Screening of Fermentation Medium for Recombinant Myceliophthora and Its Application in Weaned Piglets (Postprint)

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Abstract

This study aimed to optimize and screen the fermentation medium for recombinant plectasin and investigate the effects of dietary supplementation with recombinant plectasin on growth performance, nutrient digestibility, and intestinal microbiota in weaned piglets. The experiment utilized a 30 L fermenter for liquid fermentation of the Pichia pastoris genetically engineered strain (PPle), employing a fed-batch fermentation process to compare the effects of three different media—low-salt, basic glycerol, and basic soluble starch—on the secretory expression of recombinant plectasin. For the animal trial, 30 healthy 24-day-old "Duroc × Landrace × Yorkshire" weaned piglets were selected and randomly allocated to 5 groups according to the principle of uniform body weight: control group (CON group, basal diet), colistin sulfate group (CS group, basal diet + 0.3% colistin sulfate), antimicrobial peptide group (AP group, basal diet + 0.2% recombinant plectasin), microecological preparation group (PB group, basal diet + 0.1% microecological preparation), and combined application group (PPB group, basal diet + 0.2% recombinant plectasin + 0.1% microecological preparation), with a trial period of 21 days. The results showed that at 114 h of fermentation, the wet cell weights of the low-salt, basic glycerol, and basic soluble starch groups reached their maximum values of 450, 402, and 277 g/L, respectively. At 114 h of fermentation, the total protein concentrations in the fermentation supernatant were measured to be 0.38 g/L for the low-salt group, 3.94 g/L for the basic glycerol group, and 5.63 g/L for the basic soluble starch group. The animal trial demonstrated that compared with the CON group, the CS and AP groups significantly increased average daily feed intake (ADFI) and average daily gain (ADG), and significantly decreased feed-to-gain ratio (F/G) ($P<0.05$). Compared with the CON group, the CS and AP groups showed a tendency to reduce diarrhea rate (0.05

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Full Text

Optimal Production of Recombinant Plectasin from *Pichia pastoris* and Its Efficacy for Weaning Piglets

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Abstract

This study aimed to optimize the fermentation medium for recombinant plectasin production and evaluate the effects of dietary recombinant plectasin supplementation on growth performance, nutrient digestibility, and intestinal mi-

croflora in weaning piglets. A 30 L fermenter was used for liquid fermentation of the genetically engineered *Pichia pastoris* strain (PPle) using a batch-fed process, comparing three different media formulations: low-salt, basal glycerol, and basal soluble starch. Thirty healthy 24-day-old Duroc × Landrace × Yorkshire weaning piglets were randomly allocated to five treatment groups according to initial body weight: control (CON, basal diet), colistin sulfate (CS, basal diet + 0.3% colistin sulfate), antimicrobial peptide (AP, basal diet + 0.2% recombinant plectasin), probiotic (PB, basal diet + 0.1% probiotic preparation), and combination (PPB, basal diet + 0.2% recombinant plectasin + 0.1% probiotic preparation). The trial lasted 21 days. Fermentation results showed that at 114 h, the wet cell weights peaked at 450, 402, and 277 g/L for the low-salt, basal glycerol, and basal soluble starch groups, respectively. At 114 h fermentation, total protein concentrations in the supernatant were 0.38 g/L (low-salt), 3.94 g/L (basal glycerol), and 5.63 g/L (basal soluble starch). Animal trial results indicated that compared with the CON group, both CS and AP groups significantly improved average daily feed intake (ADFI) and average daily gain (ADG), and significantly reduced feed-to-gain ratio (F/G) ($P < 0.05$). The CS and AP groups also showed a trend toward reduced diarrhea incidence ($0.05 < P < 0.10$) compared with CON. The AP group significantly increased ileal *Bifidobacterium* content compared with all other groups ($P < 0.05$). All four treatment groups tended to have higher apparent digestibility of energy and dry matter compared with CON ($0.05 < P < 0.10$). The PB group exhibited significantly higher apparent phosphorus digestibility than the other four groups ($P < 0.01$). In conclusion, optimizing the fermentation medium enhanced recombinant plectasin expression, and dietary supplementation with recombinant plectasin antimicrobial peptide improved growth performance in weaning piglets.

Key words: recombinant plectasin; antimicrobial peptide; growth performance; nutrient digestibility; weaning piglets

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Over the past five decades, antibiotic use in animal production has generated substantial economic benefits for livestock producers. However, during the last two decades, advancing science and rising living standards have revealed concerns about antibiotic residues in animal products and the development of antimicrobial resistance. Consequently, there is an urgent need to identify green, efficient, non-polluting, and residue-free alternatives to antibiotics. Antimicrobial peptides have emerged as promising feed antibiotic substitutes. Schneider et al. [**?**] identified plectasin through analysis of a cDNA library from a saprophytic ascomycete in 2005, noting its similarity to animal defensins. *Staphylococcus aureus* infection rates in nursery pig facilities can reach 15%, with mortality rates up to 70% in infected piglets, posing significant threats to livestock production [**?**]. Meanwhile, antibiotic misuse has caused serious problems in aquaculture. Both domestic and international researchers are actively seeking novel feed additives to replace antibiotics.

Our laboratory previously cloned the plectasin gene into *Pichia pastoris*, obtaining the engineered strain PPle that secretes recombinant plectasin for *S. aureus* infection prevention and treatment. Studies demonstrated that recombinant plectasin exhibits no cytotoxicity or hemolytic activity, suggesting its potential as a non-antibiotic feed additive [**?**]. While many animal defensin-like antimicrobial peptides require low ionic concentrations for activity, plectasin—the first fungal defensin identified—maintains bactericidal activity at physiological ion concentrations and shows strong inhibitory effects against Gram-positive bacteria such as *S. aureus* and *Streptococcus suis* [**?**]. Currently, plectasin is primarily obtained from natural microorganisms, but this approach suffers from low yields and difficult purification. Our laboratory successfully constructed the PPle strain capable of high-level secretory expression of recombinant plectasin with relatively high purity. To further reduce production costs, this study compared the effects of three different media on plectasin expression in a 30 L fermenter and subsequently evaluated the application efficacy of recombinant plectasin in weaning piglets to provide foundational data for its industrial application in feed.

1.1 Experimental Materials

The recombinant plectasin-engineered *Pichia pastoris* strain PPle was constructed in our laboratory. Recombinant plectasin antimicrobial peptide was prepared as a powder with an effective content of 30 mg/kg through fermentation in this study. Colistin sulfate was purchased as a powder (20 mg/kg effective content) from Sichuan Hengtong Company. The probiotic preparation (powder form, total viable bacteria $\geq 2 \times 10^9$ CFU/g) was purchased from Vland Biotech Group.

1.2.1 Fermenter Cultivation

Fermenter sterilization involved adding medium to the fermenter with water to 20 L, heating to 120 °C for 30 min, cooling to 30 °C, and maintaining constant temperature. The pH was adjusted to 5.5 with ammonia solution, followed by aeration and agitation. Flame inoculation was used to transfer secondary seed culture into the fermenter. Carbon source feeding began after 2 h of pre-culture, with flow rate controlled based on dissolved oxygen (DO): when DO increased, carbon source feeding rate was increased accordingly, and vice versa. Methanol induction was initiated after carbon source depletion, which was indicated by a rapid DO increase, followed by 1 h of carbon starvation. A variable-speed feeding strategy was employed. Induction began when pH remained relatively stable, with DO maintained at 20–30% through adjustment of valve opening and cycle period to ensure methanol feeding balanced consumption. Methanol feeding progressed through three stages at rates of 34, 51, and 68 mL/h.

1.2.2 Fermentation Product Detection

After fermentation, the supernatant was collected by centrifugation for purification. Plectasin-containing supernatant was purified via His-affinity chromatography and stored at -20 °C for short-term use. Total protein in the fermentation supernatant was measured using the Coomassie brilliant blue method, and recombinant antimicrobial peptide expression was verified by SDS-PAGE. *Staphylococcus aureus* ATCC 25923 was inoculated into 50 mL MHB medium and cultured overnight at 37 °C in a shaking incubator. Sterilized MHA medium at 42 °C was poured into four petri dishes. After solidification, 200 L of bacterial suspension treated for 4 h with 2,500 U pepsin, 250 U trypsin, and glycine-HCl buffer (pH 2.0) was spread onto the medium. Samples were added, and plates were incubated face-up at 37 °C for 18–24 h to observe antibacterial effects.

1.3 Animal Trial Design

Thirty healthy 24-day-old Duroc × Landrace × Yorkshire weaning piglets were randomly allocated to five groups (n = 6 piglets per group, one piglet per replicate) based on initial body weight: control (CON, basal diet), colistin sulfate (CS, basal diet + 0.3% colistin sulfate), antimicrobial peptide (AP, basal diet + 0.2% recombinant plectasin), probiotic (PB, basal diet + 0.1% probiotic preparation), and combination (PPB, basal diet + 0.2% recombinant plectasin + 0.1% probiotic preparation). The trial lasted 21 days. Piglets were weighed after fasting on day 21, and daily feed intake was recorded. From days 17-21, a digestion trial was conducted using the internal indicator fecal collection method.

1.3.1 Experimental Diets

A corn-soybean meal basal diet was formulated according to NRC (2012) nutrient requirements for swine. Basal diet composition and nutrient levels are presented in Table 1 .

1.3.2 Feeding Management

The trial was conducted at the piglet facility of the Animal Nutrition Institute Research Base at Sichuan Agricultural University. Pig houses were thoroughly disinfected before the trial, and feeders and waterers were cleaned. Piglets received routine immunization and deworming. Feed was provided four times daily (08:00, 12:00, 16:00, 20:00) with small amounts added frequently, and water was available ad libitum. All treatment groups were managed under identical conditions. House temperature was maintained at 24–26 °C with relative humidity at 70–85%. Pens were kept clean and comfortable, with residual feed recorded each evening.

1.4 Sample Collection and Analysis

Feed intake was recorded throughout the trial to calculate average daily gain (ADG), average daily feed intake (ADFI), and feed-to-gain ratio (F/G). Fecal samples were collected from days 17–21 over four days. Collected feces were thoroughly mixed, oven-dried at 60–65 °C for two days, ground after drying, passed through a 40-mesh sieve, and stored in sample bags for determination of apparent digestibility of nutrients (dry matter, crude protein, ether extract, ash, energy, calcium, and phosphorus). Diarrhea incidence was recorded daily and calculated as: diarrhea incidence (%) = 100 × (number of diarrheal piglets × diarrheal days) / (total piglets × trial days).

DNA extraction from feces was performed using the Omega DNA extraction kit according to manufacturer instructions, followed by quantitative real-time PCR to quantify total bacteria, *Lactobacillus*, *Bacillus*, *Bifidobacterium*, and *Escherichia coli* in ileal and cecal digesta. Primer and probe sequences (Table 2) were adapted from Qi et al. [**?**].

1.5 Statistical Analysis

All data are expressed as means and standard errors. Preliminary statistics were performed using Excel 2010, followed by one-way ANOVA using SAS 9.1 software. Duncan' s multiple range test was used for pairwise comparisons. Significance was declared at $P < 0.05$, with $P < 0.01$ considered highly significant and $0.05 < P < 0.10$ indicating a trend.

2.1 Effects of Different Media on Recombinant *P. pastoris* Growth and Recombinant Protein Secretion

As shown in Figure 1 [Figure 1: see original paper], cell growth was very slow within the first 18 h after inoculation. Different media formulations affected the growth profile differently, with the low-salt group showing faster wet weight accumulation initially than the basal glycerol and basal soluble starch groups. At 114 h fermentation, wet cell weights peaked at 450, 402, and 277 g/L for low-salt, basal glycerol, and basal soluble starch groups, respectively. Cell growth entered stationary phase after 98 h. Fermentation was terminated at 114 h (72 h post-induction). Supernatant total protein concentrations were 0.38 g/L (low-salt), 3.94 g/L (basal glycerol), and 5.63 g/L (basal soluble starch). Based on comprehensive fermentation data, the basal glycerol group supernatant was selected for SDS-PAGE analysis and in vitro antibacterial testing. Figure 2 [Figure 2: see original paper] shows specific bands around 41 kDa appearing at 24, 48, 72, and 96 h post-induction in the basal glycerol group.

2.2 In Vitro Antibacterial Activity of Recombinant Plectasin Antimicrobial Peptide

Figure 3 [Figure 3: see original paper] demonstrates that recombinant plectasin antimicrobial peptide maintained strong antibacterial activity against *S. aureus*

after 4 h treatment with glycine-HCl buffer (pH 2.0), 2,500 U pepsin, and 250 U trypsin. The antibacterial effect after pepsin treatment was similar to the control, indicating strong pepsin tolerance and good stability against gastric degradation.

2.3 Effects of Recombinant Plectasin on Growth Performance of Weaning Piglets

As shown in Table 3 , the CS and AP groups significantly increased ADFI compared with CON ($P < 0.05$). The CS, AP, and PPB groups highly significantly improved ADG and reduced F/G compared with CON ($P < 0.01$). Both CS and AP groups showed a trend toward reduced diarrhea incidence compared with CON ($0.05 < P < 0.10$).

2.4 Effects of Recombinant Plectasin on Nutrient Digestibility in Weaning Piglets

Table 4 shows that the CS, PB, and PPB groups significantly improved apparent ash digestibility compared with CON and AP groups ($P < 0.05$), though no significant difference existed between CON and AP groups ($P > 0.05$). The PB group exhibited highly significantly greater apparent phosphorus digestibility than the other four groups ($P < 0.01$). The CON group had significantly lower apparent ether extract digestibility than the other four groups ($P < 0.05$). All treatment groups showed a trend toward higher apparent energy and dry matter digestibility compared with CON ($0.05 < P < 0.10$).

2.5 Effects of Recombinant Plectasin on Intestinal Microflora of Weaning Piglets

Figure 4 [Figure 4: see original paper] shows that the AP group significantly increased ileal *Bifidobacterium* populations compared with all other groups ($P < 0.05$), while the PB group significantly increased ileal *Lactobacillus* populations ($P < 0.05$). As shown in Figure 5 [Figure 5: see original paper], the AP group significantly reduced cecal *E. coli* populations compared with CON ($P < 0.05$), while the PB group significantly increased cecal *Bacillus* populations ($P < 0.05$).

Medium composition largely determines microbial growth rate and biomass during fermentation [**?**]. Glycerol is the most commonly used carbon source for *P. pastoris* growth, with its concentration critically affecting cell growth. Some reports indicate glucose may be more cost-effective than glycerol as a carbon source [**?**]. Soluble starch, which hydrolyzes into maltose and glucose at high temperatures, offers a cheaper alternative that could substantially reduce production costs.

The BSM basal salt medium is widely used in industrial cultivation due to its low cost, simple composition, ability to meet *P. pastoris* nutritional requirements, and successful secretion of heterologous proteins [**?**]. *P. pastoris* can express heterologous genes either intracellularly or secretively. Since the fermentation

medium contains no exogenous proteins and *P. pastoris* secretes minimal endogenous proteins, downstream purification is facilitated.

Liu [**?**] optimized pH, temperature, and methanol addition through response surface methodology, achieving recombinant human collagen expression of 19.49 g/L. Zhao et al. [**?**] increased human serum interferon expression to 215 mg/L by reducing BSM salt concentration by 25%. Even with the same expression system, different heterologous proteins show substantial expression level variations, likely due to differences in exogenous gene sequences and expression conditions.

P. pastoris growth and expression occur in two distinct phases. Phase 1 is the glycerol-based growth phase aimed at biomass accumulation. Upon carbon source depletion, Phase 2 induction begins with methanol feeding. Jiang [**?**] compared intermittent, constant-speed, and exponential feeding strategies, finding that segmented control yielded the best results with wet cell weights reaching 415 g/L, consistent with our findings. pH control during fermentation was achieved through ammonia supplementation, which both regulated acid-base balance and provided nitrogen. Reports indicate *P. pastoris* grows well at pH 3–7, with maximal exogenous protein secretion at pH 5 [**?**]. pH primarily affects enzyme activity, cell membrane permeability, and intermediate metabolite dissociation. Our results showed maximal recombinant antimicrobial peptide secretion at pH 5.5. Product activity also correlates closely with fermentation temperature. Higher temperatures accelerate metabolism and induce earlier expression, but excessive temperatures cause enzyme inactivation, cellular senescence, shortened fermentation cycles, and reduced yields. The optimal growth temperature is 28 °C, with 30 °C being ideal for induction; higher induction temperatures cause cell death [**?**]. We observed good growth and satisfactory expression at 30 °C. Our preliminary shake-flask experiments achieved only 143 mg/L expression, likely due to inability to control temperature, pH, DO, and methanol feeding rate. Based on *P. pastoris* growth characteristics and fed-batch fermentation technology, we supplemented growth-phase carbon source (glycerol), induction-phase carbon source (methanol), and nitrogen source (ammonia), adjusting feeding rates according to DO levels. Carbon source feeding began when DO rose rapidly, maintaining DO around 20%.

The slow growth within the first 18 h may be attributed to low inoculum density. During initial methanol feeding, DO dropped rapidly, requiring careful flow rate control. Although the basal soluble starch group showed the highest total protein concentration, SDS-PAGE revealed minimal target protein. Combined with an atypical growth curve, we hypothesize that starch compounds were poorly utilized, causing autolysis and artificially elevated total protein concentrations. Considering all fermentation data, the basal glycerol medium was selected as optimal.

SDS-PAGE analysis of the basal glycerol group showed minimal contaminating proteins and clear target bands, indicating high product purity. The recombinant plectasin maintained strong antibacterial activity against *S. aureus* after acid, pepsin, and trypsin treatment for 4 h, likely due to three disulfide bonds

conferring structural stability against degradation.

Numerous studies have reported effects of dietary antimicrobial peptides on weaning piglet performance. Antimicrobial peptides are attractive due to their broad-spectrum activity, low resistance potential, and rapid action. Yoon et al. [**?**] compared apramycin with different antimicrobial peptide A3 doses, finding that increasing A3 dosage significantly improved ADG without affecting ADFI, and enhanced crude protein and dry matter digestibility. Xiong et al. [**?**] reported that composite antimicrobial peptides in nursery diets significantly improved ADG and ADFI while reducing F/G. Wang et al. [**?**] found that lactoferrin antimicrobial peptide increased ADG by 41.8% and significantly reduced F/G compared with antibiotic-free controls.

Our results showed that the AP group significantly improved ADG and ADFI, reduced F/G, and tended to improve nutrient digestibility (energy, ether extract, dry matter) while reducing diarrhea incidence compared with CON. No significant differences between AP and CS groups suggest plectasin provides antibiotic-like growth-promoting effects. However, inconsistent results exist: Shan et al. [**?**] and Wu et al. [**?**] found no effect of composite antimicrobial peptides on ADFI, possibly due to differences in weaning age and body weight.

Plectasin kills pathogens by binding to negatively charged microbial membranes and inserting to form pores, causing cytoplasmic leakage. *E. coli* produces endotoxins that trigger inflammatory diarrhea. *Lactobacillus* is a well-documented beneficial bacterium that produces lactic acid, consumes oxygen, competes for mucosal binding sites, and inhibits pathogen proliferation while suppressing bacterial membrane formation. *Bacillus* produces various digestive enzymes that degrade anti-nutritional factors and improve feed utilization. *Bifidobacterium* aids in treating chronic diarrhea and constipation [**?**]. Our results demonstrate that recombinant plectasin supplementation significantly increased ileal *Bifidobacterium* populations and reduced cecal *E. coli* populations. By eliminating harmful bacteria and reducing competition for binding sites, plectasin promotes beneficial microflora proliferation and maintains intestinal microbial balance. Unlike broad-spectrum antibiotics that kill both harmful and beneficial bacteria, plectasin selectively maintains microbial equilibrium. Recombinant plectasin thus improves piglet performance and nutrient digestibility by inhibiting pathogenic growth and creating favorable conditions for beneficial microorganisms.

These findings confirm that our laboratory's recombinant plectasin antimicrobial peptide has significant potential as a feed additive, improving weaning piglet growth performance, nutrient digestibility, and intestinal microflora balance. Future research should further optimize fermentation conditions and refine processing technologies to better suit animal physiological characteristics.

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Note: Figure translations are in progress. See original paper for figures.