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IMPROVING FERMENTATION OF STEAMED STALK TO FEED USING *CANDIDA UTILIS* AND *PACHYSOLEN TANNOPHILUS*

– Research paper –

He CHEN[1], Sha MEI, Chunji DAI, Yunxia HE, Jianjun YU

*School of Food and Biological Engineering, Shaanxi University of Science & Technology, Xi'an, 710021, China

Abstract: In order to improve the protein content of straw feed and reduce the amount of nutrients added, in this paper, the cell wall structure of corn stalk was destroyed by thermophilic digestion and the polysaccharide was degraded into monosaccharide by complex enzyme, and then transformed into bacterial protein by double strain *Candida utilis* 1807 and *Pachysolen tannophilus* 1771 fermentation. Single factor experiments and orthogonal test were made to obtain the best process for the feed of double-bacteria synchronous fermentation of stalks. The optimum amount of each nutrient and the inoculation amounts of double bacteria (accounts for the percentage of the original dry straw quality): ammonium sulfate 6.79%, urea 2.72%, yeast powder 1.63%, magnesium sulfate 0.27%, *Candida utilis* 54.31% and *Pachysolen tannophilus* 54.31%; The operational parameters of fermentation process were: fermentation temperature 29℃, rotate speed 100 r/min and fermentation time 55 hours. The yield of stalk feed and crude protein was 82.04%, 23.33%, respectively. The crude protein content of stalk feed was 28.44%, which was 4.33 times of original dry stalk. The results showed that the multi-strain distribution and degradation of protein production provide important significance for corn straw bio-utilization.

Keywords: corn stalk; complex enzymes; *Candida utilis*; *Pachysolen tannophilus*; single-cell protein

INTRODUCTION

Single-cell protein (SCP), also known as microbial protein or bacterial protein, refers to proteins contained in yeast, fungi, mildew and other single-cell microorganisms, and it also contains various amino groups and vitamins (Schultz et al., 2006; Liu et al., 2013; Zepka et al., 2005). Yeast has a high nutritional value, protein content of about 55%, close to animal protein, its lysine content is higher than soybean, tryptophan content is more than 7 times higher than soybean, and it also contains B vitamins, minerals and other physiologically active substances such as Rich in enzymes, pepsin, amylase, etc (Rajoka et al., 2012). It also has high digestibility, general digestibility of 80%-90%. So, it is a multi-dimensional high-protein active yeast feed (Nigam et al., 2010). The yeast has short reproduction period (Kurcz et al., 2018).

Corn stalks are mainly composed of lignocellulose, of which coarse fiber accounts for about 66%, lignin accounts for 6.5%-13%, and contains other nutrients such as protein (Wan et al., 2013). Thus, its carbon source content is quite rich. If the corn stalks can be degraded to monosaccharide, it can be used as carbon source culture yeast (Hamza et al., 2003). By using crop stalk (corn stalk, wheat stalk, straw stalk, etc.) as raw material, and under the appropriate conditions, the single cell protein feed can be produced by microbial culture fermentation (Li et al., 2010). It not only solved the problem of recycling a lot of stalks (Abostate et al., 2014), but also increased the source of feed, improved the utilization rate of livestock and poultry for protein, produced a variety of useful metabolites in the fermentation process, which can promote the metabolism of animals and enhance their resistance, some of them also have anti-corrosion ability to feed (Saha et al., 2010; Du et al., 2013).

The structure of corn stalk is complex. Lignin used as coating protects the cellulose and hemicellulose against degradation of enzymes (Charles et al., 2009; Fanta et al., 2010). So, it should be degraded by enzyme method, and pretreating stalk is required (Chen et al., 2008). Many researchers studied simultaneous saccharification and fermentation of stalk. But the maximum active temperature of enzyme is generally between 37℃ and 60℃, so the fermentation time is long, and yeasts will waste a lot of carbon source for a long time to maintain their metabolism, or large amounts of enzymes are required. In a previous study (Chen and Zhang, 2008), the pre-treated corn

[1] Corresponding author. E-Mail address: chenhe419@gmail.com

stalk was degraded by complex enzyme for a certain time under the optimal conditions, and the reducing sugar was produced as the starting carbon source for the subsequent fermentation, and then the two kinds of yeast were inoculated. In our previous research, to achieve the purpose of saccharification and fermentation, meaning to shorten the time of stalk protein feed produced, reduce the enzyme dosage and improve the utilization of reducing sugar, the working conditions were established as being: the time of enzymatic hydrolysis = 67h; The addition of complex enzymes accounted for about 1.15% of the quality of the original dry stalk; The crude protein content of stalk feed = 27.13%. This paper intends to further improve the crude protein content and reduce the amount of nutrient added by optimizing the needed nutrient components, the two kinds yeast inoculation amounts and the process parameters required for the feed of the fermented stalk and provide a new way for the corn stalk feed use.

MATERIALS AND METHODS

Materials

Cellulase 10000U (Measured by QB2583-2003 CMC–Na, CMC-Na activity 10000U, measurement of filter paper activity, FDA 3500U. The following cellulase activities represented their filter paper activities) xylanase 10000U, β-glucanase 10000U and pectinase 30000U were from Zhaodong North Branch Enzyme Preparation Co. Ltd.Yeast extract, peptone: biochemical reagents, Beijing Aoboxing Biotechnology Co. Ltd. The rest of the regents are analytically pure.

Candida utilis (hexose consumer), *Pachysolen tannophilus* (pentose consumer) were purchased from the China Industrial Microbial Culture Collection Management Center, the number of which were 1807 and 1771.

Preparation of Steamed stalk

The fresh green corn stalks just harvested were from Wei yang District, Xi'an City, Shaanxi Province of China. After drying out, the moisture content was 7.94%. The original stalks were smashed by a high-speed pulverizer and passed through a 40-mesh sieve. Then stalks were treated by thermophilic digestion for 1.5h at 180℃ and were dried to constant weigh at 60 ℃. The obtained steamed stalks were equivalent to 83.42% of the quality of the original stalks.

Strains culture medium

Activation medium of *Candida utilis*: glucose 2.0g, peptone 2.0g, yeast extract 1.0g were added to 100mL water in triangular flask, the sterilization condition were: time 20min, temperature 121℃. A loop was put into the tube of inclining strains and cultured for 20h. The activation medium of *Pachysolen tannophilus* was the same as that of *Candida utilis*, the carbon source was xylose instead of glucose. The OD values of both seed solution diluted for 10 times were about 0.630 determined by the 722 type of spectrophotometer at 560 nm.

Analysis methods

Determination of crude protein: The method of (Lynch et al., 1999) was used to determine the crude protein. The solid was first centrifuged at 3500 r/min to obtain a solid, which was then dried at 60 ° C to a constant weight, and the crude protein was determined by a micro-Kjeldahl method.

$$\text{The yield of crude protein} = \frac{A_1}{A_2} \times 100\% \quad (1)$$

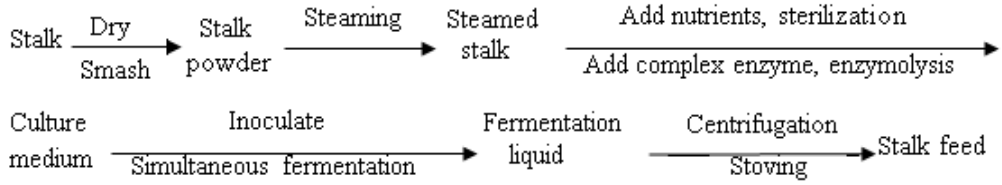
where A_1 is the amount of crude protein measured after fermentation, A_2 is the quality of the original dry stalk.

$$\text{The yield of stalk feed} = \frac{A_1}{A_2} \times 100\% \quad (2)$$

where A_1 is the quality of stalk feed dried to constant weigh at 60℃, A_2 is the quality of the original dry stalk.

Process

A more completely process was established after optimization combined with prophase researches (Chen et al., 2008); the whole process was as follows:



0.30g ammonium sulfate, 0.12g urea, 0.08g yeast extract, 0.020g magnesium sulfate, 0.020g potassium phosphate dibasic, 0.020g potassium dihydrogen phosphate and 30ml citrate buffer (pH 4.8) were added to 3.072g steamed stalks, adjusted to pH 4.8, sterilized for 20min at 121℃.

Complex enzymes (25mg cellulase, 10mg xylanase, 6mg Beta-glucanase, 1.3 mg pectinase) were added under aseptic manipulation after cooling to 50℃; enzymatic hydrolysis conditions were at 50℃ and 100 r/min for 16 hours. 2.0g *Candida utilis* broth and 2.5g *Pachysolen tannophilus* broth were inoculated under aseptic manipulation, fermentation was carried out for 53 h at 30 °C and shaking speed of 100 r/min. These basic conditions were fixed and one of them changed.

The inoculum size of *Candida utilis* seed broth and that of *Pachysolen tannophilus* seed broth, temperature, fermentation time, dosage of ammonium sulfate, urea, yeast extract, magnesium sulphate, potassium phosphate dibasic, potassium dihydrogen phosphate were determined by single factor experiments and orthogonal experiment with crude protein content as the index.

In order to analyze the effect of nitrogen sources and salts on crude protein yield, ammonium sulfate (0.10g, 0.20g, 0.30g, 0.40g, 0.50g), urea (0.03g, 0.06g, 0.12g, 0.18g, 0.24g), yeast extract (0.02g, 0.04g, 0.08g, 0.12g, 0.16g), magnesium sulfate (0.005g, 0.010g, 0.020g, 0.030g, 0.040g), potassium phosphate dibasic (0.005g, 0.010g, 0.020g, 0.030g, 0.040g) and potassium dihydrogen phosphate (0.005g, 0.010g, 0.020g, 0.030g, 040g 0.) were used. *Candida utilis* (0.5g, 1.0g, 2.0g, 2.5g, 3.0g) and *Pachysolen tannophilus* (1.0g, 1.5g, 2.5g, 3.5g, 4.0g) were inoculated. Different temperature (24℃, 26℃, 28℃, 30℃, 32℃) and different fermentation time (47h, 50h, 53h, 57h, 61h) were used to study the effect on crude protein yield.

Data processing

Each single factor test was performed in a 5 level 3 parallel test and the obtained data were plotted, standard deviation and significance analysis. Then, the obtained optimal values of each factor are subjected to the L_{27}（3^{13}）orthogonal test and the variance analysis was performed to obtain the optimal scheme. Finally, the 3 parallel verification test was performed to determine the optimal scheme.

RESULTS AND DISCUSSION

Effect of nitrogen sources and salts on crude protein yield

The effect of single factor experiment on crude protein yield was studied and the results are shown in Figure 1. The initial addition of ammonium sulfate (p<0.01), urea (p <0.01), yeast powder (p <0.01), magnesium sulfate (p<0.01) has an obvious effect on yield of crude protein. When reaching a certain degree, it not increased. The reason for this might be that the yeast proliferation was limited by the lack of sugars obtained from enzymatic hydrolysis, which affect the growth (Sánchez et al., 2010).

The addition of potassium phosphate dibasic (p >0.05), potassium dihydrogen phosphate (p >0.05) had an insignificant effect on yield of crude protein, it might be due to the presence of a certain amount of potassium and phosphorus in corn stalk (Asachi et al., 2013).

The added content of various sorts of nutrients determined from Figure 1 were shown as follow: ammonium sulfate of 0.30g, urea of 0.12g, yeast powder of 0.08g, magnesium sulfate of 0.010g, potassium phosphate dibasic of 0.005g, potassium dihydrogen phosphate of 0.005g.

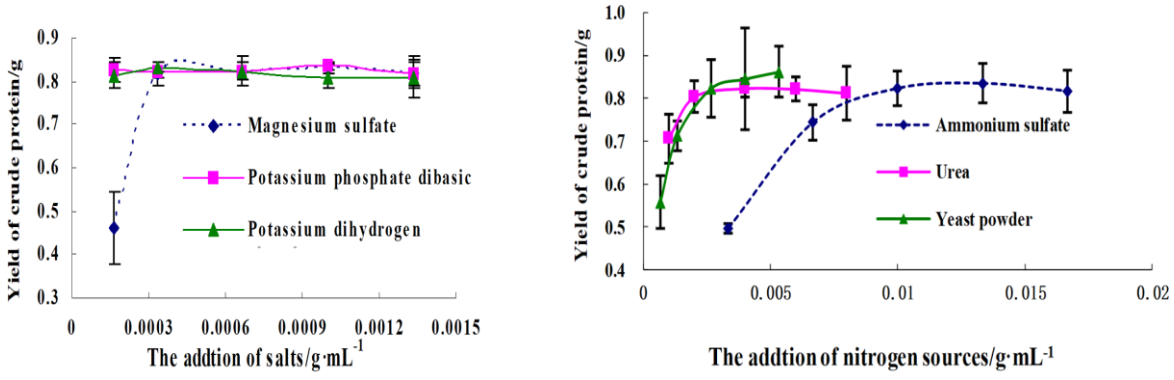


Figure 1. The relation between addition of nitrogen sources and salts and yield of crude protein

Effect of inoculum size on crude protein yield

The effect of yeast inoculum size on crude protein yield was studied and the results are shown in Figure 2. The inoculation amount of *Candida utilis* and *Pachysolen tannophilus* played an extremely remarkable effect on yield of crude protein. It was shown in figure 2 that when the inoculum size was too small, the yeast proliferation was slow, meanwhile the crude protein yield was small. When inoculum size was too large, yeasts consumed large amounts of sugar during the adaptive phase, so, the final crude protein production was low. *Candida utilis* can utilize five-carbon sugar and six-carbon sugar. Under aerobic conditions, it does not produce alcohol. It can also use molasses, wood hydrolysate to produce edible protein for animals (Rajoka et al., 2012). Corn stalk can effectively degrade to produce a large amount of xylose, but the common yeast hardly utilizes xylose (Fu et al., 2008). *Pachysolen tannophilus* can effectively utilize xylose (Seo et al., 2009). The inoculum sizes judged from figure 2 were: *Candida utilis* of 2.0g and *Pachysolen tannophilus* of 2.5g.

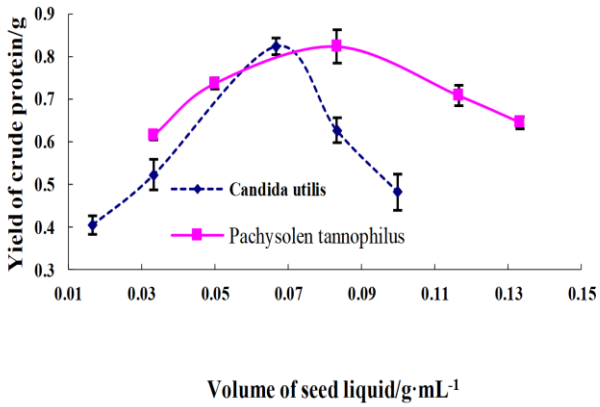


Figure 2. The relation between yeast inoculum size and yield of crude protein

3.3. Effect of temperature on crude protein yield

The effect on crude protein yield was studied at different temperature and the results are shown in figure 3. Temperature has a significant effect on crude protein yield (p <0.01). At 24 °C, the growth of yeast was limited, the crude protein production was the lowest. As the temperature increased, the yeast grew more vigorously, and the crude protein yield was higher. However, when the temperature reached more than 30 °C, the temperature was too high, the utilization of carbon source by yeast was inhibited and the yeast proliferation was affected. Kocher (2013) reported the fermentation variables for the fermentation of glucose and xylose using Saccharomyces cerevisiae and Pachysolan tannophilus, the optimum fermentation temperature was also 30℃.It was consistent with our research by using monofactorial experiment. So, the best temperature was 30℃.

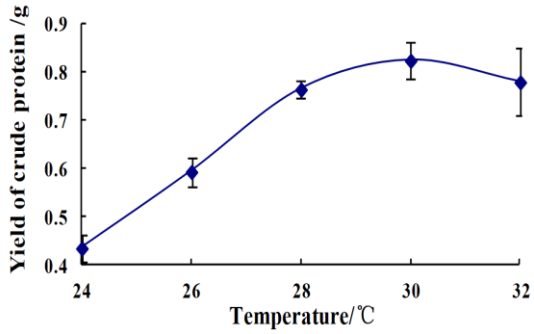


Figure 3. The relation between temperature and yield of crude protein

Effect of fermentation time on crude protein yield

The effect of different fermentation time on crude protein yield is given in figure 4. As the fermentation time (p <0.01) increases, the content of crude protein in the fermentation product increases first and then decreases.

When the fermentation time was 57h, the content of crude protein in the fermentation product was the highest. When the fermentation time is too short, the nutrients cannot be fully utilized, the crude protein content is low. But when the fermentation time was too long, the crude protein content showed a downward trend due to the decline of strains and the consumption of nutrients. Carbon source became less as the fermentation time extended (Wang et al., 2014).

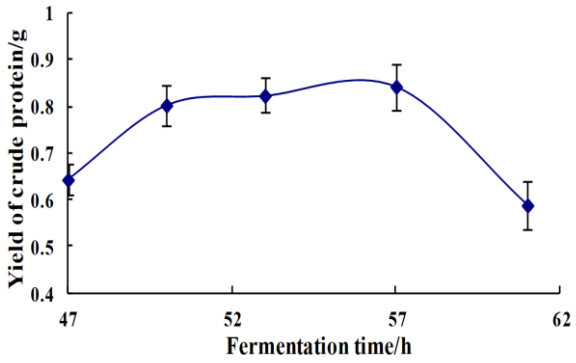


Figure 4. The relation between fermentation time and yield of crude protein

When the carbon source is not enough to maintain the yeast life, the population of yeast enters to decline phase. The yeast protein has a corresponding relationship with yeast yield, so

yield of crude protein begins to decline (Samson et al., 2017). For this cultivation, the best fermentation time was 57h, as the results in figure 4 indicate.

Optimization for orthogonal experiment of conditions for the production of protein feed by yeast fermentation

L27(3^{13}) orthogonal experiment were designed based on single factor test results, the code and unit were: A: ammonium sulfate (g), B: urea (g), C: yeast powder (g), D: magnesium sulfate (g), E: potassium phosphate dibasic (g), F: potassium dihydrogen phosphate (g), G: *Candida utilis* (g), H: *Pachysolen tannophilus* (g), I: temperature (℃), J: time (h). K, L, M were null columns. The index of experimental results was yield of crude protein (g). Factor level and results analysis of orthogonal experiment were shown in Table 1. The results of variance analysis was done for the results in Table 1 were shown in table 2.

Table 1. Factor level and analysis of the results of orthogonal experiment

Runs	A	B	C	D	E	F	G	H	I	J	K	L	M	Crude protein
1	1(0.25)	1(0.10)	1(0.06)	1(0.008)	1(0)	1(0)	1(1.5)	1(2.0)	1(29)	1(55)	1	1	1	0.7393
2	1	1	1	1	2(0.005)	2(0.005)	2(2.0)	2(2.5)	2(30)	2(57)	2	2	2	0.8208
3	1	1	1	1	3(0.007)	3(0.007)	3(2.2)	3(3.0)	3(31)	3(59)	3	3	3	0.7038
4	1	2(0.12)	2(0.08)	2(0.010)	1	1	1	2	2	2	3	3	3	0.7253
5	1	2	2	2	2	2	2	3	3	3	1	1	1	0.8525
6	1	2	2	2	3	3	3	1	1	1	2	2	2	0.7616
7	1	3(0.14)	3(0.10)	3(0.015)	1	1	1	3	3	3	2	2	2	0.7679
8	1	3	3	3	2	2	2	1	1	1	3	3	3	0.7421
9	1	3	3	3	3	3	3	2	2	2	1	1	1	0.7382
10	2(0.30)	1	2	3	1	2	3	1	3	3	1	2	3	0.8124
11	2	1	2	3	2	3	1	2	1	1	2	3	1	0.7605
12	2	1	2	3	3	1	2	3	2	2	3	1	2	0.8234
13	2	2	3	1	1	2	3	2	1	1	3	1	2	0.7352
14	2	2	3	1	2	3	1	3	2	2	1	2	3	0.7368
15	2	2	3	1	3	1	2	1	3	3	2	3	1	0.8156
16	2	3	1	2	1	2	3	3	2	2	2	3	1	0.7558
17	2	3	1	2	2	3	1	1	3	3	3	1	2	0.7411
18	2	3	1	2	3	1	2	2	1	1	1	2	3	0.8711
19	3(0.35)	1	3	2	1	3	2	1	2	2	1	3	2	0.8623
20	3	1	3	2	2	1	3	2	3	3	2	1	3	0.7323
21	3	1	3	2	3	2	1	3	1	1	3	2	1	0.8346
22	3	2	1	3	1	3	2	2	3	3	3	2	1	0.7971
23	3	2	1	3	2	1	3	3	1	1	1	3	2	0.7740
24	3	2	1	3	3	2	1	1	2	2	2	1	3	0.8023
25	3	3	2	1	1	3	2	3	1	1	2	1	3	0.7207
26	3	3	2	1	2	1	3	1	2	2	3	2	1	0.7291
27	3	3	2	1	3	2	1	2	3	3	1	3	2	0.7190
K_1	0.7613	0.7877	0.7784	0.7467	0.7684	0.7753	0.7585	0.7784	0.7710	0.7710	0.7895	0.7650	0.7803	
K_2	0.7835	0.7778	0.7672	0.7930	0.7655	0.7861	0.8117	0.7666	0.7771	0.7771	0.7708	0.7924	0.7784	
K_3	0.7746	0.7539	0.7739	0.7798	0.7855	0.7580	0.7492	0.7744	0.7713	0.7713	0.7591	0.7620	0.7608	
R	0.0223	0.0338	0.0112	0.0463	0.0200	0.0281	0.0626	0.0118	0.0061	0.0061	0.0304	0.0303	0.0195	

Table 2. The table of variance analysis

Factor	Square of deviance	Freedom	*F* ratio	*F critical-value*	Significance
A△	0.0023	2		$F_{0.05}(2,22)=3.44$ $F_{0.01}(2,22)=5.72$	
B△	0.0054	2			
C△	0.0006	2			
D	0.0102	2	4.2540		*
E△	0.0021	2			
F△	0.0036	2			
G	0.0205	2	8.5334		**

H△	0.0006	2			
I△	0.0002	2			
J△	0.0002	2			
e	0.0113	6			
e△	0.0264	22	0.0012		

The variance analysis of Table 2 shows that the effect of these factors successively are G>D>B>F>A>E>C=H>I=J. Factor D has significant influence on test, factor G has highly significant influence, so the better level D_2G_2 should be selected. Another factors had no significant influence, and each column met the requirement of adding to the error column. Therefore, level $A_1B_1C_1D_2E_1F_1G_2H_1I_1J_1$ was chosen considering cost.

Another three sets of parallel optimal conditions were verified because there were no optimum conditions in table of orthogonal experiment. The results were stable. The average weight of stalk feed was 3.021g (The yield of stalk feed was 82.04%), the color of fermentation solution was sepia. The average content of crude protein was 28.44%, which was 4.33 times of original dry stalks. The average yield of crude protein was 0.8591g (The yield of crude protein was 23.33%), which was approximately 0.0120g less than the maximum 0.8711g in the orthogonal table. The margin was razor-thin and the cost was low, so optimum condition $A_1B_1C_1D_2E_1F_1G_2H_1I_1J_1$ was selected. In order to provide the guide for production, various nutrients recruitment, the inoculum size of strains and previous research results (various component addition of compound enzyme and dosage of citrate acid buffer with a pH of 4.8) of optimization results were transformed into mass percent of the original dry stalk: ammonium sulfate 6.79%, urea 2.72%, yeast powder 1.63%, magnesium sulfate 0.27%, *Candida utilis* 54.31%, *Pachysolen tannophilus* 54.31%, cellulose 0.679%, xylanase 0.272%, β-glucanase 0.163%, pectinase 0.035%, pH 4.8 citrate acid buffer 815%, The operational parameters of fermentation process were: fermentation temperature 29℃, rotate speed 100 r/min and fermentation time 55h.

Compared with former research (Chen and Zhang, 2008), producing protein feed from corn stalks by multi-strain distributional degradation, the content of crude protein of feed in our paper increased by 1.31%, the addition of various nutrients saved above 17%, otherwise, no need to add any potassium phosphate dibasic and potassium dihydrogen phosphate.Ling (2009) reported simultaneous saccharification and fermentation conditions for production of bioethanol from steam-exploded corn stover, the fermentation time was 87 h, but in this paper it was shorter only need 55h and simple operation, little corrosion to equipment (chemical degradation without involved). From the perspective of the safety of the protein feed produced, it does not involve chemical degradation, no harmful chemical formation, it used yeast fermentation and no toxin production. From the perspective of reducing sugar utilization, double bacterium fermentation can make good use of the main reducing sugar pentose and hexose which are hydrolyzed. From the test results, the obtained effect is better, and the crude protein content exceeds the requirement of the minimum 20% crude protein in the protein feed.

CONCLUSIONS

According to the single factor test and orthogonal test, the optimal feed process of double-bacteria synchronous fermentation stalks was ammonium sulfate 6.79%, urea 2.72%, yeast powder 1.63%, magnesium sulfate 0.27%, Candida utilis 54.31%, Pachysolen tannophilus 54.31% and simultaneous fermentation for 55 hours at 29 °C. The yield of straw feed was 82.04% and the crude protein yield was 23.33%. The crude protein content of straw feed was 28.44%, which was 4.33 times of the original straw. Therefore, this technology has potential for industrializing and provides a certain guiding value for utilization of corn stalk to feed.

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