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EFFECT OF METHANOL FLOW RATE AND CASEIN ACID HYDROLYSATE TO RECOMBINANT *Pichia pastoris* FOR *Mucor pusillus* RENNIN PRODUCTION.

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Introduction. Cheese becomes one of the most favorite foods in the world and his demand increases rapidly every year. However the calf rennin supply decreases every day. *Mucor pusillus* aspartic proteinase, found by (1), is a very important substitute for bovine rennin. *This enzyme* is expressed in *Pichia pastoris* by Center of Genetic Engineering and Biotechnology (CGEB) of Cuba since 1991 using a simple fermentation process.
Subject of this work was to investigate the effect of the methanol flow rate and casein acid hydrolysate supply to the recombinant rennin production.

Metodología. **Method.** Strain: Recombinant *P. pastoris P61* Mut^+ developed by CGEB Plasmid: The pRH4 vector contains multi-copy of *Mucor pusillus* rennin gene expressing rennin enzyme under the control of AOX promoter. Medium: gycerol, $(NH4)_2SO_4$, KH_2PO_4, $MgSO_4.7H_2O$, $CaCl_2.2H_2O$, EDTA, solution of trace salts, vitamins, biotin, peptone and yeast extract. The fermentation process was operated in three phases: glycerol batch, glycerol fed-batch and methanol fed batch. The temperature was controlled at 28°C, pH was controlled at 5.5, agitation rate and aeration rate was controlled at 500 rpm and 1vvm respectively in the cultivation phase and 800rpm and 2vvm in the methanol induction phase. The factorial experiment design was established for two factors and two levels as showed in Table 1. The design consists of 2 replicates and was developed by the Software (Statgraphics Centurion XV, Colossus User). A total of 8 experiment runs with different combinations of two factors was carried out. Lowry with precipitation by acid percloric method was used for determination of total protein. The percent of rennin concentration wasestimated from the image of SDS-PAGE electrophoresisgel by the densitometry (software ImageJ 1.34s, USA).
Results and discussions. The ANOVA partitions the variability in rennin concentration into separate pieces for each of the effects. It then tests the statistical significance of each effect by comparing the mean square against an estimate of the experimental error. In this case, the P-values of the methanol flow rate is 0.0177, less than 0.05, indicating that they are significantly different from zero at the 95.0% confidence level. Whereas, the P-value of casein acid hydrolysate factor is 0.1056 and the P-value of their interaction is 0.5184, more than 0.05, meaning that they are not significantly different from zero at the 95.0% confidence level. The maximize rennin yield was estimated at 0.991g/L in the condition of 4.5g/L.h methanol flow rate without ddition of casein acid hydrolysate 40%.

Table 1. Factorial design matrix and results of rennin concentration.

Run	Block	Methanol flow rate (X_1 g/Lh)	Casein acid Hydrolysate 40% (X_2 mL)	Rennin concentration (Yg/L)
1	1	3.5	0	0.234
2	1	4.5	0	0.882
3	1	3.5	15	0.723
4	1	4.5	15	1.164
5	2	3.5	0	0.495
6	2	4.5	0	0.944
7	2	3.5	15	0.611
8	2	4.5	15	0.975

Conclusions: The recombinant rennin expressed in *P. pastoris* RP61 increased with increasing methanol flow rate. The highest rennin yield (0.991g/L) was found as the best condition (4.5g/L.h of methanol flow rate). It was demonstrated that in the *Pichia* system tightly regulating methanol feed control was critical to the high expression efficiency. In this case, casein acid hydrolysate is not necessary to add to the fermentor during the induction phase. That is a really good result to reach to the production scale, however, since this study revealed the importance of methanol feed rate in the DO-stat, future studies will determine optimal methanol levels.

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