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Optimization of Growth Parameters of *Kluyveromyces lactis* K7 for Human Lysozyme Production in Biofilm Reactor

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Abstract. Lysozyme (1,4-β-N-acetylmuramidase) is a lytic enzyme, which degrades a constituent of bacterial cell wall. Lysozyme has been of interest in medicine, cosmetics, and food industries because of its anti-bactericidal effect. Several approaches for the production of human lysozyme have been reported. *Kluyveromyces lactis* is a suitable host cell for the production of human lysozyme, because it has effective secretory capacity and crabtree negative effect in fully oxidative conditions. *K. lactis* K7 is a genetically modified organism that expresses human lysozyme. There is a need to improve the human lysozyme production by *K. lactis* K7 to make the human lysozyme more affordable. The aim of this study was to determine the best plastic composite support (PCS) and optimize the growth conditions of *K. lactis* K7 for the human lysozyme production in biofilm reactor with PCS. The best PCS type was selected according to the biofilm formation on PCS (CFU/g) and human lysozyme production (Unit/ml). After determination of the best PCS in test tubes, a bioreactor equipped with a 2 L vessel was implemented. To find the optimum combination of growth parameters, three factors Box–Behnken design was used and different temperatures (20-40°C), pH (4-7), and aeration levels (0-1.5 volume of air/volume of broth/minute) were tested. As a result, the optimum temperature for biomass production was found higher than the optimum temperature for lysozyme production. Moreover, while biomass was higher at pH 6, the lysozyme production increased as pH decreased. The aeration caused increase in biomass, but decrease in lysozyme production

Keywords. Human lysozyme, *Kluyveromyces lactis* K7, biofilm reactor, plastic composite support, growth parameters

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Introduction

Lysozyme is an enzyme that catalyzes the hydrolysis of β (1→4) glycosidic linkages between N-acetylmuramic acid and N-acetylglucosamine found in peptidoglycan. Peptidoglycan is the major component of the cell wall of both Gram-positive and Gram-negative bacteria (Schleifer and Otto, 1972). Because of its bactericidal activity, lysozyme has been of interest in medicine, cosmetics, and the food industry. The most important application of lysozyme is usage as a food preservative, such as in cheese, fish, meat, fruit, vegetables, and wine. Other potential applications of lysozyme include use in heat treated products to reduce thermal requirement, use to prevent gas formation and cracking of the cheese wheels by *Clostridium tyrobutyricum*, added as a supplement with SO_2 in wine making to prevent malolactic fermentation and spoilage, and use in baby formula to aid the immune system development (Hughey and Johnson, 1987; Gilby, 2001). Moreover, the antibacterial property of lysozyme has been exploited in a number of other applications, such as eye drops and wound healing creams. Lysozyme has also been used in treatment of gastrointestinal infections, post-radiation therapy, periodontosis, and dry-mouth (Islam et al., 2006; Jolles and Jolles, 1984; Jolles, 1996). For all these purposes, egg-white lysozyme has been commonly used, but it poses immunological problems when applied to human beings. Individuals sensitive to chicken egg have been shown to be allergic to lysozyme produced from chicken egg (Pichler and Campi, 1992). Because of this, human lysozyme is a better anti-inflammation agent and preservative for food products that will be used by humans, and it has a four-times greater specific activity than egg-white lysozyme (Choi et al., 2004). Therefore, there is an urgent need to increase the production of human lysozyme to meet its current demand.

Human milk is an important source for human lysozyme, but human breast milk is a fairly poor source for commercial production of this enzyme (Yu et al., 2006). Therefore, several approaches for the expression of recombinant human lysozyme have been reported (Muraki et al., 1985; Yoshimura et al., 1987; Choi et al., 2004). Human lysozyme was successfully produced by genetically modified *Saccharomyces cerevisiae* and *Kluyveromyces lactis*, which have been transformed by the insertion of human lysozyme gene (Choi at al., 2004; Takako et al., 2004). *K. lactis* presents several advantageous properties in comparison to *S. cerevisiae*. These include *K. lactis*'s secretory capacities, crabtree-negative effect in fully oxidative conditions, and food grade status (Swinkels et al., 1993; Gonzales-Siso et al., 2000).

To date, human lysozyme production studies using *K. lactis* K7 have been performed by Maullu et al. (1999) and Huang and Demirci (2009). Maullu et al. (1999) conducted flask studies and measured the yield of human lysozyme using cheese whey as its growth media. Huang and Demirci (2009) investigated cultivation conditions to enhance the production of human lysozyme by *K. lactis* K7 using shake flasks and suspended-cell bioreactor. However, flask studies aren't scalable and suspended cell bioreactor may not be the best reactor system due its limited cell population. Therefore, there is still need to study human lysozyme production to optimize the production by using novel approaches and make it economical for the industry.

Some researchers attempted to increase the productivities during fermentation by using techniques such as immobilized cell reactors, cell-recycle reactors, and hollow fiber reactors (Choudhury and Swaminathan, 2006; Najafpour et al., 2004; Mehaia and Cheryan, 1984). Biofilm reactor is one of immobilized cell reactors (Demirci et al., 2007). Biofilm formation occurs naturally by the attachment of microbial cells to the support without use of any chemicals agent in biofilm reactors (Qureshi et al., 2005). Biofilm reactors are more advantageous than the other novel bioreactors, such as cell-recycled reactors and hollow-fiber reactors. Biofilm reactors have lesser tendencies to develop membrane fouling and lower required capital costs (Demirci et al.,

2007). Moreover, biofilm reactors show many advantages over suspended cell reactors, especially in their higher biomass density and operation stability. Biofilm reactors are able to retain more biomass per unit volume of reactor, thereby increasing production rates, reducing the risk of washing out when operating at high dilution rates during continuous fermentation, and eliminating the need for re-inoculation during repeated-batch fermentation (Demirci et al., 2007). Moreover, the biofilm matrix contributes to high resistance of microorganisms to extreme conditions of pH and temperature, contaminations, hydraulic shocks, antibiotics, and toxic substances (Demirci et al., 2007). Plastic composite support (PCS) is an extrusion of agricultural by-products and polypropylene. While polypropylene acts as solid matrix, agricultural by-products sustain the cell growth. Advantages of PCS are providing lower nitrogen requirement of the medium, longevity and durability for long-term fermentation due to PCS's strength and slow nutrient release characteristics (Cheng et al., 2010a). Many studies showed that using PCS biofilm reactors can enhance production of ethanol, organic acids, and bacteriocins. For example; two to ten times higher ethanol productivity was achieved in PCS ring biofilm reactors (Demirci et al., 1997). Higher concentrations of lactic acid were produced by pure- and mixed-culture PCS biofilm reactors (60 and 55 g/L, respectively) in repeated-batch fermentation when compared with suspension (Demirci and Pometto, 1995). Pongtharangkul and Demirci (2006a, b, c) investigated nisin production in biofilm reactor with plastic composite support and reported that the high-biomass density of the biofilm reactor caused a significantly shorter lag time of nisin production relative to a suspended-cell reactor.

The aim of this study was to select the suitable PCS out of four different types of composition in test tubes and optimize the growth parameters of *K.lactis* K7 (temperature, pH, aeration) to achieve maximum human lysozyme production in batch biofilm reactor with selected PCS.

Materials and Methods

Plastic composite support (PCS) tubes

The PCS tubes were manufactured in the Center for Crops Utilization Research at Iowa State University using a twin-screw corotating Brabender PL2000 extruder (model CTSE-V; C.W. Brabender Instruments, Inc., South Hackensack, NJ) as described by Ho et al. (1997). The nutrition compositions of plastics composite supports, which were tested in this study, are given in Table 1.

Table 1. The nutrient composition of plastic composite supports

Support	Plastic Composite support ingredients
1	Polypropylene, soybean hulls, soybean flour, salt
2	Polypropylene, soybean hulls, soybean flour, yeast extract, salt
3	Polypropylene, soybean hulls, soybean flour, yeast extract, bovine albumin, salt
4	Polypropylene, soybean hulls, soybean flour, yeast extract, bovine red blood cell, salt

Microorganism and growth medium

Kluyveromyces lactis K7 (ATCC-MYA-413) was obtained from American Type Culture Collection (Manassas, VA). The working culture was maintained on the agar slant and transferred to a fresh sterile agar slant every 2 weeks. The culture was grown in medium containing 2% (w/v) glucose, 1% (w/v) yeast extract, and 2% (w/v) peptone at 25ºC for 20-24 hours.

Test tube fermentation for PCS selection

Four types of PCS with different compositions were evaluated for both biofilm formation and human lysozyme production using test tube fermentation with three replicates. For each replicate, 3 g of PCS disks was sterilized dry in capped 50 ml culture tubes for 1 h at 121°C. Ten milliliters of sterilized medium was added aseptically to the sterile PCS disks and each test tube was inoculated with 1% (v/v) of 24-h grown *K. lactis* K7. Five repeated-batch fermentations were performed for biomass formation. Then, samples were analyzed after repeated-batch fermentation, which took 48 hours at 25 °C. The medium was composed of 0.67% (w/v) yeast nitrogen base, 9 % lactose, and 1% casamino acid, which were determined as optimum medium compositions for human lysozyme in suspended cell bioreactor by Huang and Demirci (2009). A control fermentation without PCS was performed simultaneously.

Batch fermentation in bioreactor

After determination of the best plastic composite support in test tubes, a Sartorious Biostat B Plus bioreactor (Allentown, PA) equipped with a 2 L vessel was implemented. For the biofilm reactor, 12 PCS tubes (6.5 cm long) were bound to the agitator shaft in a gridlike fashion, with six rows of two parallel tubes (Figure 1). The reactor vessel with PCS was autoclaved with water at 121°C for 90 min. Then, 1.5 L of sterilized medium (0.67% (w/v) yeast nitrogen base, 9% (w/v) lactose, and 1% (w/v) casamino acid) was added aseptically to the sterile reactor vessel with PCS. For biofilm formation, a 24-h grown culture of *K. lactis* K7 (1% v/v) was inoculated and pre-fermentation conditions were maintained as no pH control, 25ºC, 100 rpm agitation, and no aeration, which are the optimum conditions in suspended cell bioreactor determined by Huang and Demirci (2009). Five repeated batch pre-fermentations were carried out to establish biofilm on PCS supports. Then, batch fermentation was conducted for each level determined by Box-Behnken design for three days. Different temperatures (20-40 °C), pH (4-7) and aeration levels (0-1.5 volume of air/volume of broth/minute) were tested. To find the best combination of growth parameters, three factors Box–Behnken design was used. The response variables were biomass and lysozyme production.

Analysis

i) Biomass. Biomass was determined by measuring optical density (OD) using a spectrophotometer (Beckman Coulter, Fullerton, CA) by obtaining absorbance at 600 nm, which will then converted to biomass concentration by using equation: Biomass (g/l)=0.564 × OD_{600} as suggested by Huang and Demirci (2009).

ii) Biofilm cell population. The stripping sand method was used to determine the relative biofilm population on the supports (Ho et al., 1997). The PCS discs in the 50 ml test tubes were washed in 100 ml of sterile 0.1% (w/v) peptone water by turning the tubes upside-down 10 times. Then, PCS discs were aseptically transferred into a 50 ml test tube. The test tube contained 5 g of sterile sand and was autoclaved at 121°C for 30 min. After vortexing the tube

three times in 30-s intervals, the samples were serially diluted, and 10^{-2} to 10^{-5} dilutions were spread plated onto agar plates by using Autoplate 4000 (Spiral Biotech, Norwood, MA). The medium composition of agar plates was 2% (w/v) glucose, 1% (w/v) yeast extract, and 2% (w/v) peptone, 2% (w/v) agar. Then the agar plates were incubated at 25°C for 48 hours. The results were expressed in CFU/g PCS.

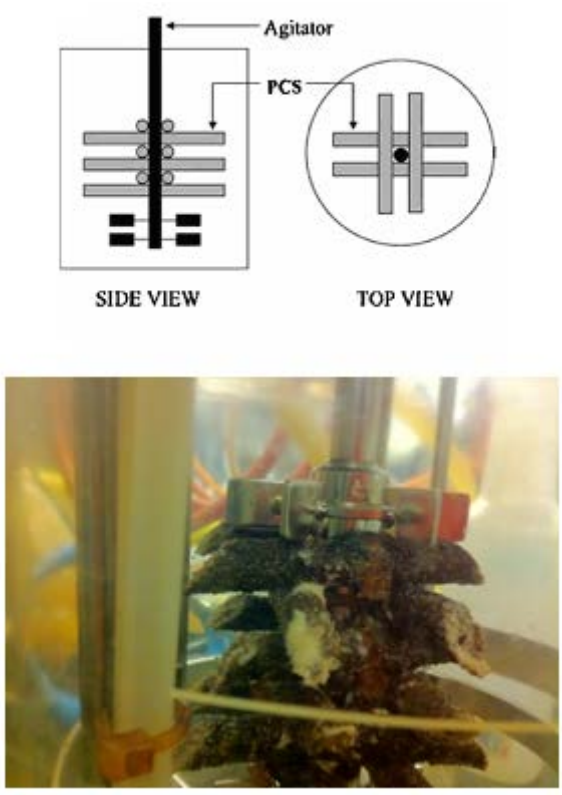

Figure 1. Diagram of the PCS biofilm reactor

iii) Lactose and glucose concentration. Lactose and glucose concentration determinations were conducted using high pressure liquid chromatography equipped with a refractive index detector (Waters, Milford, MA). Lactose and glucose were separated using Bio-Rad Aminex HPX-87H column (300×7.8 mm; Bio-Rad, Richmond, CA) with 0.8 ml/min of 0.012 N sulfuric acid as mobile phase. The detector temperature and column temperature were set up as 35 and 65ºC, respectively. The analysis was performed in duplicate.

iv) Lysozyme activity. Lysozyme activity analysis was carried out using a method adapted from Richard et al. (1965). The procedure for lysozyme assay is as follows: A 0.5 mg/ml of *Micrococcus lysodeikticus* cell suspension (Sigma-Aldrich, St. Louis, MO) was prepared using potassium phosphate buffer at pH 6.2. A 300-mM sodium chloride was prepared with deionized water. The assay solution consists of 600 µl of 0.5 mg/ml *M. lysodeikticus* cell suspension, 200 µl of 300 mM sodium chloride, and 400 µl sample. The absorbance was set at 540 nm, and the optical density readings were recorded every 10 s for 1 min. One unit of lysozyme activity is the amount of lysozyme that produces a 0.001 A540 change per minute. The slope of the linear regression line refers to the speed of lysis of *M. lysodeikticus* suspension, and the slope estimates the concentration of human lysozyme in unit per milliliter.

Statistical analysis

The statistical analyses were performed by using MINITAB Statistical Software package (Version 15, Minitab Inc.; State College, PA). Analysis of variance (ANOVA) was performed for investigating statistically significant differences between production amounts at different PCSs and growth parameters. A p value of <0.05 was considered to be significant.

Results and Discussion

The results of PCS selection for human lysozyme production in biofilm reactor were summarized in Figure 2. The biomass formation on PCS ranged from 7.62 to 7.04 log CFU/g. The biomass produced in the medium was ranged 1.69 to 1.59 g/L in the test tubes which includes PCS. The lysozyme production was found to be significantly higher in the test tube, which includes soybean hull, soybean flour, yeast extract, bovine albumin, and salts, than the others and the test tube, which includes only suspended cells ($p < 0.05$). The nitrogen content per g PCS may cause differences in the production of lysozyme in test tubes (Cheng et al., 2010b). Therefore, PCS consisting of soybean hull, soybean flour, yeast extract, bovine albumin, and salts was selected for human lysozyme production.

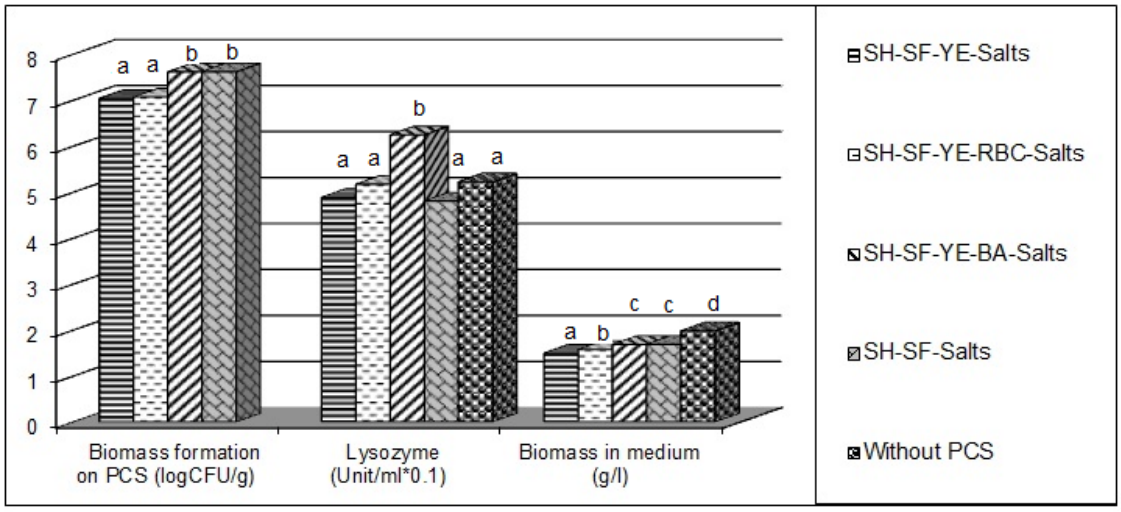

Figure 2. Effects of different PCS blends on the number of viable attached cells on the PCS, lysozyme production in test tubes (n =3) (Different letters above the columns mean statistical significance based on Tukey test, $p < 0.05$).

Table 2 shows the Box-Behnken design, experimental and predicted values for lysozyme and biomass production as a result of batch fermentation. By the application of multiple regression analysis on the experimentally determined values, the following second order polynomial equations were obtained by Minitab software. The sample variations of 99.6% of biomass production and 99.3% of human lysozyme production were attributed to the factors stated in the models for biomass and human lysozyme production in biofilm reactor with PCS.

Lysozyme (Unit/ml) = 109.42 – 87.19 × Aeration – 0.08 × Temperature × Temperature + 5.4× Aeration × Aeration + 0.43 × Temperature × pH + 0.73 × Temperature × Aeration + 5.4 × pH × Aeration ($R^2 = 0.993$)

Biomass (g/L) = - 10.65 + 0.74 × Temperature + 8.39 × Aeration – 0.01 × Temperature × Temperature – 2.68 Aeration × Aeration – 0.05 × Temperature × Aeration (R^2= 0.996)

Table 2. Effect of different growth parameter combinations on biomass and lysozyme production in Biofilm reactor with PCS.

Temperature		Aeration	Experimental		Predicted	
(^{o}C)	pH	(vvm)	Biomass (g/L)	Lysozyme (Unit/ml)	Biomass (g/L)	Lysozyme (Unit/ml)
30	7.0	0.00	1.93	43.50	2.08	41.25
30	5.5	0.75	5.85	38.00	5.85	38.00
30	5.5	0.75	5.85	38.00	5.85	38.00
20	5.5	1.50	6.32	20.00	6.47	19.19
20	7.0	0.75	5.35	18.00	5.37	17.94
30	4.0	1.50	6.40	24.00	6.26	26.25
30	7.0	1.50	6.66	19.00	6.49	19.88
20	5.5	0.00	1.48	61.50	1.32	63.81
40	4.0	0.75	3.49	22.00	3.47	22.06
40	7.0	0.75	3.61	15.00	3.62	16.44
20	4.0	0.75	5.15	51.00	5.14	49.56
40	5.5	1.50	3.81	18.00	3.97	15.69
40	5.5	0.00	0.55	37.50	0.40	38.31
30	4.0	0.00	1.77	73.00	1.94	72.13
30	5.5	0.75	5.85	38.00	5.85	38.00

As a result of application of regression analysis in Minitab, the optimum lysozyme production was achieved as 80 U/ml when the aeration level, pH, and temperature decreased to 0 vvm, 4, and 25ºC, respectively. On the other hand the optimum biomass production was found as 6.89 g/L when the temperature, aeration level, and the pH increased up to 27ºC, 1.33 vvm, and 6, respectively. For the verification of the model, batch fermentation was conducted at optimum levels for biomass and lysozyme production in duplicate. The results were in agreement with the predicted values (Table 3).

Table 3.Optimum conditions for lysozyme and biomass production and verification

	Suggested Optimum Conditions	Experimental*	Predicted
Biomass (g/L)	27^{o}C, pH 6, 1.33 vvm	6.53±0.25	6.89
Lysozyme (Unit/ml)	25^{o}C, pH 4, 0 vvm	83.75±0.77	80.06

*Average±standard deviation, (number of replicates = 2)

Conclusion

In conclusion, the PCS, which contains polypropylene, soybean hulls, soybean flour, yeast extract, bovine albumin, salt, was selected based on human lysozyme and biomass production. Temperature at 25°C, pH at 4 and no aeration were determined as optimum growth parameters of *K. lactis* K7 for human lysozyme production. The production level was predicted as 80 Unit/ml at these optimum conditions. Temperature at 27°C, pH at 6 and aeration at 1.33 vvm were determined as optimum growth parameters of *K. lactis* K7 for biomass production. The biomass level was predicted as 6.89 g/L at these optimum conditions.

As a future research, aeration and pH shift strategies will be evaluated to increase the biomass and then secrete the lysozyme to enhance the production. Moreover, different nitrogen sources and salts will be tested and the fermentation medium will be optimized to increase the human lysozyme production by *K. lactics* K7 in biofilm reactor. Fed-Batch and continuous fermentation modes will also be tested.

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