2SO_4$ (0.5), KH_2PO_4 (1), pH 7.0. The moisture content was measured using the vacuum oven method. PH of Solid-water suspension (1:1) was determined using a pH meter. Reagent of digested CMF is H_2SO_4–H_2O_2. The total N K_2O P_2O_5 content was determined by the Kjeldahl method, potassium tetraphenylborate gravimetric method, molybdenum antimony anti-colorimetry respectively. The organic matter was digested and tested with H_2SO_4–$K_2Cr_2O_7$ method.

2.5 Statistical Analysis

Variance (ANOVA) of experimental data was analysed by a significance value of $p < 0.05$ and to Duncan's multiple range test (Duncan's) using SPSS 18.

3 Results and Discussion

3.1 Isolation and Breeding of Bacillus Subtilis Xue-113168

Bacillus subtilis Xue-113168 was isolated from a corn rhizosphere and breed by UV, UV+LiCl, natural selection, breed genealogy is showed in Fig. 1. Sieved by feldspar as the only source of potassium and colloid chitin for carbon. We use monosodium glutamate as osmotic protection, culture the stress-tolerance bacteria with a stepwise increase of KCl concentration, which are different from sorbitol [9], NaCl, glycerol and glucose as osmotic protection [10]. The biofertilizer are manufactured by *B. subtilis* Xue-113168 for dissolve potassium mineral substance in soil. Potassium dissolving microorganisms (KSM) is able to solubilize 'unavailable' forms of K-bearing minerals, such as micas, illite and keldspar, by excreting organic acids that either directly dissolves rock K or chelate silicon ions to bring the K into solution [11].

B. subtilis Xue-113168 has a high potassium dissolving rate (41%), also produces chitinase. Due to superiority alive in 8% K_2O of *Bacillus subtilis* Xue-113168, CMF had the combining advantage of both chemical fertilizer and biological fertilizer.

B. subtilis Xue-113168 colon morphology changes into opaque, *B. subtilis* Xue-10311 still transparent and *B. subtilis* Xue-103116 stay thickness and viscous (Fig. 2). The changes of colon morphology indicate the potential increase of stress-tolerant capacity.

Morphology of *B. subtilis* Xue-113168 is Gram-positive, rod shape showed in Fig. 3.

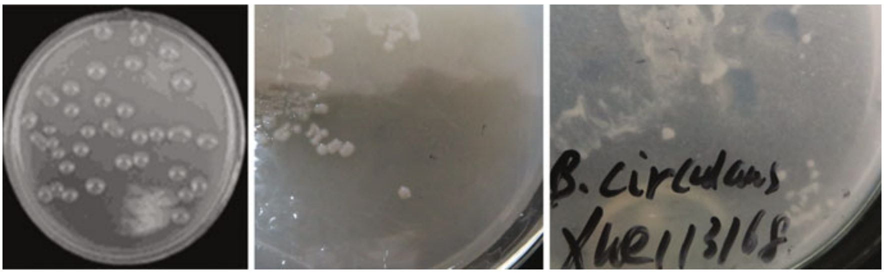

Fig. 2 Colon morphology of *B. subtilis* Xue-10311 (*left*), *B. subtilis* Xue-11316 (*middle*), *B. subtilis* Xue-113168 (primitive name *B. circulans*) (*right*)

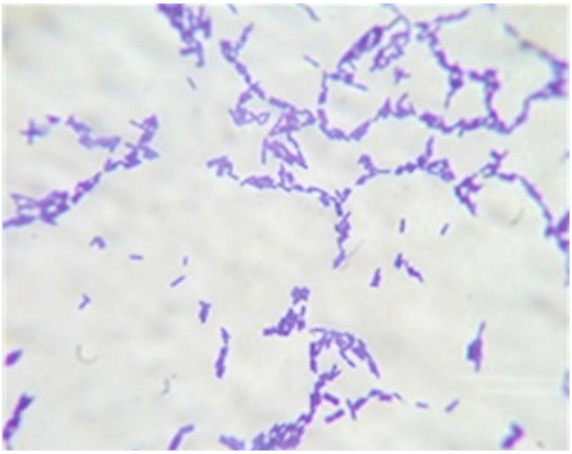

Fig. 3 Morphology of *B. subtilis* Xue-113168(*1600) by Gram Stain

3.2 Formulation of the Biofertilizer Produced by the SSF

The component of formulation is a key factor of the SSF and influences the overall cost significantly.

The formulation of SSF could be similar to that of a submerged fermentation (SMF) [12]. But sucrose was replaced with wheat flour for the SSF in this study and the spore formation rate of SSF is higher than SMF because these two processes have state and component of substance. The combination of corn bran, shrimp shell, and spent mushroom substrate was the best medium for the SSF. The K-dissolving rate (43.00%) and spore formation rate (83.18%) achieved were the best among all the groups, and the live bacteria amount of 9.16 lg (CFU) was second only to the spent mushroom substrate group (10.08 lg(CFU)).

The fermentation formulation for obtain a higher concentration of spores and potassium dissolving capacity for *B. subtilis* Xue-113168 was initially determined through a single factor experiment, then further through an orthogonal experiment. The range of $(NH_4)_2SO_4$ concentrations was the most important variable, suggesting that in the *B. subtilis* Xue-113168 culture, the effects of the three factors of the potassium dissolving ratio were $(NH_4)_2SO_4$ content > $MgSO_4$ content > wheat flour content by range analysis. The above three factors have evident effects on potassium dissolving ratio ($P < 0.05$) and the optimal conditions were: wheat flour content, 0.75%; $(NH_4)_2SO_4$ content, 0.1%; and $MgSO_4$ content, 0.2%.

As for the spore concentration, it can be concluded that the ranges of $(NH_4)_2SO_4$ and wheat flour concentrations were larger compared to the $MgSO_4$ concentration. Thus, the effects of the three factors of the spore concentration were $(NH_4)_2SO_4$ content > wheat flour content > $MgSO_4$ content, $MgSO_4$ is not significant factor for spore concentration ($p > 0.05$). Optimal conditions were: wheat flour content, 0.75; $(NH_4)_2SO_4$ content, 0.1%; and $MgSO_4$ content, 0.2%. The effect of ingredient composition and concentration in medium for spore formation is the same with potassium dissolving condition. The K-dissolving rates are different between the SMS and Corn bran + Shrimp shell + SMS in the SSF. The humic acids in the SMS can also dissolve the K-feldspar, which is complementary to the effect of *B. subtilis* Xue-113168.

Organic matter is 250 g kg^{-1} for the *B. subtilis* Xue-113168 high decompose performance to food waste. Organic matter reach 532 g kg^{-1} by adding humic acid.

3.3 Process Index of SSF

We observed some changes in pH in the reactor with different medium during the experiment. The pH decreased from 7.0 to 5.0 during the first day, recovered to 5.0–6.0 in a few days and then increased and maintained around 7.6 to the end of the experiment.

The Spore formation was affected by varietal spectrum of humidity and temperature. Controlling the moisture content of the SSF process was performed as follows: Initially the moisture content was adjusted to approximately 60–65%, at the end of the fermentation, the moisture content was 40–50%. The moisture content was controlled by environmental moisture and also by controlling the temperature in three phrases. During the prophase, the temperature was 33–35 °C, whereas, at the metaphase, the temperature dropped to 30–33 °C. Finally, it increased to 33–35 °C during the anaphase.

The fermentation time was also an important parameter. By the time that the rate of spores is 80%, the processes had typically ended. An appropriate temperature and humidity ensured the formation of the spores. When the fermentation last for 4 days, the potassium dissolving rate was 24.09%. After the fermentation time was extended to 7 days, the potassium dissolving rate increased to 41.53%.

The process of potassium dissolving from feldspar is a synthesized multifaceted result. There is no single gene that encodes for a potassium dissolving ability in bacteria, which is similar to the process of dissolving phosphate. It is well known that there are many differences between SSF and SMF in physiology, such as Aw (water activity) and metabolite. The pH range for *B. subtilis* Xue-113168 is between 5 and 9, and the temperature range is 28–37 °C. Moreover, the indispensable moisture required for SSF was low. All the characteristics described above make *B. subtilis* Xue-113168 suitable for SSF. Sheng demonstrated that the potassium dissolving rate of Bacillus edaphicus is low, which also has been proven to have a low potential for commercial use [13]. Tan reported that 9–28% of the potassium that they measured was released by humic acids [14]. It has been hinted that the potassium dissolving rate (41.53%) of *B. subtilis* Xue-113168 may include dissolved potassium from K-feldspar through the action of humic acids. Consistent and sustained product of SSF can be achieved in different growing media as long as the following variables are being controlled: consistency of the parent organic material, moisture content, carbon to nitrogen ratio, and process parameters.

During SSF with feldspar, spore concentrations can reach 2×10^9 cfu/g and soluble potassium content is 1%, *B. subtilis* Xue-113168 ferments and dissolves K simultaneously. *B. subtilis* Xue-113168 displayed the highest K-solubilizing activity, as high as 41.53% after 7d in culture with K-feldspar powder. This cycle is shorter than Lian and Ciceri [4, 15], who performed SSF and SMF for 15 and 35 d respectively. Bioleaching combined with SSF is more advantageous, more economical and more timesaving. Stephanie. K gets mutant bacteria for a high efficient bioleaching also [16].

3.4 Quality Index of the CMF

The quality index plays a vital role in measuring the efficiency of a fertilizer. The indexes are consistent with the Compound Microbial Fertilizer Standard (NY/T 798-2015, China): $N + P_2O_5 + K_2O \geq 80\text{–}250$ g kg^{-1}, number of viable cells is 0.2 (one hundred million/g) [17]. Compound Microbial Fertilizer (CMF), formulated by humic acid and K_2HPO_4 with biofertilizer, have a quick and long-lasting effect. The stress-tolerance towards K_2HPO_4 of *B. subtilis* Xue-113168 is a key factor, the humic acid's chelating functions also lessens the damage to bacteria done by K_2HPO_4. Product Quality index of the CMF were examined by Quality supervision, inspection and Testing Center for microbial products of Ministry of Agriculture in WuHan in December 2016. Provisional registration certificate of CMF is No. 3878 by Ministry of Agriculture of the People's Republic of China in March 15, 2017. Product Quality index of the CMF is different from the that of pilot sample showed in Table 1, such as content of P_2O_5, inferring phosphorus as massive element in cell is less harmful to microorganism. Function of PGPR dependent on the clone in zone of root [18]. Organic matter in CMF offer nutrient, shelter to adverse situation for PGPR in field.

Table 1 Results of the quality index determination experiment

Item	Effective viable cells a hundred million (g)	Total primary nutrient (g kg^{-1})	Organic matter (g kg^{-1})	N Nitrogen (g kg^{-1})	P_2O_5 (%)	Available potassium (g kg^{-1})
NY/T798-2015	0.2	80–250	200			
CMF (pilot)	2	130	250	12	38	80
CMF (product)	2.8	174	532	35	91	48

4 Conclusions

This CMF is formulated by humic acid, K_2HPO_4, and biofertilizer which is SSF by chemical fertilizer-tolerant feldspar-solubilizing *B. subtilis* Xue-113168. Provisional registration certificate of CMF is No. 3878 by Ministry of Agriculture of the People's Republic of China in March 15, 2017.

Bacillus subtilis Xue-113168 converts K-feldspar, corn bran, shrimp shell, and spent mushroom substrate to a biofertilizer that have a 41% potassium dissolving rate. The CMF used in this study can enhance the efficiency of fertilizers and utilize recycled waste resource. It also promotes a sustainable agriculture industry, complex formulations and process of the SFF are suitable for factory, especially farmer uses in the fields.

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