

# Co-Production of Nattokinase and Poly ( $\gamma$ -Glutamic Acid) Under Solid-State Fermentation Using Soybean and Rice Husk

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## ABSTRACT

The aim of this work was to study the co-production of nattokinase and poly ( $\gamma$ -glutamic acid) by *Bacillus subtilis* natto with soybean and rice husk under solid-state fermentation (SSF). The results showed that the size of soybean particle and rice husk significantly improved the co-production of nattokinase and poly ( $\gamma$ -glutamic acid), yielding 2503.4 IU/gs and 320 mg/gs, respectively in the improved culture medium composed of 16.7% soybean flour and 13.3% rice husk with 70% water content. The yields increased by approximate 7- and 2-fold factor relative to their original ones. Thus, the co-production of nattokinase and poly ( $\gamma$ -glutamic acid) under SSF could be considered as an efficient method to exploit agro-residues for economical production of some higher-value products.

**Key words:** Co-production, Nattokinase, poly ( $\gamma$ -glutamic acid), Rice husk, Solid-state fermentation

## INTRODUCTION

Intravascular thrombosis, which results from blood clot fibrin in arteries, is a bad disease against human health. As compared to the clinical thrombolytic drugs (urokinase and streptokinase), nattokinase (NK) with many advantages such as confirmed efficacy, prolonged effects, cost effectiveness and preventative use is a potent thrombolytic enzyme *in vitro* and *in vivo* (Zheng et al. 2005). For example, it has four times fibrinolytic activity over plasmin (Sumi et al. 1992), and the activity can be retained in the blood for more than 3 h (Fujita et al. 1995). In view of the traits like safe, low cost, and easy oral administration, NK has been considered as an excellent functional food for thrombosis therapy.

Poly ( $\gamma$ -glutamic acid) or  $\gamma$ -PGA is a homopolymer of D- and L-glutamic acid units, which is biodegradable, edible and nontoxic

toward humans and environment (Shih and Van 2001). Accordingly, it is a promising environmental-friendly biomaterial with broad industrial application in the fields of medicine, foods, plastics, farming and many others (Shih and Van 2001; Wang et al. 2008; Zhu et al. 2010).

*Bacillus subtilis* has been usually applied to produce NK under submerged fermentation (SmF) (Deepak et al. 2008) and to manufacture  $\gamma$ -PGA under solid-state fermentation (SSF) (Wang, et al. 2008; Yao et al. 2012; Zeng et al. 2013). This strain has been applied to co-produce multiple biomaterials containing  $\gamma$ -PGA under SSF (Wang et al. 2008; Yao et al. 2012; Zeng et al. 2013).

There are a large number of agricultural and forestry residues produced world-wide whose disposal is a serious problem in China. However, these could be effectively used in SSF for enzyme production (Jana et al. 2012; Selwal and Selwal 2012; Beniwal et al. 2013; Wang et al. 2013).

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Soybean and rice husk are low-cost and abundant in China. Rice husk, in particular, is an excellent solid support for SSF in view of its uniform shape of the particles with fluffy and elastic characteristics. On the other hand,  $\gamma$ -PGA fermentation broth under SmF is highly viscous and exhibits non-Newtonian rheology along with a serious barrier against mixing, heat transfer and oxygen supply (Richard and Margaritis 2003). Impurities in the liquid fermentation increase the cost for  $\gamma$ -PGA purification. As compared to SmF, SSF can not only exploit cheap and easily available raw agro-residues but also offers economical and engineering advantages such as low processing cost, less energy expenditure, simple equipment and facilities (Pandey 2003; Chen et al. 2005).  $\gamma$ -PGA, an important composition of natto, can promote mineral substance to be utilized. It also can preserve the moisture of solid culture medium and further improve NK production under SSF. Accordingly, it is considered that co-production of NK and  $\gamma$ -PGA under SSF should be an interesting work. To our knowledge, the co-production, especially with soybean and rice husk as solid matrix, has not yet been reported. In this work, soybean and rice husk were utilized as solid substrate and support for SSF to co-produce NK and  $\gamma$ -PGA using *Bacillus subtilis* natto.

## MATERIAL AND METHODS

### Material

NK-producing strain was obtained from the commercial *Bacillus subtilis* natto powder and maintained in 4°C. Casein was purchased from Sinopharm Chemical Reagent Co., Ltd. (SCRC), China. Thrombin, cattle fibrinogen and urase were bought from the National Institute for the Control of Pharmaceutical and Biological Products (NICPBF). Rice husk and Chinese northeast soybean were bought from the local market.

### CO-production of NK And $\gamma$ -PGA Under SSF

A dosage of 500  $\mu$ L *Bacillus subtilis* natto solution was inoculated in 150 g boiled Chinese soybeans in a plastic case ( $\Phi$  20 cm). After being mixed evenly, the solid culture medium was covered with three layers of gauzes, then the case was sealed with foodstuff preservative film with six holes ( $\Phi$  2 mm) and incubated at 37°C for 24 h.

Afterwards, the whole contents were preserved at 4°C for 24 h.

### Extraction of Crude NK And Determination Its Activity

Sixteen gram fermented matrix was added in 40 mL normal saline in 250 mL flask, mixed at 100 rpm for 1 h and centrifuged (10,000 rpm, 10 min). The above extraction protocols were carried out two times. The supernatant was collected and defined as crude NK. NK activity was determined using the method of Wei et al. (2007). Briefly, 2.0 mL 0.5% casein solution and 1.0 mL crude NK samples were incubated at 30°C for 5 min, then the NK sample was mixed with the casein solution uniformly and incubated at 30°C for 10 min. Afterwards, 3.0 mL 10% trichloroacetic acid was added in the mixture and incubated for 10 min, then filtered. A control was prepared by mixing 1.0 mL crude NK sample with 3.0 mL 10% trichloroacetic acid uniformly and then 2.0 mL 0.5% casein solution was added in the mixture. Other protocols were the same as the above mentioned. Then 1.0 mL NK sample and 1.0 mL the control were added in two tubes containing 5.0 mL 0.55 mol/L sodium carbonate and 1.0 mL phenol, respectively and incubated at 30°C for 15 min after mixing well. The absorbance of the sample and the control was read by an ultraviolet spectrophotometer (SP-754, Shanghai Spectrum Instruments Co Ltd, China) at 680 nm, which were defined as  $A_s$  and  $A_c$ , respectively. NK activity was calculated based on tyrosine calibration curve and its achievable model (NK activity =  $K \cdot (A_s - A_c) \cdot N \cdot 6/10$ , where K was the slope reciprocal of tyrosine calibration curve, N was the distilled fold). The unit of NK activity was defined as the NK dosage required for 1.0  $\mu$ g tyrosine formation in 1 min. All the experiments were carried out in three sets.

### Extraction of Crude $\gamma$ -PGA And Determination Its Yield

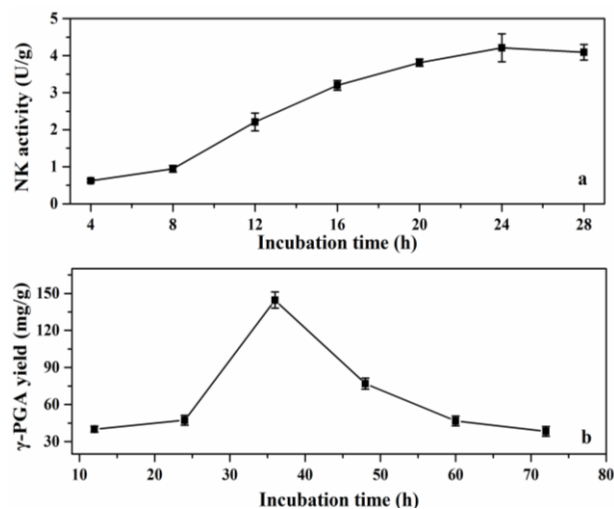
The extraction method in this work was modified based on the method by Bajaj et al.(2008). Twenty gram fermented substrate was added in 80 mL normal saline in 250 mL Bunsen beaker and mixed at 30°C on a rotary shaker (200 rpm) for 1 h, then the mixed solution was filtered by two-layer gaze. The filtrate was centrifuged at 10000 rpm for 15 min. Clarified supernatants were collected for  $\gamma$ -PGA purification.

$\gamma$ -PGA was purified by the method reported by Goto and Kuniko(1992). Ten milliliters of supernatant was poured in 40 mL methanol and kept at 4°C for 12 h. Crude  $\gamma$ -PGA was collected by centrifugation at 10000 rpm and 4°C for 30 min and then it was dissolved in distilled water. Any insoluble impurity was removed by centrifugation. The aqueous  $\gamma$ -PGA solution was desalted by dialysis (molecular weight cut off 3500) against 1 L distilled water for 12 h with water exchange three times.  $\gamma$ -PGA yield was determined by UV assay according to the reported method previously (Zeng et al. 2012). All the experiments were carried out in three sets.

## RESULTS AND DISCUSSION

### Time courses of NK and $\gamma$ -PGA production

Studies on finding the optimal time for NK and  $\gamma$ -PGA production showed the maximum NK yield, 4.21 U/g dry substrate (U/gds or U/g) at 24 h (Fig. 1A). Afterwards, the yield began to decrease. As shown in Figure 1B, the  $\gamma$ -PGA yield exhibited a trend from ascent to descent with the increment of incubation time and the maximum yield was 144 mg/g at 36 h. The optimal time of  $\gamma$ -PGA production was shorter than the previous report, 72 h (Bajaj et al. 2008).

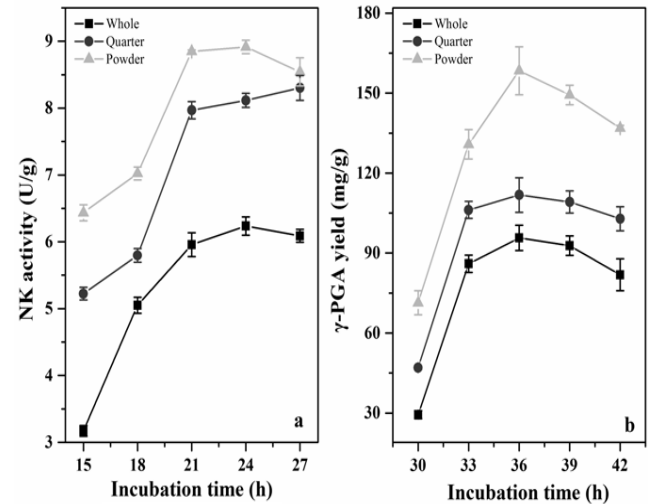


**Figure 1** - Schedule for NK (A) and  $\gamma$ -PGA (B) production.

### Effect of particle size of Soybean on NK and $\gamma$ -PGA Production

The effect of the particle size of solid substrate (whole bean, quarter bean, and bean flour) on co-production of NK and  $\gamma$ -PGA showed that the

maximum yields of NK and  $\gamma$ -PGA were obtained with bean flour as substrate. It was inferred that NK and  $\gamma$ -PGA production could be enhanced by the small size of substrate particles.



**Figure 2** - Effect of substrate particles size on NK (A) and  $\gamma$ -PGA (B) production.

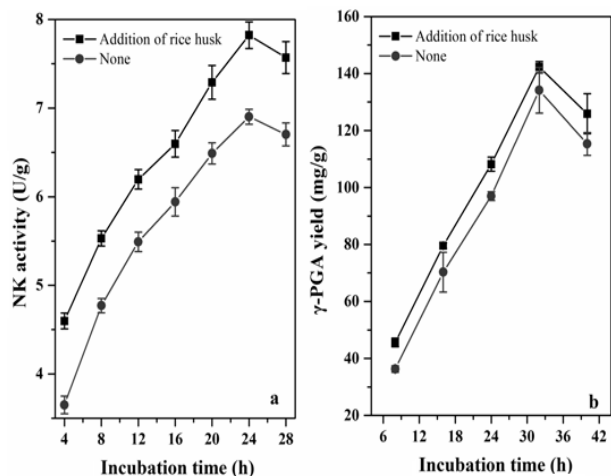
“Whole” referred to whole soybean as sold substrate under SSF. “Quarter” referred to smaller particle size of soybean residue, which was one quarter, the size of whole soybean. “Powder” referred to soybean flour. Inoculum size and water size were 4 and 70%, respectively. The other conditions were same as described in the “Material and methods” section.

Smaller size of solid substrate particles provides larger surface area. The enlarged area is helpful for mass transfer by enhancing the affinity of substrate to microbial cells. Thus, the substrate containing small size particles gave higher yields of NK and  $\gamma$ -PGA by meeting the rapid requirement of microbial metabolism for nutrients, particularly at the latter stage of metabolite synthesis. However, the small size effect could also inhibit the metabolite synthesis by blocking air to flow fluently. It was deduced that nutrient rather than oxygen was even more necessary for the microbial metabolism at the latter stage. Nevertheless, the poor oxygen concentration available in solid matrix has a harmful effect on microbial metabolism. Therefore, it is important that a desired balance between particle size and air-flow rate is established in SSF processes.

### Effect of Rice Husk on NK And $\gamma$ -PGA Production

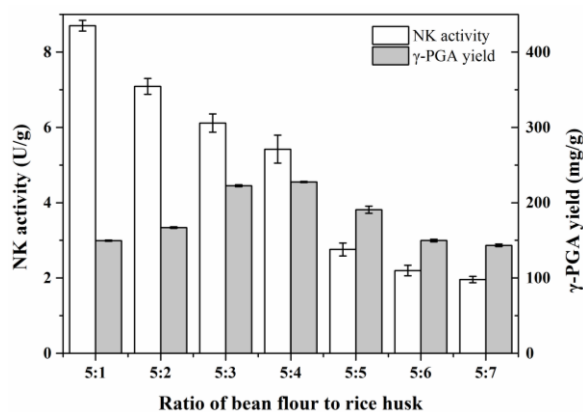
The effect of particle size of rice husk on air-flow was studied, the result indicated that rice husk can improve the strain in co-producing NK and  $\gamma$ -PGA. The improvement was more prominent for

NK production than for  $\gamma$ -PGA production (Fig. 3). Figure 4 showed that the NK yield kept on decreasing with the increment of rice husk content. On the contrary, the yield of  $\gamma$ -PGA kept on increasing until the ratio of rice husk to soybean grew up to 5:4, when the maximum yield (227.6 mg/g) was achieved.



**Figure 3** - NK (a) and  $\gamma$ -PGA (b) production using rice husk.

In this diagram, “Addition of rice husk” refers to direct addition 30 g rice husk in the solid fermentation medium containing 150 g soybean flour, “None” refers to the solid fermentation medium without any rice husk. Inoculum size and water size were 4 % and 70%, respectively. The other conditions were seen in the “Material and methods” section.



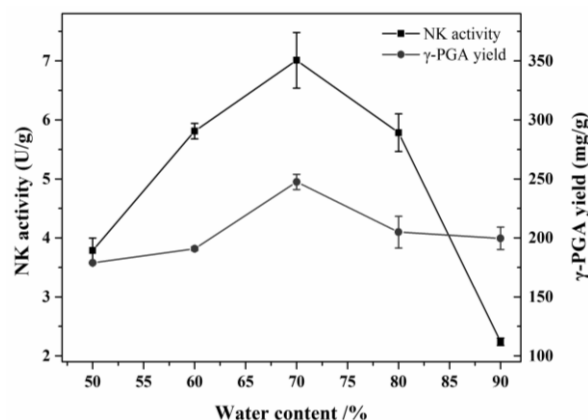
**Figure 4** - Effect of the ratio of bean flour to rice husk on NK and  $\gamma$ -PGA production.

In this experiment, the solid fermentation medium was composed of 30 g soybean flour and different amount of rice husk decided by the ratio of bean flour to rice husk. Inoculum size and water size were 4 % and 70%, respectively. NK activity was assayed after the strain was incubated for 24 h, and  $\gamma$ -PGA yield was determined after the strain was incubated for 36 h. The other conditions were seen in the “Material and methods” section.

Accordingly, it was concluded that the enhancement in air flow could enhance the co-production of NK and  $\gamma$ -PGA. In view of its fluffy and elastic nature, rice husk could make the compact culture medium (composed by fine soybean particles) so fluffy that countless new “air-lines” are can be constructed. Besides, rice husk can enlarge the binding area of microbial cell to substrate by making an even and extensive distribution of microorganism in solid culture medium. This not only improved the air permeability and heat dissipation in the solid medium but also speeded up mass transfer of substrate and product. The fermented rice husk could be utilized to improve organic fertilizer in water retention and nutrients (Chen et al. 2005). This was in agreement with the present increasing emphasis on exploiting agro residues. Therefore, rice husk could be regarded as an excellent leavening agent for SSF.

#### Effects of Water Content and Inoculum Size on NK and $\gamma$ -PGA Production

In view of moisture retention of  $\gamma$ -PGA, different water contents (50-90%) were investigated to find the optimum of moisture level. Figure 5 indicated that the water content at 70% was the best initial moisture level for NK and  $\gamma$ -PGA production.

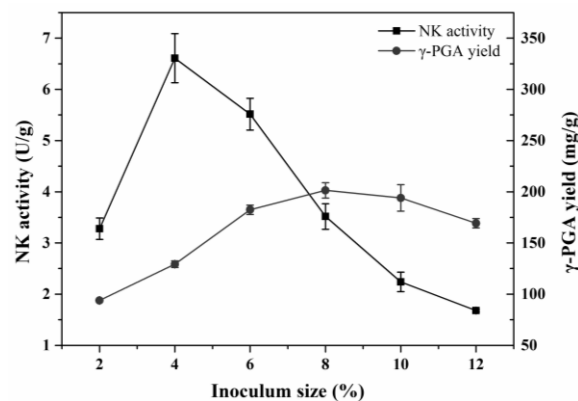


**Figure 5** - Effect of the water content on NK and  $\gamma$ -PGA production.

In this experiment, the solid fermentation medium was composed of 30 g soybean flour and 18 g rice husk. Inoculum size was determined as 4%. NK activity was assayed after the strain was incubated for 24 h, and  $\gamma$ -PGA yield was determined after the strain was incubated for 36 h. The other conditions were seen in the “Material and methods” section.

Water content in solid culture medium has a direct correlation with the water activity, which is a critical factor for microbial growth and metabolism under SSF. Poor water can limit some nutrients to be dissolved and transferred so as to inhibit microbial growth, while excessive water can inhibit the microbial growth by making substrate clustered and preventing air permeability and heat dissipation (Yadav et al. 2008). It's worth noting that  $\gamma$ -PGA has a capability of water retention to reduce the evaporation of the moist culture medium during incubation. On the other hand, water in the fluffy culture medium composed of rice husk could evaporate faster than that in the compact medium. Therefore, the optimization of water content in the culture medium is essential. Generally, the inoculum size has a dramatic effect on microbial biomass and metabolites synthesis. The effect of inoculum size (2-12%) was explored on the co-production of NK and  $\gamma$ -PGA. Figure 6 indicated that the maximum yields of NK and  $\gamma$ -PGA were achieved with the inoculum sizes of 4 and 8%, respectively. Excessive inoculum size could inhibit NK

synthesis, but less inoculum size would not stimulate  $\gamma$ -PGA production completely. Accordingly, the inoculum size at 6% was chosen in further studies.



**Figure 6** - Effect of inoculum size on NK and  $\gamma$ -PGA production.

In this experiment, the solid fermentation medium was composed of 30 g soybean flour and 18 g rice husk. Water content was determined as 70%. NK activity was assayed after the strain was incubated for 24 h, and  $\gamma$ -PGA yield was determined after the strain was incubated for 36 h. The other conditions were seen in the "Material and methods" section.

**Table 1** - Analysis of the difference of improved NK and  $\gamma$ -PGA production from the original ones along with reaction time.

Reaction time (h)	Improved					Original			
	NK <sub>s</sub> (U/g)	$\gamma$ -PGA <sub>s</sub> (mg/g)	NK <sub>p</sub> (%)	$\gamma$ -PGA <sub>p</sub> (%)	R	Top NK <sub>s</sub> (U/g)	Top $\gamma$ -PGA <sub>s</sub> (mg/g)	R <sub>NK</sub>	R <sub>PGA</sub>
9	1.67	65.6	52.22	45.40	1.15			0.40	0.45
18	3.14	126.2	98.58	87.34	1.13			0.75	0.87
24	6.69	147.25	209.70	101.90	2.06	4.21		1.59	1.02
27	6.08	177.8	190.49	123.04	1.55			1.44	1.23
36	3.70	222.6	116.13	154.05	0.75		144.5	0.88	1.54
45	3.57	194.8	111.95	134.81	0.83			0.85	1.35
54	1.99	153	62.24	105.88	0.59			0.47	1.06
63	1.13	127.6	35.51	88.30	0.40			0.27	0.88
72	0.77	85.2	24.23	58.96	0.41			0.18	0.59
Mean	3.19	144.45							

NK<sub>s</sub> and  $\gamma$ -PGA<sub>s</sub> denote the yields of NK and  $\gamma$ -PGA, respectively. NK<sub>p</sub> and  $\gamma$ -PGA<sub>p</sub> are their percents relative to their corresponding means, respectively. R equals to the ratio of NK<sub>p</sub> to  $\gamma$ -PGA<sub>p</sub>. Top NK<sub>s</sub> and Top  $\gamma$ -PGA<sub>s</sub> are the top yields of NK (incubated for 24 h) and  $\gamma$ -PGA (incubated for 36 h) before improvement, respectively. R<sub>NK</sub> and R<sub>PGA</sub> are the ratio of NK<sub>s</sub> to top NK<sub>s</sub> and the ratio of  $\gamma$ -PGA<sub>s</sub> to top  $\gamma$ -PGA<sub>s</sub>, respectively. In the improved experiment, the solid fermentation medium was composed of 30 g soybean flour and 18 g rice husk. Inoculum size and water content were designed as 6% and 70%, respectively. The other conditions were seen in the "Material and methods" section.

Inoculum size is very crucial for metabolite synthesis under SSF. Poor inoculum size can defer the synthetic progress of metabolite by prolonging the time course of microbial growth, while superfluous inoculum size can give rise to major nutrients of culture medium to be exhausted in microbial growth (Kashyap et al. 2002). Sufficient

inoculation is beneficial to both the cell growth and product synthesis (Zeng et al. 2013). Accordingly, it is important for achieving a promising yield of metabolite to keep the balance between the biomass proliferating and available substrate (Pandey et al. 2000; Kashyap, et al. 2002). Rice husk and the small size effect of

soybean have a positive effect on microbial distribution, and this reasonably accelerates the growth of microbial cell in this work. It was concluded that the inoculum size depended on not only the dispersability of culture medium but also microbial distribution.

*B. subtilis* natto was incubated in an optimized culture medium for 9-72 h based on the above results. It was found that NK and  $\gamma$ -PGA yields at 24-45 h were higher than their corresponding values at 9-72 h. NK and  $\gamma$ -PGA yields at 24 and 27 h surpassed the maximum ones without optimization. The ratio of NK<sub>p</sub> to  $\gamma$ -PGA<sub>p</sub> (R) at 27 h was closer to one relative to that at 24 h (Table 1). Consequently, the optimum incubation time was considered as 27h for the co-production of NK and  $\gamma$ -PGA, where NK and  $\gamma$ -PGA yields were 6.08 U/g and 177.8 mg/g, respectively, and the corresponding fibrinolytic activity of NK was 1564.4 IU/g (assayed by the standard fibrin plate method (Astrup and Müllertz 1952)). The fibrinolytic activity of NK and  $\gamma$ -PGA yield was converted to 2503.4 IU/g for soybean (IU/gs) and 320 mg/gs, increasing by approximate 7 and 2-fold factors relative to their original yields (342.98 IU/gs and 144.5 mg/gs), respectively, when soybean instead of total solid supports was regarded as a measurement baseline. Furthermore, the  $\gamma$ -PGA yield in this work was higher than that (51.3 mg/g) in co-production of iturin A and  $\gamma$ -PGA (Yao et al. 2012). Also, the NK and  $\gamma$ -PGA yields were higher than the ones reported by Zeng et al. (2013). Troublesomely, it is difficult to extract NK from the co-production ferment including  $\gamma$ -PGA because high concentration of  $\gamma$ -PGA could disturb NK separation (Wei et al. 2012). Microbial  $\gamma$ -PGA with relatively high molecular weight can make the ferments too viscous to be separated easily. Therefore, it is very necessary to construct an efficient method for the departure of NK from  $\gamma$ -PGA in the further work.

## CONCLUSIONS

In summary, the smaller size of the substrate particles significantly improved the co-production of NK and  $\gamma$ -PGA by *B. subtilis* natto. Rice husk enhanced the soybean utilization and decreased the detrimental effect of the smaller size of the substrate particles related to aeration. The fermented solid medium could be utilized to

prepare organic fertilizer. These findings suggested that the co-production of NK and  $\gamma$ -PGA under SSF with agro-residues as substrate could be considered as an efficient method to exploit agro-residues for economical production of higher-value products.

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