RESEARCH

# *A* Pichia pastoris *fermentation process to express* Mucor pusillus *rennin*

Toan Hong Phuoc[1], ✉ Mariela Pérez[2], Julio C Dustet[3], Inalvis Herrera[4], Sheila Padrón[4], Jorge Valdés[2]

[1]Biotechnology Center of Ho Chi Minh City
176 Hai Ba Trung Street , District 1, Ho Chi Minh City, Vietnam
[2]Fermentation Department, Unit of Technological Development,
Center for Genetic Engineering and Biotechnology, CIGB
Ave. 31 / 158 and 190, Playa, PO Box 6162, Havana, Cuba
[3]Faculty of Chemical Engineering, "José Antonio Echevarría" Higher Polytechnic Institute
114 St, 11901, Havana, Cuba
[4]Analytical Department, Unit of Technological Development,
Center for Genetic Engineering and Biotechnology, CIGB
E-mail: mariela.perez@cigb.edu.cu

**ABSTRACT**

Rennin from *Mucor pusillus* (MPR) is an efficient substitute of bovine rennin used as milk clotting enzyme for cheese manufacture. A new fermentation process in the laboratory scale was developed to produce MPR by means of the strain RP61 Mut$^s$ from *Pichia pastoris* containing multiple copies of the gene for MPR expression. This process includes three phases, the first one in batch mode for growth on glycerol with MYPG medium supplemented with 10 g/L peptone and 20 g/L yeast extract, the second one a fed-batch phase with glycerol; and the last one with methanol for protein expression. Growth, yield of MPR and milk clotting activity were used to define the best conditions achieved with a methanol feed flow of 4.5 g/L·h, pH of 5.5 at 28 °C. As a result, the yield obtained for MPR was 0.95 g/L at 112 hours of fermentation with milk clotting activity of 565 IU/mL. On the other hand, the volumetric productivity obtained for rennin production was 8.5 mg/L·h, which means 1.7 times higher than those obtained in other works before.

*Keywords*: rennin, fermentation, *Pichia pastoris*

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**RESUMEN**

**Proceso de fermentación en *Pichia pastoris* para la expresión de renina de *Mucor pusillus*.** La enzima renina de *Mucor pusillus* (MPR), constituye un sustituto eficiente de la renina bovina que se usa como coagulante de la leche en la fabricación de quesos. En este trabajo se desarrolló un nuevo proceso de fermentación en la escala de laboratorio para producir MPR por medio de la cepa *Pichia pastoris* RP61 Mut$^s$ que contiene copias múltiples del casette de expresión con el gen MPR. El proceso incluye tres fases, una de crecimiento en glicerol en modo discontinuo con el medio MYPG suplementado con 10 g/L de peptona y 20 g/L de extracto de levadura, otra en modo incrementado con glicerol y una última fase de incremento con metanol para la expresión de la proteína. Las mejores condiciones para el crecimiento, el rendimiento de MPR y la actividad coagulante en leche se lograron con un flujo de alimentación de metanol de 4.5 g/L·h, un pH de 5.5 y 28 °C. El rendimiento final obtenido de MPR fue 0.95 g/L en 112 horas de fermentación con una actividad coagulante en leche de 565 IU/mL. La productividad volumétrica alcanzada para la producción de renina fue de 8.5 mg/L·h, 1.7 veces mayor que la obtenida en trabajos anteriores.

*Palabras clave*: renina, fermentación, *Pichia pastoris*

## *I*ntroduction

Cheese is one of the most favourite nutritional foods in the world. Animal rennin (bovine chymosin) is conventionally used as a milk-clotting agent in the manufacture of cheeses with good flavour and texture. An increase of cheese demand together with reducing calf rennin supply has encouraged scientists in searching for animal rennin substitutes [1, 2].

*Mucor pusillus* aspartic proteinase, found by Arima K [3], is a very important substitute for bovine rennin, possessing relatively high milk clotting activity. The gene for this enzyme has been cloned in many different microorganisms, including *Pichia pastoris.*

In 1991, Morales *et al*. [4] obtained the strain MR3 from *P. pastoris* with a single copy of the *Mucor pusillus* rennin (MPR) gene. The average yield of MPR obtained in a fermentation process was 587 mg/L at 120 hours with a volumetric productivity of 4.9 mg/L·h [5].

Due to the ambition to fill the domestic demand and achieve the international market, a new strain from *P. pastoris* (RP61 Mut$^s$) was developed in 1999 [6]; containing multiple copies of expression cassettes integrated to the genome including MPR *gene*, AOX1 promoter (p*AOX1*), GAP gene terminator (tGAP*),* the HIS3 selection marker, the secreting signal peptide SUC2 of *Saccharomyces cerevisiae* and a non-codified region 5' and 3' of *AOX1* gene needed for the homologous crossover at the end of the cassette. Antibiotic resistance genes or other markers were not included. This strain has a potential to achieve a very high yield of recombinant MPR (rMPR). However, the fermentation process has not been optimized because the best results obtained with this strain were 0.91 g/L of MPR at 160 h with a volumetric productivity of 5.7 mg/L·h. Consequently, the objective of our study is to define a

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✉ Corresponding author

new fermentation process at laboratory scale to obtain rMPR with a higher volumetric productivity than the process designed before.

## Materials and methods

### Strain

*P. pastoris* RP61 Mut$^s$

#### *Culture Medium*

The MPYG medium with different amounts of peptone and yeast extract was used for the study in shake flask. This medium contains 20 g/L Glycerol, 3 g/L $(NH_4)_2SO_4$, 4.3 g/L $KH_2PO_4$, 3.2 g/L $MgSO_4 \cdot 7H_2O$, 0.22 g/L $CaCl_2 \cdot 2H_2O$, 0.4 g/L EDTA, trace salt solution 1000X, vitamin solution 400X and biotin solution 4000X as well as different amounts of peptone and yeast extract according to the design of experiments used.

### Experiment design to study the effects of peptone and yeast extract on growth and rMRP expression

This experiment was carried out using a $2^2$ factorial designs. Shake flask total volume was 2.0 L and 200 mL of working volume. The two experiment factors were designated A for peptone and B for yeast extract. Two replicates were running. A negative control without peptone and yeast extract was also carried out. These experiments were started with the same amount of biomass. The initial glycerol concentration was 1%. After 32 hours of cultivation, methanol 1% (v/v) was added to induce the protein expression; later, another pulse of methanol 1% was added every 24 hours. The whole time for the experiment was 80 hours. The pH of the culture medium was maintained over 5.0 by adding 2.4 g/L of succinic acid. In this experiment, the rMPR yield and the yield coefficient rMPR-biomass ($Y_{P/X}$) were the response variables.

### The new fermentation process for *P. pastoris* RP61

In this scheme, we have established a new fermentation process including three phases: glycerol batch, glycerol fed-batch and methanol fed-batch phases. Glycerol batch and glycerol fed-batch phases were used to reach a high cell density in the range of 250 to 300 g/L of wet biomass. The glycerol batch phase was initiated with MYPG medium, adding 40 g/L of glycerol. Fermentation conditions like temperature, pH, agitation and aeration, were automatically controlled at 28 ± 2 ºC, 5.5 ± 0.2, 500 ± 50 min$^{-1}$ and 1 vvm respectively. After 13 hours of cultivation, the agitation and aeration rates were controlled at 800 min$^{-1}$ and 2 vvm to supply enough oxygen. Once the pH was increased, finished the glycerol batch phase and started the glycerol fed-batch phase. The glycerol concentration in the fed was 50 g/L. Glycerol flow rate in this phase was calculated by the following equation [7]:

$$F = \frac{V_1 X_1 \mu}{Y_{x/s} (S_0 - S_1)} \quad (1)$$

Considering, F as the glycerol feeding flow rate (L/h), $X_1$ the desired dry biomass (g/L), $V_1$ the desired volume of culture medium, $Y_{X/S}$ the yield coefficient of biomass-glycerol, $S_0$ the concentration of glycerol in the fed stream (g/L), $S_1$ the final concentration of glycerol (assumed as 0 g/L).

In the methanol fed-batch phase, the methanol flow rate was investigated together with casein acid hydrolysate concentration using a $2^2$ factorial design consisting of 2 replicates, as described in table 3. Protein expression was induced with methanol 1% (v/v) added as pulse. Agitation and aeration were controlled during this phase at 800 min$^{-1}$ and 2 vvm respectively in other to maintain a high oxygen transfer. The fermentations were stopped when the rMPR yield was about 1.0 g/L.

### Correlation between dry cell weight and wet cell weight

This experiment was implemented in the fermentor. Samples were taken at different time points (4 h, 7 h, 10 h, 13 h and 16 h) to weigh wet and dry cells. Each sample was carried out twice.

Wet cell weight (WCW) was determined as follows: A volume (V = 10 mL) of sample was taken to a tube known weight ($m_0$), centrifuged at 3000 rpm for 30 min. Then, the supernatant was removed. The tube containing cells pellet was weighed again to get $m_1$. The wet cell weight was calculated by the equation:

$$WCW\ (g/L) = (m_1 - m_0) * 100 \quad (2)$$

Dry cell weight (DCW) was determined as follows: The cell pellet was washed with twice-distilled water and resuspended to 1.0 mL with distilled water and distributed to the aluminum plates known weight ($m_i$). The plate was dried at 105 ºC until the weigh was constant ($m_2$). The wet cell weight was calculated by the equation:

$$DCW\ (g/L) = (m_2 - m_i) * 100 \quad (3)$$

The correlation curve between the dry cell weight and the wet cell weight was obtained by the programme Curve Expert (version 1.34).

$$DCW\ (g/L) = 0.21\ WCW\ (g/L) - 0.26 \quad (4)$$

Correlation coefficient = 0.9984

### Protein identification and qualification

In this study, recombinant rennin was identified by SDS-PAGE electrophoresis [8] and its biological activity by the Arima's method. Lowry method [9] with precipitation by acid perchloride was used for determining the total protein. The percentage of rMPR was estimated by the software Image J 1.34s, national Institutes of health, USA.

### Milk clotting activity assay

Milk clotting activity was determined in terms of international units (IU) according to published procedures [10]. One IU (equivalent to 400 SU, Soxhlet rennet units) was defined by Arima [3] as the amount of enzyme which clotted 10 mL of milk solution containing 1 g skim milk powder and 0.0173 g $CaCl_2$ at 1 min and 35 °C. Then, milk clotting activity was calculated by the following equation:

$$Activity\ (IU/mL) = 6000/t * Dilution \quad (3)$$

t: milk clotting time (s)

5. Pérez T, Ramos E. Dustet J C. Producción a escala de laboratorio de la proteasa aspártica del hongo *Mucor pusillus* expresada en la levadura *Pichia pastoris*. Rev Cub Ciencia Agr 2001;35(4):387-91.

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The enzyme reactions were carried out in at least three replicates. The tube was well agitated until milk clots appeared. The milk clots were visualized by turning the tubes upside down. Milk clotting time was carefully noted which should be from 3 to 6 minutes, if not, it should be diluted. The commercial bulk enzyme Cuamexyme (Industries Cuamex, S.A de C.V), Mexico, was *used* as a positive control and the negative control was the fermentation supernatant of *P. pastoris* for Hepatitis B vaccine.

### Statistical analysis

Statgraphics Centurion XV, version 15.2.05, colossus user was used for statistical analysis.

## Results and discussion

### Effect of peptone and yeast extract on the rMPR expression

The summary of experimental design and responses at 80 hours are given in table 1. The rMPR yield was estimated from the SDS-PAGE electrophoresis (Figure 1) and $Y_{P/X}$ was calculated by dividing rMPR yield to final biomass. In the case of negative controls, rMPR was only 1.2 mg/L. This value was much lower than the other ones resulting that the addition of peptone and yeast extract was important to improve the rMPR yield. Recombinant rennin appeared in the range from 45 to 66 kDa (Figure 1), stated in the results reported by Beldarrain *et al.* [11].

The ANOVA tables for rMPR yield response (Table 2) and yield coefficient product-biomass ($Y_{P/X}$) (Table 3) showed yeast extract and the interaction between peptone and yeast extract have P-values less than 0.05, indicating that they are significantly different from zero at the 95.0% confidence level (P < 0.05). It means that the combination of peptone 10 g/L and yeast extract 20 g/L was the best combination for rMPR production.

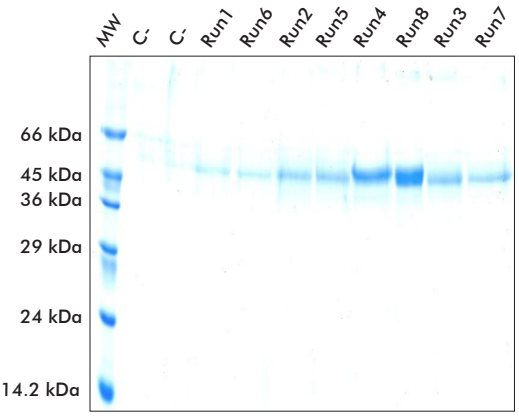


Figure 1. SDS-PAGE electrophoresis for the final fermentation broths. Effect of peptone and yeast extract on the rMPR yield and the yield coefficient product-biomass $Y_{P/X}$.

The average values of $Y_{P/X}$ with different combinations of A and B are showed in figure 2. The combination 10 g/L peptone and 20 g/L yeast extract is promoting the highest value of $Y_{P/X}$.

### Effects of peptone and yeast extract on the growth

All combinations of peptone and yeast extract stimulated a cell concentration higher than the negative control (samples 1 and 2) (Figure 3). The variable cell concentration was analysed in the glycerol phase (beginning at hour 0 up to hour 32 when the substrate was all consumed) (Table 4) and for the whole pro-

11. Beldarraín A, Acosta N, Montesinos R, Mata M, Cremata J. Characterization of *Mucor pusillus* rennin expressed in *Pichia pastoris*: enzymic, spectroscopic and calormetric studies. Biotechnol Appl Biochem 2000;31:77-84.

Table 1. Factorial design matrix and responses. Effect of peptone and yeast extract on the rMPR yield and the yield coefficient product-biomass $Y_{P/X}$. Fermentation conditions: shake flask at initial pH 5.0, temperature at 28 °C and 200 $min^{-1}$

| Run | Replicates | A(g/L) | B(g/L) | rMPR yield (mg/L) | $Y_{P/X}$ (mg/g) |
|---|---|---|---|---|---|
| 1 | 1 | 10 | 10 | 6.68 | 0.107 |
| 2 | 1 | 20 | 10 | 8.83 | 0.133 |
| 3 | 1 | 20 | 20 | 22.74 | 0.302 |
| 4 | 1 | 10 | 20 | 23.92 | 0.378 |
| 5 | 2 | 20 | 10 | 11.45 | 0.171 |
| 6 | 2 | 10 | 10 | 4.89 | 0.077 |
| 7 | 2 | 20 | 20 | 22.77 | 0.318 |
| 8 | 2 | 10 | 20 | 25.94 | 0.410 |

Table 2. ANOVA for the effect of peptone and yeast extract on rMPR yield

| Source | Sum of Squares | Df | Mean Square | F-Ratio | P-Value |
|---|---|---|---|---|---|
| A:Peptone | 2.3762 | 1 | 2.3762 | 1.18 | 0.3567 |
| B:Yeast extract | 504.349 | 1 | 504.349 | 250.58 | **0.0005** |
| AB | 21.3205 | 1 | 21.3205 | 10.59 | **0.0473** |
| Blocks | 1.0368 | 1 | 1.0368 | 0.52 | 0.5248 |
| Total error | 6.0381 | 3 | 2.0127 | | |
| Total (corr.) | 535.12 | 7 | | | |

R-squared = 98.8716 percent.
R-squared (adjusted for d.f.) = 98.0254 percent.
Standard Error of Est. = 1.4187.
Mean absolute error = 0.8.
Durbin-Watson statistic = 2.77724 (P = 0.8109).
Lag 1 residual autocorrelation = -0.554028

Table 3. ANOVA for the effect of peptone and yeast extract on yield coefficient product-biomass ($Y_{P/X}$)

| Source | Sum of Squares | Df | Mean Square | F-Ratio | P-Value |
|---|---|---|---|---|---|
| A:Peptone | 0.000288 | 1 | 0.000288 | 0.61 | 0.4923 |
| B:Yeast extract | 0.105800 | 1 | 0.105800 | 223.52 | **0.0006** |
| AB | 0.010368 | 1 | 0.010368 | 21.90 | **0.0184** |
| Blocks | 0.000392 | 1 | 0.000392 | 0.83 | 0.4299 |
| Total error | 0.00142 | 3 | 0.000473 | | |
| Total (corr.) | 0.118268 | 7 | | | |

R-squared = 98.7993 percent
R-squared (adjusted for d.f.) = 97.8988 percent
Standard Error of Est. = 0.0217562.
Mean absolute error = 0.011.
Durbin-Watson statistic = 2.48662 (P=0.6699)
Lag 1 residual autocorrelation = -0.442254

cess (Table 5). The ANOVA results showed that there is a statistically significant difference between the means of the 5 groups at the 95.0% confidence level ($P < 0.05$) in both cases (the glycerol growth phase and the whole process). For the glycerol growth phase, the best combination was 10 g/L of peptone and 20 g/L of yeast extract. However, for the whole process the best combination was 20 g/L for peptone and 20 for yeast extract. The cell concentration was 73.5 g WCW/L with this last combination

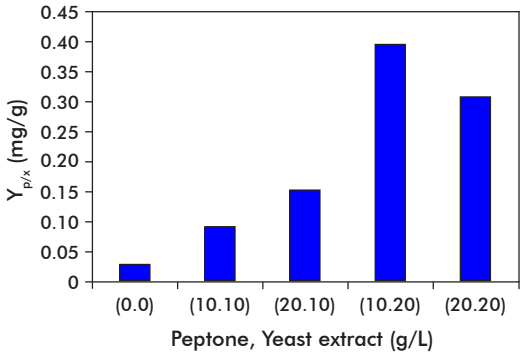


Figure 2. Average value of yield coefficient product-biomass ($Y_{P/X}$) for different combinations of peptone and yeast extract concentrations (g/L) used in the shake flask experiments.

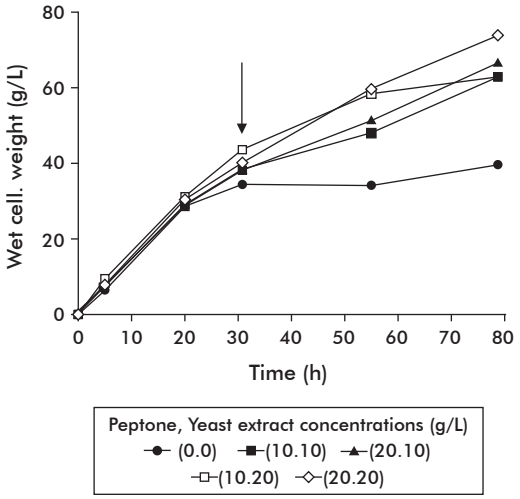


Figure 3. Biomass concentration as function of time for different combinations of peptone and yeast extract. Fermentation conditions: pH 5.0, temperature 28 °C, 200 rpm. The arrow indicated the time of induction (32 h). Error bars represent the standard deviation.

Table 4. ANOVA for the effect of peptone and yeast extract on the growth of *P. pastoris* RP61 in glycerol phase

| Source | Sum of Squares | Df | Mean Square | F-Ratio | P-Value |
|---|---|---|---|---|---|
| Between groups | 95.294 | 4 | 23.8235 | 16.84 | **0.0042** |
| Within groups | 7.075 | 5 | 1.415 | | |
| Total (Corr.) | 102.369 | 9 | | | |

Following the main purpose in the production process of rMPR, we take the combination of 10 g/L peptone and 20 g/L yeast extract as the best one for the next study in the fermentor.

## The new fermentation process for *P. pastoris* RP61

### *High cell density cultivation on glycerol*

In the fermentation process before [5, 6], the growth observed in the methanol phase was slowly. Therefore, the new scheme studied here possessed a glycerol fed-batch to obtain a higher specific growth rate to reach a high cell density before rennin expression could begin. The process was started in batch mode with 40 g/L initial concentration of glycerol. An initial concentration over 40 g/L could inhibit growth in glycerol batch phase [7]. At around 19-20 hours, the pH increased meaning that glycerol batch was finished. The cell concentration at the end of glycerol batch was approximate 120 g WCW/L. The yield coefficient biomass-substrate $Y_{X/S}$ was 0.60.

Subsequently, the glycerol feed batch was initiated and maintained for 7 hours to reach a cell concentration between 230 and 250 g WCW/L, *i.e*. the biomass increased two times (Table 6). The glycerol (50%

Table 5. ANOVA for the effects of peptone and yeast extract on the growth of *P. pastoris* RP61 in methanol phase

| Source | Sum of Squares | Df | Mean Square | F-Ratio | P-Value |
|---|---|---|---|---|---|
| Between groups | 1333.83 | 4 | 333.457 | 226.07 | **0.0000** |
| Within groups | 7.375 | 5 | 1.475 | | |
| Total (Corr.) | 1341.2 | 9 | | | |

Table 6. Biomass concentration in glycerol batch and fed batch phases

| Time (hours) | Description | Wet cell weight (g/L) | | |
|---|---|---|---|---|
| | | Replicate 1 | Replicate 2 | Replicate 3 |
| 0 | | 6.1 | 5.7 | 9.07 |
| 19 | Glycerol batch | 115.5 | 122.5 | 128.2 |
| 26 | Glycrol fed-batch | 231.5 | 253 | 244 |

concentration) flow rate was maintained at 21 mL/h. This scheme was used in the next experiment. In the process designed before, the growth stop-ped when the cell concentration was 182 g WCW/L due to oxygen limitation [5]. Whereas, in the process designed by Flores [6] the cell growth reached the value of 240 g WCW/L but at 160 hours ( at the end of the process).

### *Effect of methanol flow rate and casein acid hydrolysate on the growth and rMPR expression*

The entire experiment design and responses are summarized in table 7. All conditions reached up to quite high cell density (Figure 4). However, the growth in methanol fed-batch phase looked slowly because of an increase in working volume [12]. In this scheme, the rMPR was also expressed in a very high percentage ~90%. Figure 5 shows the formation of rMPR in different conditions. After 40 hours, the rMPR was formed rapidly and the conditions 1, 2, 5 and 7 reached about 1.0 g/L of rMPR at 112 hours.

The ANOVA analysis for final biomass response indicated that none of the effects had P-values less than 0.05, meaning that they were not significantly different from zero at the 95.0% confidence level (Table 8). According to the rMPR yield $Y_{P/X}$ and the milk clotting activity, ANOVA gave the same results, only the methanol flow rate had P-values less than 0.05, indicating that it was significantly different from zero at the 95.0% confidence level (Table 9).

From the above results is concluded that to obtain the best rMPR yield, it is necessary to operate at the methanol flow rate of 4.5 g/L·h (higher than in the fermentation process before: 3.5 g/L·h, [5]). In this case, casein acid hydrolysate was an insignificant effect on all the responses that is why, the addition of casein acid hydrolysate was not necessary. The best rMPR yield was 0.95 g/L (this value was given by the software Statgraphics using multiple response optimi-

12. Peter F Stanbury, Whitaker A, Stephen J. Principles of Fermentation Technology. 2nd ed. BPC Wheaton: Butterworth-Heinemann Publisher; 1999.

Table 7. Factorial design matrix and responses in the study of the effect of methanol flow rate and casein acid hydrolysate on the growth and rMPR expression

| Run | Replicates | Methanol flow rate (g/L h) | Casein acid hydrolysate (%) | Final biomass (g/L) | rMPR yield (g/L) | $Y_{P/X}$ (mg/g) | Milk clotting activity (IU/mL) |
|---|---|---|---|---|---|---|---|
| 1 | 1 | 4.5 | 0 | 271.8 | 0.882 | 3.25 | 561 |
| 2 | 1 | 4.5 | 2.5 | 241.8 | 1.013 | 4.19 | 585 |
| 3 | 1 | 3.5 | 0 | 235.0 | 0.234 | 1.00 | 120 |
| 4 | 1 | 3.5 | 2.5 | 284.2 | 0.723 | 2.54 | 292 |
| 5 | 2 | 4.5 | 0 | 225.8 | 0.944 | 4.18 | 567 |
| 6 | 2 | 3.5 | 0 | 260.5 | 0.495 | 1.90 | 250 |
| 7 | 2 | 4.5 | 2.5 | 266.2 | 0.975 | 3.66 | 555 |
| 8 | 2 | 3.5 | 2.5 | 284.6 | 0.611 | 2.15 | 399 |

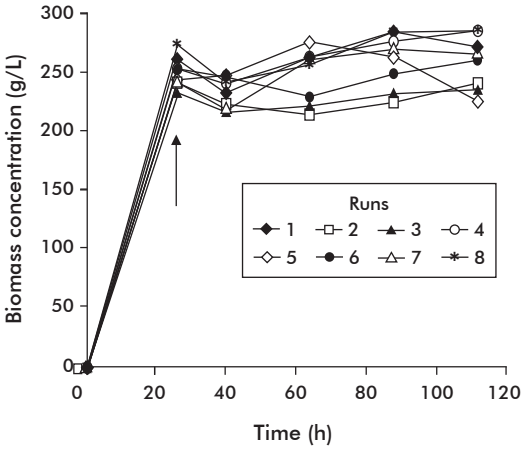


Figure 4. Biomass concentration as function of time for different combinations of methanol flow rate and casein acid hydrolysate. Fermentation conditions: 28 ± 2 °C, pH 5.5 ± 0.2, agitation rate 800 ± 50 $min^{-1}$ and aeration rate 2 vvm. The arrow indicates the time of induction.

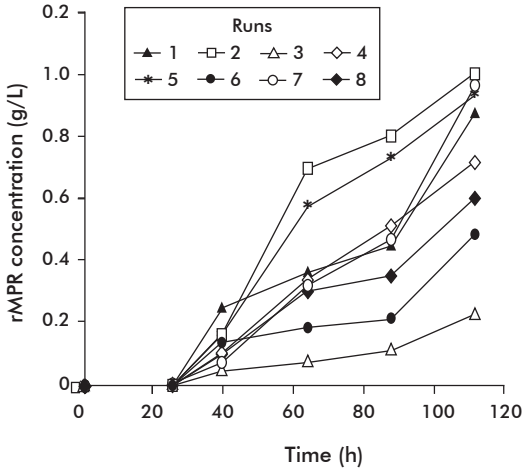


Figure 5. rMPR concentration as function of time for different combinations of methanol flow rate and casein acid hydrolysate. Fermentation conditions: 28 ± 2 °C, pH 5.5 ± 0.2, agitation rate 800 ± 50 rpm and aeration rate 2 vvm.

zation procedure). Therefore, we have increased the rMPR yield compared to the proposed process previously. A shorter fermentation time (112 h) is needed to obtain the rMPR yield. The volumetric productivity has had a value of 8.5 mg/L h which means 1.5 and 1.7 times higher compared to the obtained by [5] and [6] respectively). Also, our process has a higher volumetric productivity than this designed by Zang [13] to produce buffalo rennin in *P. pastoris*.

In the new process, the glycerol batch and fed-batch were operated to generate a high density of biomass before inducing with methanol. Moreover, the average milk clotting activity in the new process was also higher than in the proposed process before.

Another advantage of the new process is the opening of possibilities to produce the rMPR in a multiproduct plant because it reduces the total time of production.

## Conclusions

MYPG medium containing 10 g/L peptone and 20 g/L yeast extract was suitable for the growth phase in glycerol of *P. pastoris* RP61. Best fermentation conditions for rMPR expression in *P. pastoris* RP61 included three phases, a glycerol batch phase up to 19 hours, a Glycerol fed-batch phase with a glycerol (50% concentration) flow rate of 21 mL/h maintained during seven hours, and a methanol fed-batch phase with a methanol flow rate of 4.5 g/L h. The other conditions were temperature at 28 °C, pH of 5.5, agitation rate at 500 $min^{-1}$ and aeration rate of1 vvm, after 13 hours of fermentation, it is necessary to increase agitation rate to 800 $min^{-1}$ and aeration rate to 2 vvm. The whole fermentation process at laboratory scale took 112 hours to obtain a desirable rMPR yield of approximate 1.0 g/L, with the volumetric productivity of 8.5 mg/L h.

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Table 8. ANOVA for the effects of methanol flow rate and casein acid hydrolysate on final biomass

| Source | Sum of Squares | Df | Mean Square | F-Ratio | P-Value |
|---|---|---|---|---|---|
| C: Methanol flow rate | 430.711 | 1 | 430.711 | 0.77 | 0.4449 |
| D: Casein acid hydrolysate | 875.711 | 1 | 875.711 | 1.57 | 0.2996 |
| CD | 494.551 | 1 | 494.551 | 0.88 | 0.4165 |
| Blocks | 2.31125 | 1 | 2.31125 | 0.00 | 0.9528 |
| Total error | 1678.57 | 3 | 559.525 | | |
| Total (corr.) | 3481.86 | 7 | | | |

R-squared = 51.7909 percent.
R-squared (adjusted for d.f.) = 15.634 percent.
Standard Error of Est. = 23.6543.
Mean absolute error = 11.9375.
Durbin-Watson statistic = 2.01911 (P=0.3919).
Lag 1 residual autocorrelation = -0.174614.

Table 9. ANOVA for the effects of the methanol flow rate and casein acid hydrolysate on rMPR yield

| Source | Sum of Squares | Df | Mean Square | F-Ratio | P-Value |
|---|---|---|---|---|---|
| C: Methanol flow rate | 0.38325 | 1 | 0.38325 | 26.94 | 0.0139 |
| D: Casein acid hydrolysate | 0.0735361 | 1 | 0.0735361 | 5.17 | 0.1076 |
| CD | 0.0245311 | 1 | 0.0245311 | 1.72 | 0.2805 |
| Blocks | 0.000300125 | 1 | 0.000300125 | 0.02 | 0.8937 |
| Total error | 0.0426764 | 3 | 0.0142255 | | |
| Total (corr.) | 0.524294 | 7 | | | |

R-squared = 91.8602 percent.
R-squared (adjusted for d.f.) = 85.7554 percent.
Standard Error of Est. = 0.119271.
Mean absolute error = 0.0621875.
Durbin-Watson statistic = 1.97452 (P=0.3147).
Lag 1 residual autocorrelation = -0.213716.

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