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# Coupling aerobic biodegradation of methanol vapors with heterologous protein expression of endochitinase Ech42 from *Trichoderma atroviride* in *Pichia pastoris*

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ABSTRACT

Methanol is included among the most hazardous air pollutants, and an effort of vapors biofiltration by using microbial consortiums has been reported. The aim of this work was to couple the methanol vapors biodegradation with the production of recombinant endochitinase (*ech42*) from *Trichoderma atroviride* in *Pichia pastoris* transformed with the pPIC-*ech42* plasmid. After carrying out batch experiments at 0.5% (w/v) of methanol concentration, the recombinant *P. pastoris* Mut$^+$ strain was selected because it showed methanol biodegradation rates similar to those of wild type GS115 strain (39 g/m$^3$ h), but 15% higher than the transformed Mut$^S$ strain. In addition, the recombinant Ech42 protein production was higher in Mut$^+$ than Mut$^S$. After various methanol vapor concentrations were evaluated, the maximum recombinant protein recovery was 317 mg/l and the volumetric methanol consumption rate was 88.7 g/m$^3$ h at 0.5% (w/v) of methanol concentration. This research underlines the promising application of linking methanol vapors biodegradation with the production of recombinant protein with high biotechnological interests.



## 1. Introduction

Biological techniques for volatile organic compounds (VOCs) treatment involve the oxidation of pollutants to water and carbon dioxide by the activity of microorganisms (Devinny et al., 1999). The biofiltration of highly soluble VOCs has been a research subject for decades, i.e. methanol biofiltration has been widely reported (Devinny et al., 1999; Shareefdeen et al., 1993; Arulneyam and Swaminathan, 2003; Cornabé et al., 2002; Prado et al., 2006; Avalos-Ramirez et al., 2008, 2009). Although methanol has relatively low toxicity, it is included in the list of most hazardous air pollutants (EPA, 1990). Most emissions of methanol (65%) come from the pulp and paper industry which generates approximately 66,550 tons of methanol per year (Avalos-Ramirez et al., 2009). Several microorganisms have the ability to metabolize methanol vapors including yeast genera such as *Pichia*, *Torulopsis*, *Kloeckera*, *Rhodotorula,* and *Candida*. Specifically, the methylotrophic yeast *Pichia pastoris* has been consolidated as an efficient expression system for the production of recombinant proteins with biotechnological applications (Calik et al., 2010; Mayson et al., 2003; Skoko et al., 2003; Pérez-Martínez et al., 2007). *P. pastoris* is a eukaryotic organism that has an efficient growth rate at high cell densities in economical culture media, and the recombinant protein produced by this strain can be exported to the culture medium, facilitating the protein recovery process (Cereghino and Cregg, 2000).

The endochitinase gene *ech42* from *Trichoderma atroviride* was previously cloned and expressed in a constitutive *P. pastoris* (pGAP) system, reaching a production of 186 mg/l of endochitinase after 96 h in shaking flask cultures (Pérez-Martínez et al., 2007). In the present study, an inducible pPIC9-*ech42* vector was constructed and two *P. pastoris* transformants (Mut$^+$ and Mut$^S$) strains were obtained and tested on their ability to consume methanol vapors coupled with the production of recombinant Ech42 protein in batch experiments.

## 2. Methods

### 2.1. Strains and culture conditions

*Escherichia coli* DH5α strain (Invitrogen, Carslbad CA, USA) was used for routine plasmid amplification. Plasmid pPIC9 K (Invitrogen), carrying *kan* gene, which confers Geneticin resistance, and a functional version of *his4* gene, was used for heterologous protein

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production. *P. pastoris* GS115 (*his4*⁻) strain and restriction enzymes were purchased from Invitrogen. *P. pastoris* strain was grown on YPD (10 g/l yeast extract, 20 g/l dextrose, 20 g/l peptone, and 10 g/l bacto agar) plates. Transformed *P. pastoris* pPIC-*ech42* strains were maintained on YPD containing 100 μg/ml Geneticin (Invitrogen).

### 2.2. Construction of expression vector

The restriction sites *Eco*RI and *Not*I were added to the *ech42* gene by PCR amplification with the forward primer 5′-GAATTCGCCAGTGGATACGCA-3′ and reverse primer 5′-GCGGCCGCGTTGAGACCGCTTCG-3′. The resulting 1100 pb cDNA sequence was cloned in frame at *N*-terminal with the α-mating factor signal peptide of pPIC9 K vector (Fig. 1A). *E. coli* DH5α was transformed with pPIC-*ech42* construction by heat shock and plated into low-salt concentration LB plates (10 g/l NaCl, 10 g/l yeast extract, 10 g/l bactotryptone containing 100 μg/ml ampicillin). Plates were incubated overnight at 37 °C. Plasmid was recovered using Quiaprep spin Plasmid MiniKit (Quiagen, Hildgen, Germany).

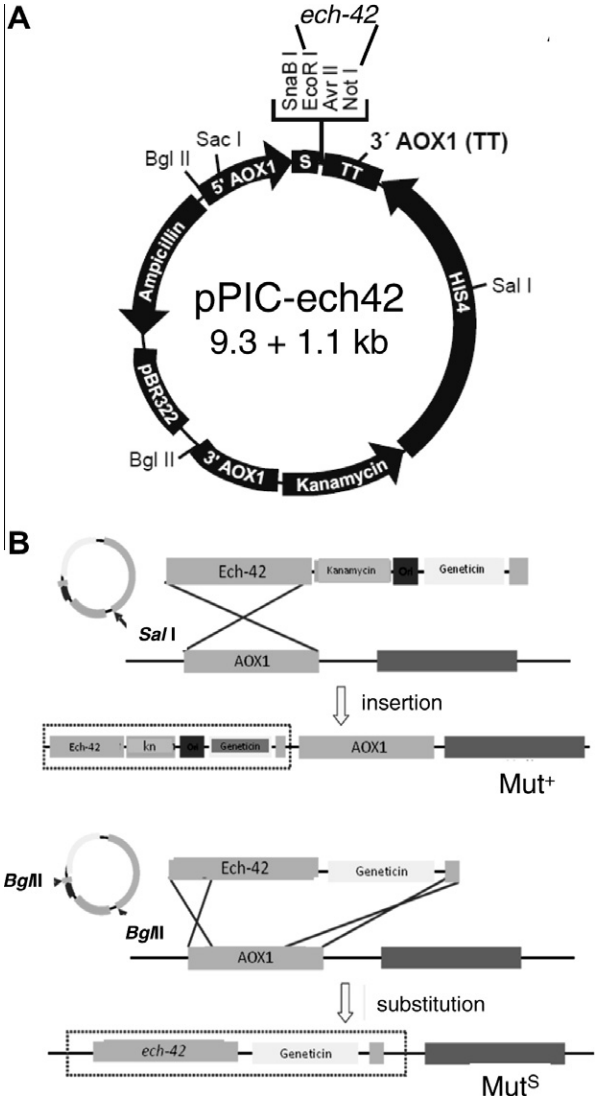


**Fig. 1.** (A) Construction of the recombinant plasmid pPIC-*ech*42. Location of restriction sites *Bgl*II and *Sal*I for plasmid linearization are indicated with arrows. (B) Liner vector is used for *P. pastoris* transformation. *Sal*I digested results in Mut⁺ strain conserving intact the AOX1 and AOX2 genes, and *Bgl*II generated the Mut$^S$ strain, where AOX1 gene is eliminated.

### 2.3. Transformation of P. pastoris with pPIC-ech42

The plasmid pPIC-*ech42* was digested with *Sal*I or *Bgl*II, for the generation of the Mut⁺ and Mut$^S$ mutants, respectively (Fig. 1B). Competent *P. pastoris* GS115 cells were transformed with 10 μg of linearized plasmid by electroporation (Electrocell Manipulator ECM 630, BTX) at 1500 mV, 200 μF and 50 Ω. Transformed cells were plated on RDB media (34 g/l YNB, 20 g/l dextrose, 8 mg/l biotine, 10 mg/l aminoacids (except of histidin), 182.2 g/l sorbitol, and 10 g/l agar). The plates were incubated for 4 days at 28 °C. Selected clones were re-plated on YPD with Geneticin at 250 μg/ml. Integration of plasmid into *P. pastoris* genome was confirmed by PCR amplification, using the specific α-factor and 3′AOX1 primers (Invitrogen). Individual clones of recombinant *P. pastoris* Mut⁺ and Mut$^S$ strains were isolated.

### 2.4. Methanol induction of recombinant protein expression in P. pastoris

Pre-inocula of Mut⁺ and Mut$^S$ strains were grown at 28 °C overnight in 5 ml YPD medium. Fresh YPD medium (200 ml) was inoculated with the pre-inocula and incubated at 28 °C and 250 rpm until reaching an optical density of 0.5. Samples were centrifuged for 5 min at 13,000 rpm and cells pellet were resuspended in fresh YPD medium. The induction of protein expression was carried out by the addition of 0.2% methanol and 0.1% glycerol (w/v). The flasks were incubated for 16 h at 28 °C and 250 rpm.

### 2.5. Batch experiments (Microcosms)

Batch experiments were carried out in 120 ml serum bottles sealed with Mininert Teflon valves. Twelve milliliter of YPD medium at pH 5.5 were inoculated with 5 ml of preinoculum as described above. Methanol was injected at a concentration of 0.2, 0.5, 0.8 and 1% (w/v) with a 200 μl gas tight syringe (Vici, Precision Sampling, Co. Inc., Baton Rouge, LA). Four bottles of each methanol concentration were prepared and marked as 24, 48, 72 and 96 h. Bottles were incubated with agitation at 250 rpm at 28 °C. About 100 μl of gas and 1 ml of liquid samples were taken every 24 h from the corresponding bottle. The methanol concentration in the gas phase and the concentration of the recombinant protein and methanol in the liquid phase were measured. The liquid samples were first centrifuged at 13,000 rpm for 3 min at 4 °C and filtrated through 0.22 μm Polyvinylidene fluoride membrane (Millipore, Ireland) before their analysis. The concentration of methanol was obtained by a mass balance between the concentration in the gaseous and aqueous phase values. All experiments were done in triplicate.

### 2.6. Gas chromatography analysis

The methanol concentration in the gas and liquid phases was analyzed by gas chromatography following the procedure and conditions reported elsewhere (De León-Rodríguez et al., 2006; Santana-Olguin, 2004) in a gas chromatograph HP 6890N (Agilent Technologies, Palo Alto, CA, USA) coupled with a flame ionization detector (FID) and using a capillary HP-Innowax 30 m × 0.25 mm column (Agilent Technologies).

The carrier gas was helium at a flow rate of 1.5 ml. The temperatures of the injector, oven, and detector were 220, 35, and 250 °C, respectively.

The calibration curves for methanol in gas and liquid phases were carried out with hermetically sealed bottles. In the gas phase

different concentrations of methanol (2, 4, 6, 8, and 10 μl) were added, and incubated at 28 °C. Aliquots of 100 μl were taken from gas phase and injected into a gas chromatograph. The calibration curve in the liquid phase was done using methanol solutions at different concentrations (122.9, 245.8, 368.7, 491.6, 737.4, and 983.2 mmol/l). The internal standard was isopropanol at 196.2 mmol/l used at 1:1 (v/v). The samples were kept at 4 °C.

### 2.7. Protein quantification

After 24 h induction with methanol, the culture medium was centrifuged at 13,000 rpm for 3 min at 4 °C. The supernatant was recovered and soluble protein was quantified using the Protein Assay kit (Bio-Rad, Hercules, CA) using BSA (Bovine serum albumin) as standard.

Extracellular proteins were subjected to electrophoretic separation. In brief, 10 μg of sample were mixed with 5 μl of Laemmli buffer and electrophoresis was carried out (Laemmli, 1970) in a MiniProtean III (Bio-Rad, Hercules, CA, USA). Gels were stained with Sypro Ruby (Invitrogen).

The zymogram assay was carried out as reported previously (Pérez-Martínez et al., 2007). The samples were prepared with Laemmli buffer without reducing agent. After electrophoretic separation, the gel was washed twice for 15 min with buffer A (25% isopropanol, 2 mM EDTA), 30 min with buffer B (2 mM EDTA, 40 mM Tris–HCl, pH 8.0), and final wash with reaction buffer (10 mM EDTA, 5 mM sodium acetate, pH 5.5). The gel was covered with 1% (w/v) agarose melted in reaction buffer and the substrate 4-methyl-umbelypherone (4-MU, Sigma–Aldrich, USA) at a final concentration of 1 μg/ml. The gel with the substrate was incubated for 10 min at 40 °C; fluorescence was detected with an UV-transiluminator (Bio-Rad).

## 3. Results

### 3.1. Generation of Mut$^S$ and Mut$^+$ P. pastoris strains

The expression vector pPIC9 K including the *ech*42 gene from *T. atroviride* was linearized with *Sal*I or *Bgl*II (Fig. 1B) and *P. pastoris* GS115 was transformed. Two variations of recombinant *P. pastoris*; the Mut$^+$ or methanol utilization type + (which has intact AOX1 and AOX2 genes), and the Mut$^S$ or methanol utilization type slow (which possesses only intact AOX2 gene) were obtained (Cregg et al., 1993). The identity of the two strains was confirmed by PCR amplification.

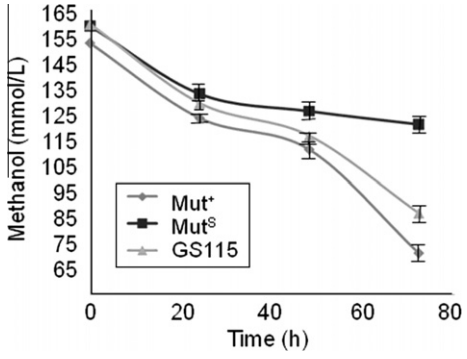


**Fig. 2.** Kinetics of methanol consumption from GS115 (—▲—) wild type and two recombinant *P. pastoris*, Mut$^+$ (—◆—), and Mut$^S$ (—■—) strains. The initial concentration of methanol was 156 mmol/l.

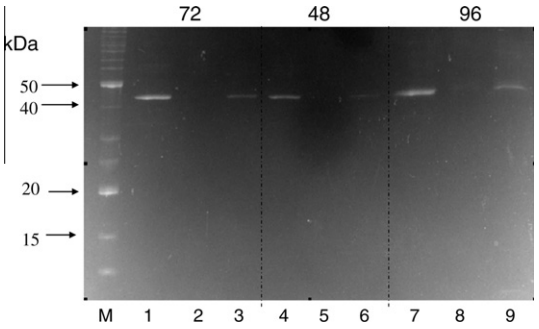


**Fig. 3.** SDS–PAGE of the *P. pastoris* culture medium after different expression times. The *P. pastoris* Mut$^+$ (lanes 1, 4, 7); GS115 (lanes 2, 5, 8), and Mut$^S$ (lanes 3, 5, 9) strains were grown at 1% w/v methanol, culture medium was collected at: 72, 48 h, and 96 h. Lane M = molecular weight markers.

**Table 1**
Effect of methanol concentration on Ech42 expression and on the volumetric consumption rate.

| Initial methanol concentration (%, w/v) | Volumetric methanol consumption rate ($V_{max}$) (g/m$^3$h) | Total protein concentration produced (mg/l) |
|---|---|---|
| 0.2 | 23.6 ± 0.1 | 283 ± 1.1 |
| 0.5 | 88.7 ± 0.2 | 317 ± 1.5 |
| 0.8 | 35.8 ± 0.1 | 310 ± 1.1 |
| 1.0 | 36.1 ± 0.1 | 297 ± 1.4 |

### 3.2. Kinetics of methanol consumption of P. pastoris wild and transformants strains

From our preliminary work, it was defined that the optimal methanol concentration for the start point of growing of *P. pastoris* was 156 mmol/l. As shown in Fig. 2, the higher methanol consumption (73 mmol/l) was observed with the Mut$^+$ strain, this value corresponds to the 47.4% of methanol consumed. The Mut$^S$ strain had a methanol consumption of 34.3 mmol/l (the 21.6%). The GS115 strain had a similar methanol consumption as that of Mut$^+$ (41.2%).

The expression of recombinant protein was assayed at 0.5% of methanol concentration. The culture media was analyzed as shown in Fig. 3, in a main band of 45 kDa was observed in *P. pastoris* strain Mut$^+$ (lanes 1, 4, 7), and Mut$^S$ (lanes 3, 6, 9). As expected, no band was observed in the wild type GS115 strain (lanes 2, 5, 8). The highest protein production was observed at 96 h with the Mut$^+$ strain. For this reason, the Mut$^+$ was selected for microcosms experiments and protein expression experiments.

### 3.3. Kinetics of methanol consumption in microcosms

The kinetics of methanol consumption of *P. pastoris* Mut$^+$ at different methanol concentrations (0.2, 0.5, 0.8 and 1%) is shown on Table 1. The higher methanol consumption of 88.7 g/m$^3$h, was found when the initial methanol in the media was 0.5% (w/v), these data are in agreement with data reported in our previous work (Pérez-Martínez et al., 2007).

The volumetric consumption ($V_{max}$) obtained at a methanol concentration of 0.5% (88.7 g/m$^3$h) was at least 60% higher than the values obtained among the concentrations tested (23.6–36.1 g/m$^3$h). The concentration of recombinant Ech42 produced at 0.5% methanol was 317 mg/l, similar values of recombinant protein, 310 and 297 mg/l, were obtained for 0.8% and 1% of methanol, respectively (Fig. 4A). The zimogram (Fig. 4B) shows that recombinant endochitinase was active at all methanol concentrations.

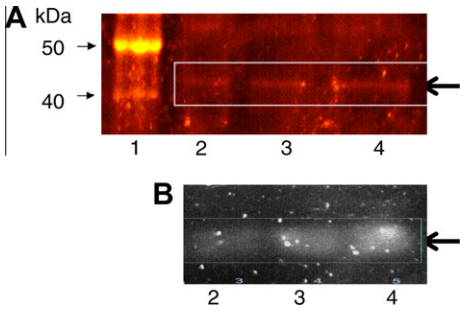


**Fig. 4.** (A) SDS–PAGE of culture media after 96 h of induction time at different methanol concentrations. Lane 1 = Molecular weight marker. Lanes 2–4, methanol concentrations of 0.5%, 0.8%, and 1%, respectively. (B) Zimogram corresponding to the gel on figure A.

## 4. Discussion

Recombinant *P. pastoris*, Mut$^+$ and Mut$^S$ were obtained, in both cases the heterologous gene expression is controlled by AOX1 promoter (Cregg et al. 1993). Mut$^+$ phenotype had higher methanol consumption (47.4%) than Mut$^S$ (21.6%), but similar values compared with the GS115 wild type strain (41.2%). From these results, the Mut$^+$ strain was selected to perform the studies of vapors methanol biodegradation coupled to heterologous protein production.

Some authors have described that even though the Mut$^S$ variant has a diminished maximal growth rate compared with Mut$^+$, the final concentration of the functional protein is occasionally higher in the case of Mut$^S$ fermentations (Chen et al., 1997; Loewen et al., 1997; Pla et al., 2006), but still a reliable correlation describing the specific product formation rate as the function of the specific growth rates was not established in case of Mut$^S$ cells (Kupcsulik and Sevella, 2004). In this work, we have observed that Mut$^+$ has a slightly higher protein expression than Mut$^S$ (317 mg/l and 297 mg/l, respectively) as observed for other authors (Qian et al., 2006; Orman et al., 2009). The time of 96 h to obtain the maximum protein production found in this work, was reported by Xie et al. (2004) for the maximum production of heterologous angiostatin using controlled systems of dissolved oxygen.

Since methanol acts as an inducer and carbon source, its close regulation is a crucial factor in achieving optimal fermentation conditions (Pla et al., 2006). For this reason, the effect of initial methanol concentration was analyzed on Mut$^+$. We have found that at a methanol concentration of 0.5% (w/v) the highest consumption rate (88.7 g/m$^3$h) was obtained for *P. pastoris*. This value was lower than the value reported of 190 g/m$^3$h for the biofiltration of methanol vapors by *P. methanolica*, wild type, which does not produces recombinant protein (Santana-Olguin, 2004). *P. methanolica* strain is better adapted to use methanol as a carbon source (Gruzman et al., 1996), but is less characterized as an expression system for the scale-up processing (Cregg et al., 2000).

Up to now, only one study has focused on the production of proteins during VOC vapors biofiltration (Cornabé et al., 2002). In this study, the biofiltration of methanol vapors was conducted using a transformed strain *P. pastoris* BN21 for the production of Laccase. The maximum methanol consumption rate and Lacasse activity reached were 175 g/m$^3$h and 916 U/l, respectively. The $V_{max}$ obtained by Cornabé et al. (2002) is approximately two times greater than the value obtained in the present study. These results suggest that when easily biodegradable air pollutants are treated (i.e. methanol) oxygen transfer limitation may occur. Also, it is important to consider that the present study was conducted in batch systems which are commonly limited by oxygen transfer, while in biofilters, a continuous air flow passes through the bed (Devinny et al., 1999). On the other hand, it has been indicated that implementation of robust control system during protein production are required to improve, in this case, methanol consumption to avoid oxygen transfer limitations and to scale-up the process (Cos et al., 2005; Zhang et al., 2002).

The value of 317 mg/l of Ech42 production obtained at 0.5% (w/v) of methanol and at 96 h, can be compared with a previous fed-batch study conducted by Mayson et al. (2003) in which 450 mg/l of the protein transferrin were obtained at a methanol concentration of 0.7% (v/v) after 72 h. Also, it can be compared with a more recent study carried out by Calik et al. (2010), which reported a maximum production of 270 mg/l of the recombinant human growth hormone (rhGH) in a fed-batch reactor using methanol as a carbon source and the strain *P. pastoris*. Further studies should be done to scale-up the process in biofilter or biotrickling filters, where oxygen and nutrients should not be limiting and then resulting in higher protein expression (Cos et al., 2006).

## 5. Conclusions

The present study shows that in batch test it is possible to carry out the VOC vapors biodegradation coupled with the recombinant protein production. We can conclude that the process of linking protein production with the biofiltration of easily biodegradable compounds like methanol vapors represent a promising application for production of several proteins of high biotechnological interest, due to the fact that we can take advantage of the high level of methanol biodegradability and the high emissions of methanol from the pulp and paper industry.

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