Optimization Of Capsaicin Acylase Production From *Streptomyces Mobaraensis* In Bench-Top Reactor

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Capsaicin, the major pungent principle in hot pepper fruit, can be hydrolyzed enzymatically to vanillylamine (a natural precursor of vanillin) using a specific acylase from *Streptomyces mobaraensis*. Production of this enzyme using strain DSM40847 was studied under batch fermentation conditions in stirred tank (STR) and airlift (AR) bioreactors. The process performance in both fermentation devices was different with respect to biomass, enzyme concentration and specific yield (enzyme activity/biomass content); in particular the specific yield was lower in the AR (5.7 mU/g of biomass) than in the STR (6.25 mU/g of biomass). Experiments carried out in STR bioreactors at controlled (DO = 20% of saturation) and uncontrolled dissolved oxygen concentration, and at constant stirrer speeds (300, 450 and 600 rpm) demonstrated that the DO level has no remarkable effect on the production of the capsaicin-hydrolyzing enzyme, which is mainly produced in a cell-associated form.

1. Introduction

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is the major component of natural vanilla, which is one of the most widely used and important flavouring ingredient worldwide. Since current production of natural vanilla is not sufficient to meet the increasing demand for this flavour compound, vanillin has been a target for biotechnological production by several approaches: use of enzymes to release or generate vanillin from *Vanilla* and other plant material, development of tissue cultures, genetic modification and, finally, use of microbial cultures (Priefert *et al*., 2001; Walton *et al*., 2003). Capsaicin (8-methyl-N-vanillyl-6-nonenamide), the major pungent principle in hot pepper fruit (*Capsicum annuum L.*), is a potentially attractive and cheap feedstock for the production of vanillin. Capsaicin can be hydrolyzed enzymatically to vanillylamine (4-hydroxy-3-methoxybenzylamine), a natural precursor of vanillin, by either specific bacterial acylases (Koreishi et al, 2006; Flagan and Leadbetter, 2006) or mammalian carboxylesterases (Oi et al., 1992). So far, the only one bacterial acylase, which efficiently hydrolyzes capsaicin and has been characterized, is the capsaicin acylase from *Streptomyces mobaraensis*. This enzyme is an heterodimeric protein that consists of two dissimilar subunits (61 and 19 KDa) and has a specific activity toward capsaicin 100-10000 times higher than that of capsaicin-hydrolyzing enzymes found in mammalian (Koreishi *et al.*, 2006). The vanillin production from capsaicin as natural source using a bi-enzymatic process (with mammalian and bacterial enzymes) has been

previously described (van den Heuvel *et al*., 2001), but it was never translated into a commercial process because the mammalian enzyme used to obtain vanillylamine was too expensive. The discovery of microbial acylases that efficiently hydrolyze capsaicin provides a valuable opportunity to develop a cost-effective process for enzymatic synthesis of vanillin. We are examining different strategies for enhancing fermentative production of capsaicin acylase from *S. mobaraensis* strain DSM40847 in the effort to reduce the cost of acylase enzymes suitable for vanillin production.

2. Materials and Methods

2.1 Chemicals

All chemicals and HPLC solvents were of the highest purity commercially available and were purchased from Fluka (Buchs, Switzerland) and Carlo Erba (Milan, Italy).

2.2 Microorganism cultivation

S. mobaraensis DSM40847 was used in this work. Batch fermentation were carried out in duplicate in Erlenmeyer flasks on an orbital shaker at 180 rpm or in stirrer tank reactors Applikon (Schiedam, The Netherlands) and in air lift reactors, unless indicate otherwise, under the following conditions: incubation temperature 30°C, aeration rate, 1vol/vol min^{-1}; stirrer speed, 450 rpm; silicone antifoam 1ml/L.

2.3 Culture media

The following media were used in the present work:
Pre-inoculum medium: glucose (10.0 g/L), dextrin (10.0 g/L), N-z amine (5.0 g/L), yeast extract (5.0 g/L), $CaCO_3$ (1.0 g/L).
Medium A: soluble starch (40.0 g/L), polypeptone (20.0 g/L), beef extract (40.0 g/L), $MgSO_4$ (20.0 g/L), K_2HPO_4 (2.0 g/L).
Medium C: glucose (20.0 g/L), yeast extract (5.0 g/L), asparagine (1.5 g/L), $CaCO_3$ (5.0 g/L), NaCl (1.0 g/L), $MgSO_4 \times 7\ H_2O$ (0.5 g/L), $CaCl_2 \times 2H_2O$ (0.1 g/L), 1 ml of mineral solution (boric acid 0.5 g/L; $CuSO_4 \times 5\ H_2O$ 0.04 g/L; KI 0.1 g/L; $FeCl_3 \times 6\ H_2O$ 0.2 g/L; $MnSO_4 \times H_2O$ 0.4 g/L; $FeSO_4 \times 7\ H_2O$ 0.4 g/L; ammonium molybdate 0.2 g/L).
Medium S/BIS: glucose (10.0 g/L), peptone (4.0 g/L), yeast extract (4.0 g/L), $MgSO_4 \times 7\ H_2O$ (0.5 g/L), K_2HPO_4 (4.0 g/L).
Medium M8: meat extract (2.0 $g \cdot l^{-1}$), yeast extract (2.0 $g \cdot l^{-1}$), casein hydrolysate (4.0 g/L), glucose (10.0 g/L), soluble starch (20.0 g/L), $CaCO_3$ (3.0 g/L).
AF/Ms: glucose (20.0 g/L), soybean meal (8.0 g/L),yeast extract (2.0 g/L), NaCl (1.0 g/L).
AUR/M: maltose (20.0 g/L), dextrin (10.0 g/L), yeast extract (2.0 g/L), meat extract (4.0 g/L), peptone (4.0 g/L), soybean meal (15.0 g/L),$CaCO_3$ (2.0 g/L).
Medium T: glucose (5.0 g/L), meat extract (4.0 g/L),yeast extract (1.0 g/L), peptone (4.0 g/L), soybean meal (10.0 g/L), $CaCO_3$ (1.0 g/L), NaCl (2.5 g/L).

2.4 Analytical assays

Biomass concentration was determined as dry weight; broth samples were recovered by filtration on pre-weighed Whatman GF/A discs, the filters were dried at 105°C for 24 h, cooled in desiccator and weighed. Acylase activity was determined by a

spectrophotometric method using a double enzymatic reaction with DAO and POD. Total protein content was measured using the Bradford method.

3. Results and Discussion

3.1 Effect of media composition on acylase production

Fermentations in a number of different liquid media were carried out using shaken flasks to find a medium that promoted production of capsaicin hydrolytic enzymes. Selected media investigated are shown in Table 1. Medium A, which contains soluble starch, polypeptone and beef extract, produced the highest titres of biomass and capsaicin acylase and was chosen for future investigations.

Table 1: Effect of medium composition on biomass and acylase production by *S. mobaraensis* DSM40847 in shaken cultures after 144 hours of growth.

	Biomass (g/L)	**Acylase (U/ml)**
Medium A	32 ± 0.9	0.02
Medium C	9.29 ± 0.2	0.00053
***Medium S/BIS*:**	8.4 ± 0.3	0.003
Medium M8	12.9 ± 0.7	0.0081
AF/Ms	8.12 ± 0.5	0
AUR/M:	9.94 ± 0.2	0.008
Medium T	4.93 ± 0.06	0.015

3.2 Effect of agitation speed

Since there was no literature on the effect of agitation speed on the production of capsaicin acylase by *S. mobaraensis* in a stirred tank reactor (STR), the fermentations were carried out at the constant temperature of 30°C and aeration rate of 1.0 vvm, but with different agitation speeds of 300, 450 and 600 rpm, respectively. The maximum level of acylase activity was achieved at 450 rpm after 168 hours of fermentation (Fig.1). When the agitation speed was higher or lower than 450 rpm, we observed a reduction in the bacterial growth that was attributed to the effect of shear stress at the higher agitation speed (600 rpm) and of oxygen limitation at the lower speed (300 rpm). Time profiles of biomass dry weight, secreted proteins and enzyme production at 450 rpm are shown in Figure 1. Biomass concentration increased rapidly (up to 7.3 g [dry weight]/L) during the first 24 hours of fermentation and continued to increase, albeit at a lower rate, until the end of the experiment. Dissolved oxygen concentration rapidly dropped at values near zero during the first 24 hours, remained unaltered for the next 120 hours and then increased till the end of the fermentation. Surprisingly, production of acylase activity rapidly increased between 144-168 hours of fermentation, when an increase in dissolved oxygen levels was measured, and reached a maximum level (0.17 U/ml) at the end of the fermentation. Under optimal culture conditions in STR the production of acylase could be increased 8-fold when compared with shake flask.

3.3 Effect of dissolved oxygen tension

The influence of different percent of air saturation on cell growth and acylase production was investigated by controlling DO at constant concentration of 20%. The effects of DO on the fermentation are shown in Figure 2. At 20% of air saturation, we could not observe a significant increase of dry cell weight which indicated that the availability of molecular oxygen in the growth medium is not a limiting factor. The highest acylase yield was obtained in the experiments at uncontrolled dissolved oxygen concentration. In both case the maximal acylase production was obtained at the end of fermentation when the DO levels raised up.

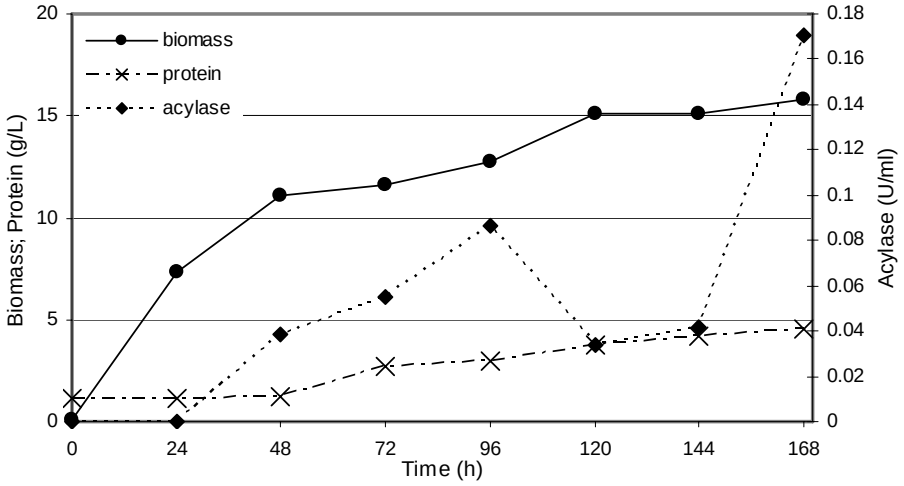

Figure 1. Time courses of biomass, proteins and acylase production during STR (450 rpm) fermentation of *S. mobaraensis* DSM40847.

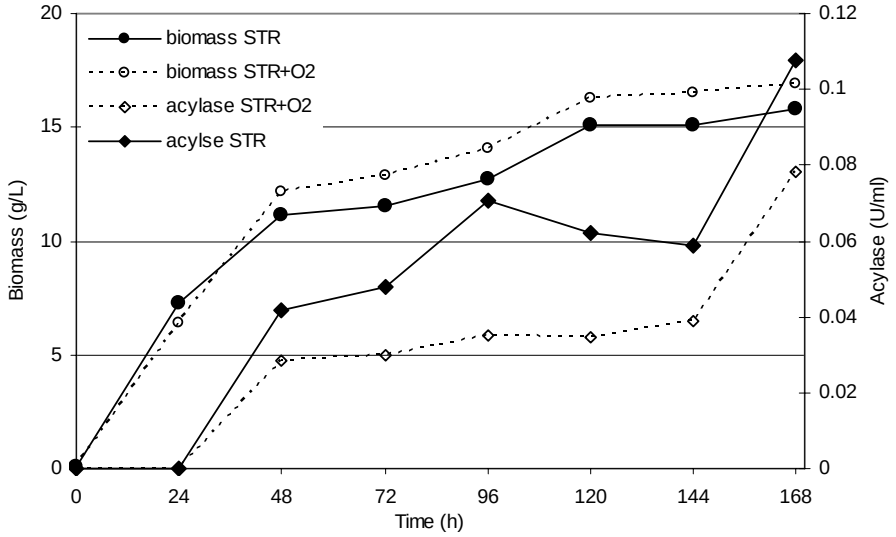

Figure 2. Time profiles of cell growth and acylase production of batch cultures of *S. mobaraensis* DSM40847 maintained at controlled (STR+O_2; DO=20% of saturation) and uncontrolled (STR) dissolved oxygen concentration.

3.4 Comparison of different bioreactor systems

Since the shear force arising in STR bioreactors caused undesired effects on the growth of *S. mobaraensis*, the possibility to cultivate the microorganism in air lift (AR) reactors was also evaluated. Biomass and acylase production in the AR were higher than that achieved in the STR (Table 2). However, the increase in acylase levels was not proportional to changes in biomass concentration and consequently the specific yield (enzyme activity/biomass content) in the AR (5.7 mU/g of biomass) was lower than that achieved in the STR (6.25 mU/g of biomass).

Table 2: Maximal acylase and biomass production obtained in STR and AIR LIFT (AR) reactors.

	Acylase (U/ml)	**Biomass (g/L)**
STR	*0.10*	*16*
AR	*0.16*	*28*

3.5 Acylase localization

Analysis of acylase activities associated with *S. mobaraensis* DSM40847 showed the presence of a cell associated enzyme active against capsaicin. At the end of the fermentation, capsaicin-hydrolytic activity of DSM40847 cells cultured in STR reactors was about 6-7 U/g of biomass. Approximately 90% of this enzymatic activity could be extracted with phosphate buffer containing 0.8 M KCl, suggesting that the enzyme is

probably associated with cell walls of *S. mobaraensis*, primarily by ionic-type interactions.

5. Conclusion

Production of acylases with capsaicin hydrolytic activity is possible by batch fermentation of *S. mobaraensis* using different reactor systems (STR and AR). Strain DSM40847 was able to produce a cell-associated enzyme active on capsaicin. The optimization of culture conditions permitted to obtain a total (extracellular plus cell-associated) acylase activity of 7 U/g of biomass.

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6. References

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