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# Optimization of Biomass Production by *Ustilago maydis* in Submerged Culture using Taguchi Experimental Design

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**Abstract:** The aim of this study was to optimize the submerged culture conditions in baffled Erlenmeyer flasks for biomass production by *U. maydis* FB12 by $L_8$ Taguchi orthogonal array experimental design using YPD broth (yeast extract-peptone-dextrose) as a basal medium. The optimized conditions (glucose 40 g $L^{-1}$, [Cu] 0.005 g $L^{-1}$, pH 7, orbital shaking speed 200 rpm, temperature 32°C) predicted a final biomass concentration of 13.88 g $L^{-1}$ after 48 h. When these conditions were tested experimentally, a real final biomass concentration of 15.67 g $L^{-1}$ was obtained and the microorganism presented a specific growth rate $\mu = 0.03\ h^{-1}$.

**Key words:** Biomass, optimization, submerged culture, Taguchi, *Ustilago maydis*

## INTRODUCTION

The phytopathogenic and dimorphic fungus *Ustilago maydis* is the causal agent of the corn smut disease (Valverde *et al*., 1995; Ruiz-Herrera and Martínez-Espinoza, 1998). However, this microorganism has also been grown in submerged culture to produce different useful metabolites such as siderophores (Drews and Kraume, 2002), glycolipids (Spoeckner *et al*., 1999; Hewald *et al*., 2005) and several enzymes (proteases, nucleases, lipases) (Hellmich and Schauz, 1988; Mercado-Flores *et al*., 2003). In many of these processes, a high amount of biomass is necessary, so the optimization of its production in submerged culture is a very important step. Most published optimization procedures involve response surface methodology (Cui *et al*., 2006; Tari *et al*., 2007), however, when the number of variables is high, the number of experimental runs is also very high. Reports are available on the use of Taguchi orthogonal matrix method (Li *et al*., 2001; Kim *et al*., 2005; Prasad *et al*., 2005; Korbekandi *et al*., 2008) in biotechnology. This method examine the effects of several process variables and identify the factors which have major effects on the process using just a few experiments (Seyedeh *et al*., 2007). The objective of this research was to identify the culture conditions that could lead to improved biomass production by *Ustilago maydis*.

## MATERIALS AND METHODS

This study was conducted in 2007 at the Escuela Nacional de Ciencias Biológicas of the Instituto Politécnico Nacional in Mexico City, Mexico.

**Microorganism:** The *Ustilago maydis* strains FB1 (a1 b1), FB2 (a2 b2) and FBD12 (a1/a2 b1/b2) were provided by Dr. Flora Banuett, University of California at San Francisco, USA and grown in YPD broth (1% yeast extract, 2% casein peptone and 2% dextrose).

**Strain selection:** The three strains were grown in YPD broth at 32°C on an orbital shaker incubator at 200 rpm until the stationary phase was reached (48 h) and the biomass (dry weight) was measured after vacuum filtration through a Whatman 5 filter paper and drying at 65°C for 96 h to a constant weight. The inoculum was standardized so an initial concentration of 3 g $L^{-1}$ was obtained and the net growth was calculated by subtracting the initial from the final concentration. The strain with the highest biomass production was selected for the optimization study.

**Optimization of biomass production using the $L_8$ Taguchi orthogonal array experimental design:** The design for the $L_8$ ($2^7$) orthogonal array was developed and analyzed

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using the Design Expert 7.0.3 software (Stat-Ease Inc., Minneapolis, MN 55413). The experimental variables were: the concentrations of dextrose (20 and 40 g $L^{-1}$), copper ion (0 and 0.005 g $L^{-1}$), Tween 80 (0 and 1 g $L^{-1}$) and 2,5-xylidine (0 and 0.05 g $L^{-1}$), pH (4 and 7), temperature (28 and 32°C) and orbital shaking speed (150 and 200 rpm). Copper (Levin *et al*., 2002) and 2,5-xylidine (Alves-García *et al*., 2006) have been shown to be beneficial for the growth of ligninolytic fungi, so they were included in the experimental design. Tween 80 was reported to increase the biomass production in *Penicillium citrinum* (Chakravarti and Sahai, 2002), so it was also included in the design. All the runs were performed in baffled Erlenmeyer flasks with cotton plugs (Table 1). The biomass was collected by vacuum filtration and determined as dry weight as described above. All the results were expressed as the average of three determinations. A kinetic study of biomass production using the optimal culture conditions was performed in order to calculate the specific growth rate of the selected strain of *U. maydis*. The pH of the optimal medium was also recorded after the 48 h fermentation. The use of shake flasks with cotton plugs involves the fact that oxygen supply may become a limiting factor, so the volumetric oxygen transfer coefficient ($k_LA$) value and the time in which the liquid phase oxygen concentration is equal to zero were evaluated by the method of van Suijdam *et al*. (1978).

## RESULTS

**Strain selection:** After an incubation period of 48 h at 32°C and 200 rpm agitation speed, the biomass concentrations were 7.9, 10.9 and 11.1 g $L^{-1}$ for strains FB1, FB2 and FBD12 respectively in YPD broth. The last strain was then selected for the optimization experiments.

**Optimization of biomass production:** The results for biomass production by the strain FBD12 of *U. maydis* under the different experimental conditions are shown in Table 1.

The most important factors for biomass production were pH, shaking and glucose with contributions of 48.19, 23.12 and 13.5%. Tween 80 and 2,5-xylidine had a negligible contribution to the process and the model (Fig. 1).

The results were analyzed with the Design Expert 7.0.3 software and it predicted the following model.

Biomass = 7.89 + 2.03*pH + 1.41*speed + 1.08*glucose + 0.72*temperature + 0.75*$CuSO_4$ $R^2$ = 1.000

The coefficients for Tween 80 and 2,5-xylidine were not significant ($p>0.05$), so these ingredients were not included in the optimized culture medium.

Copper and temperature with contributions of 6.58 and 6.02% to the growth of *U. maydis* were also important for the biomass production.

The optimal conditions predicted by the program for the production of *U. maydis* biomass were: 40 g glucose $L^{-1}$, 0.005 g Cu $L^{-1}$, pH 7, 200 rpm shaking speed and a temperature of 32 °C (Table 2).

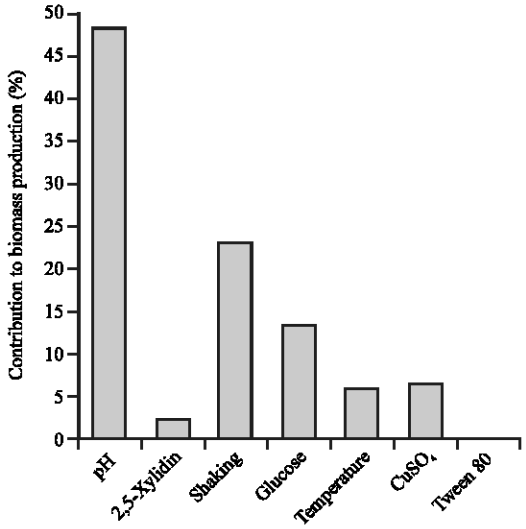


Fig. 1: Contribution (%) to the *Ustilago maydis* strain FBD12 biomass production of each of the experimental design factors

Table 1: $L_8$ Taguchi orthogonal array experimental design for *U. maydis* strain FBD12 biomass production

| | Experimental variables | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Run | pH | 2,5-xylidine (g $L^{-1}$) | Shaking (rpm) | Glucose (g $L^{-1}$) | Temperature (°C) | Copper (g $L^{-1}$) | Tween 80 (g $L^{-1}$) | Biomass (g $L^{-1}$) |
| 1 | 7 | 0.05 | 150 | 20 | 32 | 0.005 | 0 | 8.50 |
| 2 | 7 | 0.00 | 200 | 40 | 28 | 0.005 | 0 | 12.97 |
| 3 | 4 | 0.00 | 150 | 20 | 28 | 0.00 | 0 | 2.43 |
| 4 | 4 | 0.00 | 150 | 40 | 32 | 0.005 | 1 | 7.41 |
| 5 | 4 | 0.05 | 200 | 20 | 28 | 0.005 | 1 | 5.70 |
| 6 | 7 | 0.00 | 200 | 20 | 32 | 0.00 | 1 | 10.64 |
| 7 | 7 | 0.05 | 150 | 40 | 28 | 0.00 | 1 | 7.60 |
| 8 | 4 | 0.05 | 200 | 40 | 32 | 0.00 | 0 | 7.90 |

Table 2: Impact and optimized values for the seven factors tested in the Taguchi design for the biomass production in *Ustilago maydis*

| Factor | pH | 2,5 xylidine ($g\ L^{-1}$) | Agitation speed (rpm) | Glucose ($g\ L^{-1}$) | Temperature (°C) | $CuSO_4$ ($g\ L^{-1}$) | Tween 80 ($g\ L^{-1}$) |
|---|---|---|---|---|---|---|---|
| Percentage of contribution to biomass production | 48.19 | 2.56 | 23.12 | 13.5 | 6.02 | 6.58 | 0.037 |
| Optimized value | 7.00 | 0.00 | 200.00 | 40.0 | 32.00 | 0.005 | 0.00 |

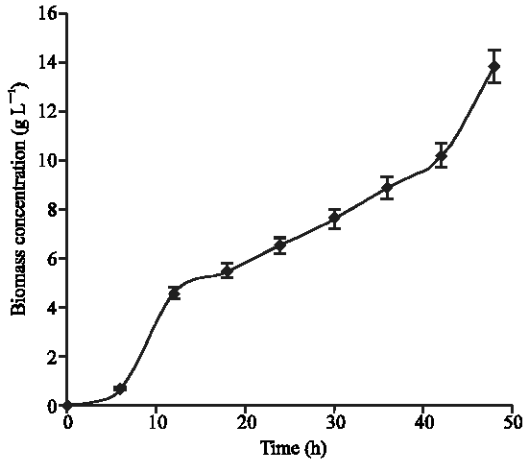


Fig. 2: Time course of *Ustilago maydis* FBD12 biomass production in the optimal culture conditions (40 g glucose $L^{-1}$, 0.005 g Cu $L^{-1}$, pH 7, 200 rpm orbital shaking speed and 32°C)

The model predicted for these conditions a final biomass concentration of 13.86 g $L^{-1}$ after 48 h. When these conditions, which were not originally used, were tested experimentally, a real final biomass concentration of 15.67 g $L^{-1}$ was obtained so the validity of the prediction was confirmed. In the kinetic study under the optimal culture conditions, the exponential phase was detected between the 12 and 48 h of fermentation (Fig. 2). The specific growth rate ($\mu$) was evaluated by means of an exponential regression analysis of the biomass production with time. The regression had a determination coefficient $R^2 = 0.9884$ with $\mu = 0.03\ h^{-1}$. The pH of the culture medium dropped only slightly from 7.0 to 6.6 after 48 h and the volumetric oxygen transfer coefficient ($k_LA$) had an estimated value of 0.1072 $sec^{-1}$. According to these results, the oxygen concentration in the liquid phase would be a growth-limiting factor only after 97 h of fermentation. This was not the case in our study, since we observed the start of the stationary phase after 48 h of fermentation.

## DISCUSSION

Taguchi methodology is a very good option for the optimization of biotechnological processes involving yeasts and fungi. In this case, the influence of seven factors on the biomass production of *Ustilago maydis* could be tested with only eight runs with the consequent savings in time and money. A similar factorial design would have included 128 runs, that is, 16 times more runs.

In this study, *U. maydis* reached the stationary phase after 48 h, which is slightly earlier than other yeasts like *Saccharomyces cerevisiae* (54 h) as reported by Rosenfeld *et al.* (2004). The most important factors for *U. maydis* biomass production were pH, shaking and the initial glucose concentration. The optimal initial pH for the production of fungal biomass was found to be pH 4.0 in the case of the basidiomycete *Agrocybe cylindracea* (Kim *et al.*, 2005), however in the case of *U. maydis*, the growth at pH 4.0 was low compared with the growth at pH 7.0 (Table 1). Agitation speed was also found to be a significant parameter for maximal biomass production in the case of *Aspergillus sojae* ATCC 20235 (Tari *et al.*, 2007).

Carbon source have been found to be a very important factor in the production of biomass by different fungi (Kim *et al.*, 2005; Cui *et al.*, 2006; Tari *et al.*, 2007). When glucose was used as the carbon source, a similar concentration (45.2 g $L^{-1}$) was found by Cui *et al.* (2006) for the production of biomass by the basidiomycete fungus *Grifola frondosa*.

Levin *et al.* (2002) found that the addition of copper (up to 0.064 g $L^{-1}$) did not affect the growth of *Trametes trogii* but strongly stimulated the production of ligninolytic enzymes. In our case, the addition of copper (0.005 g $L^{-1}$) was important for the production of biomass. Growth temperature for maximal biomass production was also different in the case of *U. maydis* (32°C) compared with *A. cylindracea* (25°C) indicating that there is a great variability in this parameter among different basidiomycetes.

A maximal amount of 15.67 g of biomass/L was obtained in 48 h with the culture conditions predicted by the model. The value of the specific growth rate obtained in this study (0.03 $h^{-1}$) fell in the range of $\mu$ values reported by Drews and Kraume (2002) for the strain FB1 of *U. maydis* grown batchwise in 5 L fermentors with sucrose as the main carbon source. They reported $\mu$-values in the range of 0.01 to 0.19 $h^{-1}$. In summary, this is the first report on the use of the Taguchi methodology in the optimization of the culture conditions for the biomass production of *U. maydis*. A maximal amount of biomass can be harvested in the optimized medium after

a culture time of only 48 h. It is recommended that the production of some important metabolites be tested under the optimized conditions.

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