

## Production of Poly(arginyl-histidine) by *Verticillium kibiense* E18

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### Introduction

Poly(amino acid)s are useful material for antimicrobial agents and biodegradable polymer. Many kinds of poly(amino acid)s have been chemically synthesized to be investigated for a wide variety of technical applications. On the other hand, some microorganisms produce poly(amino acid)s such as poly-ε-lysine (ePL), poly-γ-glutamic acid (PGA), and cyanophycin (multi-L-arginyl-poly-L-aspartic acid). The ePL has been used as a food additive, and PGA has potential as a material for biodegradable polymer. Recently, it has been reported that an ergot fungus *Verticillium kibiense* (formerly *Epichloe kibiensis*) E18, which was newly isolated as ePL producer, also produced poly-(arginyl-histidine) (PRH) [1]. The PRH is a decapeptide consisting of L-arginyl-D-histidine units (Fig. 1), and a potential novel antimicrobial agent [1,2]. However, PRH productivity of *V. kibiense* E18 is very low (approximately 1~5 mg/l). In this report, we investigated the cultivation conditions for production of PRH by *V. kibiense* E18.

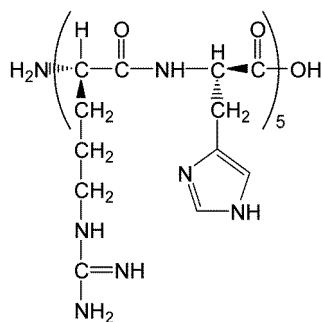


Fig. 1. Structure of poly(arginyl-histidine)

### Results and Discussion

*V. kibiense* strain E18 was cultivated in potato dextrose agar plate for stock culture, and in the SG liquid medium<sup>1</sup> containing glycerol instead of galactose for poly(arginyl-histidine)(PRH) production, because strain E18 grew with glycerol faster than with galactose. To produce PRH, strain E18 was grown in 30 ml of SG medium in 100 ml of baffled flask. The culture was then transferred into a fresh 120 ml of 2SG liquid medium which contained carbon and nitrogen source at twice the concentration of SG medium in a 500 ml Sakaguchi flask. It was cultivated under various conditions, i.e. media composition, shaking speed, initial pH, temperature, and cultivation days. The basal conditions for preculture were as follows: medium, SG (pH7.0); rotary shaking, 120 rpm; temperature, 28 °C; cultivation days, 5 days. The basal conditions for PRH production were as follows: medium, 2SG (pH5.0);

reciprocal shaking, 120 rpm; temperature, 28 °C; cultivation days, 7 days. The PRH secreted into the culture was filtered through a micropore membrane and purified by solid phase extraction (SPE) using a Sep-Pak CM cartridge (a cation-exchange type, Waters, Milford, MA). The samples were subjected to HPLC equipped with a column of μBondasphere C18 (Waters). The peptides were monitored by measuring absorbance at 200 nm and 220 nm. The concentration of carbon source was measured by HPLC equipped with a column of Shim-pack SCR-101H (Shimadzu, Kyoto, Japan) and detectors of UV (210nm) and RI.

The PRH production of strain E18 is unstable under basal cultivation conditions. We first investigated the shaking speed and temperature of production culture for

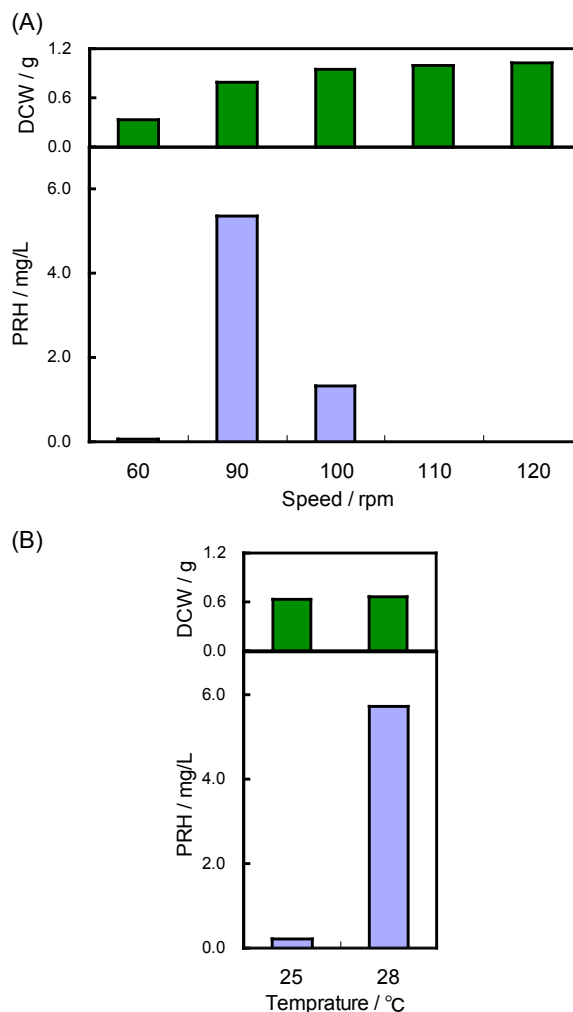


Fig. 2. Effect of shaking speed (A) and temperature (B)

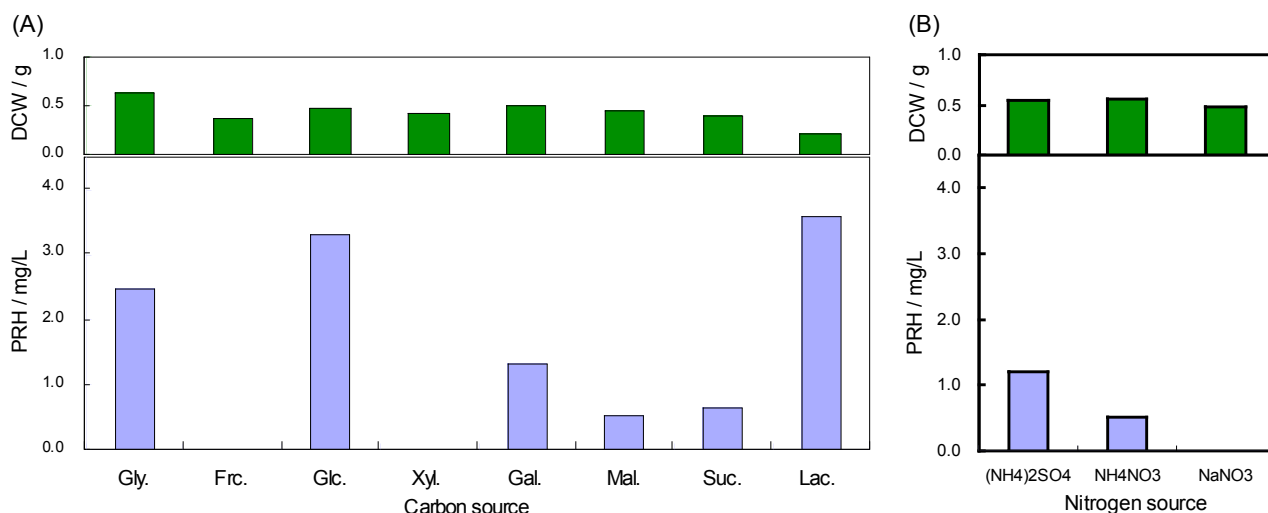


Fig. 3. Effect of carbon (A) and nitrogen (B) source

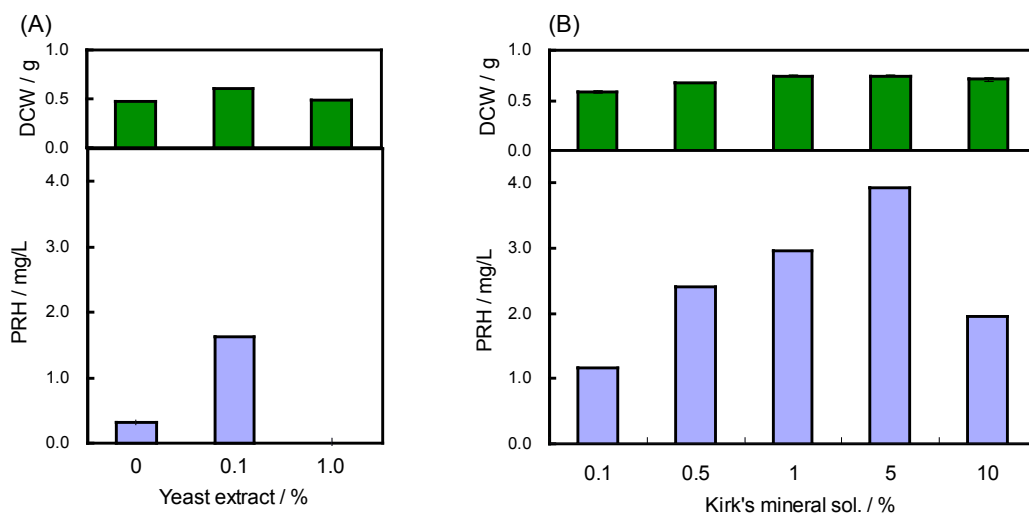


Fig. 4. Effect of concentration of trace element

PRH. As shown in Fig.2, the optimal shaking speed and temperature are 90 rpm and 28 °C, respectively. The PRH production was unstable in the culture with reciprocal shaking at 120 rpm, but became stable at 90 rpm. The filament shape of the cell was observed in the culture with 120 rpm, but not 90 rpm. Cell growth was increased with shaking speed. Therefore, PRH production might be related to cell damage and growth. Although the cell growth at 25 °C and 28 °C was at same level, PRH production was significantly declined in the culture at 25 °C. In addition, the cultivation at 30 °C for PRH production was not performed, because strain E18 was weakly grown at 30 °C in preculture. Hereafter, the basal condition modified with reciprocal shaking at 90 rpm in production culture was used as a modified basal cultivation condition. Although the PRH productivity under modified basal condition was still altered, each experiment was performed in duplicate, it was confirmed that the error rate in each experiment was small and the each data was reliable.

Because it was predicted that PRH production might be related to cell damage and growth in the preculture, the

condition of preculture was also investigated as for all combination of medium (SG or 2SG), shaking speed (90 or 120 rpm), and cultivation days (5 or 7 days). As a result, the optimal condition was as follows: medium, 2SG; shaking speed, 90 rpm; cultivation days, 7 days (data not shown).

Effect of medium component was investigated under the modified basal culture condition in production culture. The PRH production of cultivation with glycerol, fructose, glucose, xylose, galactose, maltose, sucrose or lactose as a carbon source was measured. As shown in Fig. 3(A), PRH was produced at same level in the culture with glycerol, glucose and lactose. No PRH was produced in the culture with fructose and xylose. Strain E18 was best grown in the culture with glycerol. Strain E18 was grown at same level in the culture with glucose and galactose, but PRH in the culture with glucose was produced more than that with galactose. On the other hand, in the culture with lactose, strain E18 was slightly grown, but PRH was produced at same level in the culture with glycerol and glucose. This phenomenon is interesting, but the cause is unclear

The PRH production of cultivation with  $(\text{NH}_4)_2\text{SO}_4$ ,  $\text{NH}_4\text{NO}_3$ ,  $\text{NaNO}_3$ ,  $\text{NaNO}_2$ , peptone, tryptone, soybean flour, soybean meal, soybean oil or corn steep liquor as a nitrogen source was also measured. As shown in Fig. 3(B), PRH was only produced with ammonium salt. On the cultivation with other nitrogen source, pH of these cultures were higher than that with ammonium salt. Therefore, the PRH production might be related to pH.

Furthermore, the optimal concentration of trace element, which was constituent of SG medium except carbon and nitrogen source, was determined. These results showed that PRH was best produced at the concentration of 0.1% yeast extract and 5% Kirk's mineral solution as shown in Fig. 4. As for yeast extract, cell growth was also best in the culture with 0.1% of yeast extract. It is interesting that the excess amount of yeast extract inhibited PRH production and cell growth. As for Kirk's mineral solution, the PRH production and cell growth were increased with increasing the concentration up to 5%.

As mentioned above, PRH was highly produced in the cultivation with glycerol, glucose, and lactose. Therefore, the PRH production was measured under the modified basal culture condition in production culture with glycerol, glucose or lactose as a carbon source for 7 and 14 days in detail. As for glycerol, strain E18 consumed most of glycerol for 7 days as shown in Fig. 5. Although cell growth was increased with the course of cultivation days, PRH production was declined on 14 days. PRH may be utilized by strain E18 for its growth. As for glucose, half of initial amount was remained on 7 days, strain E18 produced twice amount of PRH on 7 days for 14 days. The cell growth in the culture with glucose was lower than that with glycerol on both of 7 and 14 days, PRH production was higher. The cultivation with glucose may be adequate to produce PRH. On the other hand, as for lactose, almost no lactose was utilized, strain E18 was slightly grown. These results indicated that the relationship of cell growth and amount of residual carbon source is important to improve PRH production.

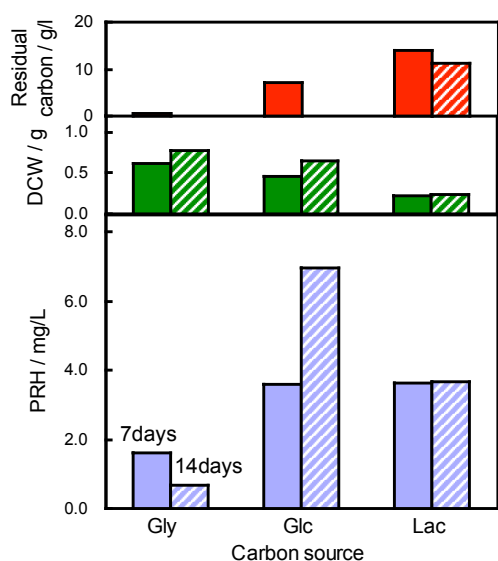


Fig. 5. Effect of carbon source

The PRH production was measured under a condition combined the optimum mentioned above. The preculture was performed in 2SG medium at 28 °C with rotary shaking at 90 rpm for 7 days. And the production culture was performed in 2SG medium containing 5% Kirk's mineral solution with glycerol or glucose at 28 °C with reciprocal shaking at 90 rpm for 14 days. During 14 days cultivation under this optimal condition, strain E18 produced approximately 20 mg/l and 40 mg/l of PRH with glycerol and glucose, respectively, as shown in Fig. 6. Histidine was synthesized from phosphoribosyl pyrophosphate (PRPP) via pentose phosphate pathway. In metabolic pathway, PRPP from glucose might be synthesized easier than that from glycerol, because the synthesis of PRPP from glycerol required the

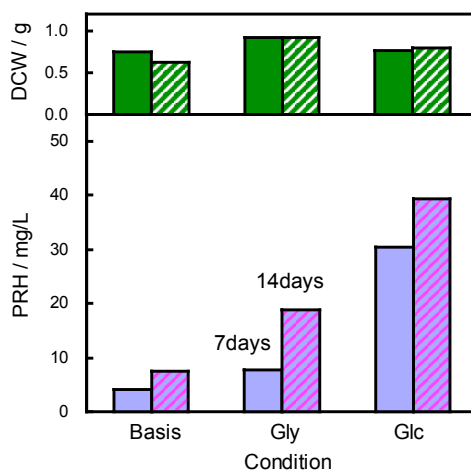


Fig. 6. Effect of carbon source

gluconeogenesis.

In conclusion, the PRH productivity was significantly affected by shaking speed and temperature, and also by the composition of culture media. Furthermore, PRH production stability was affected by pre-culture conditions. Under optimal conditions with glycerol, *V. kibiense* E18 produced approximately 20 mg/l of PRH for 14 days, and then approximately 40 mg/l with glucose. PRH production was successful in improving at 10 fold over that of basal condition.

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#### References

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