# PRODUCTION OF CHITINOLYTIC ENZYMES BY A STRAIN (BM17) OF *PAENIBACILLUS AMYLOLYTICUS* ISOLATED FROM CRAB SHELLS SAMPLES COLLECTED IN THE EAST SECTOR OF CENTRAL TYRRHENIAN SEA.

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## Abstract

Ninety-five bacterial isolates, obtained from water and sediment samples collected in the East sector of Central Tyrrhenian Sea, were plate screened for 12 extracellular enzyme activities. Among them, 19 strains, found positive for chitinolytic activity, were shaken cultured in media containing chitin as carbon source and different additional nitrogen sources. Strain BM17 showed the highest activity (200 UI/l) in medium containing Chitin (1%) and Yeast Nitrogen Base (YNB) (0.5%). Partial analysis of the gene encoding the 16S rRNA and comparison to sequences in databases showed that strain BM17 likely belongs to the specie *Paenibacillus amylolyticus*. Time course of chitinolytic activity by the selected strain shows that the enzyme activity started to be released in the culture media after 12-24 h. However, exponential enzyme production have been recorded from the 24th h and lasted till the 96th h of incubation where enzyme activity peaked to decrease thereafter. Further experiments have been carried out testing cheaper nitrogen-containing substrates and preliminary medium optimisation was carried out by Response Surface Methodology (RSM) considering the effects of colloidal chitin, corn steep liquor (CSL) and yeast extract (YE). BM17 chitinolytic activity appeared to be induced by chitin. However, the increase of its concentration did not have significant affects on enzyme activity. On the contrary, the nitrogen source, YE in particular, strongly affected the enzyme production. Time course of chitinolytic enzyme production on the optimised medium showed that the maximal enzyme activity was not statistically different from that obtained in the medium containing YNB even if it was slightly delayed (96 and 144 h, respectively). Further media optimisation, sequencing of the full rRNA amplicon and characterisation of *P. amylolyticus* chitinolytic system are in progress.

## Introduction

Marine environment is a source of biological and chemical diversity of huge importance and a great resource of less exploited microorganisms (Sponga et al., 1999). The majority of marine species have yet to be discovered (Malakoff, 1997; Pomponi, 1999). The seas represent a virtually unexploited resource of new substances. Most of these bioproducts have a potential market value of many billions of dollars (BioScience, 1996). The commercial value of enzymes increased in the past 20 years mainly in traditional fields but it is still growing particularly in environment depollution and bioremediation, medical applications, biosensors and so on. Current estimation for the global enzyme market is around US$ 1.5 billions (Munn, 2004). The search of novel or improved enzyme activities is a very lucrative business for which marine environment should not be overlooked (Faukner, 1994; Tulp and Bohlin, 2004). In spite of this, only few works have been carried out in order to screen marine microorganisms' ability to

produce extracellular enzymes (Christian and Karl, 1995; Zaccone et al, 2002).

Degradation of chitin in the aquatic biosphere is an efficient process mainly carried out by bacteria. In spite of the enormous quantity ($10^{11}$ metric tons) of chitin produced annually, marine sediments contain only trace of this polysaccharide (Keyhani and Roseman, 1999, Ramaiah et al., 2000). Application of chitinolytic enzymes (chitinases) in biotechnology is wide. Traditionally these hydrolases find uses in chitin hydrolysis, production of chitin derivates, protoplast formation, biocontrol of pathogenic organisms, and so on (Shaikh and Deshpande, 1993; Aloise et al., 1996; Deshpande, 1998; Patil et al, 2000). Some unconventional application of chitinases in food and wine industries have been successfully tested (Fenice et al. 1999, Esti et al., 2005). In this context, the search of new chitinolytic organisms and/or activities is still of interest.

Preliminary, 95 isolates (from water and sediments samples collected in the East Sector of Tyrrhenian Sea) have been plate-screened for the production of 12 extracellular enzyme activities; among them, 19 strains were found positive for chitinolytic activity (unpublished results).

In this work, the 19 strains were shaken cultured in various media containing chitin as carbon source and different additional nitrogen sources in order to verify their production of chitinolytic enzymes. After preliminary molecular classification, media optimization was carried out for the best producer by Response Surface Methodology (RSM). Time course of the production of chitinolytic enzymes by the selected strain under optimized conditions is also reported.

## Materials and methods

*Microorganisms and culture conditions*

Pure cultures of the isolates were conserved on Plate Count Agar (PCA) slants at 4 °C and subcultured every 3-4 months. Media for Media for RSM optimisation were as reported in table 1. Colloidal chitin preparation and chitinolytic enzyme assay were as already reported (Fenice et al. 1997). Media pH was adjusted at 7.0 before sterilisation (121 °C, 20 min). Bacterial growth was determined by viable counts. Media for chitinolytic activity detection in shaken cultures (20 °C; 200 rpm) were as follows were as follows: MA: colloidal chitin 10 g/l, YNB 5 g/l; MB: colloidal chitin 10 g/l, CSL 5 g/l; MC: colloidal chitin 10 g/l, YE 5 g/l, $(NH_4)_2SO_4$ 1 g/l, $KH_2PO_4$ 1.36 g/l, $MgSO_4$ x $7H_2O$ 0.3 g/l.

*Microorganism identification*

Strain BM17 was identified by analysis of the sequence of the gene encoding the 16S rRNA and comparison to sequences in databases. Primers fD1 and rD1 (Weisburg *et al.* 1991) were used to amplify almost the full length of the 16S rRNA gene as reported by Vinuesa *et al.* (1998). PCR products were run on 1% agarose gels and the bands were purified using the Quiaex II kit (Quiagen, Germany). The nucleotide sequence of the purified bands was determined using the ABI-PRISM kit and automated sequencer ABI PRISM 3100 (Applied Biosystems). Sequence data were analyzed and compared to sequences in EMBL bank using the BLASTn software (Altschul *et al.*, 1997).

*Statistical analysis of data*

One-way analysis of variance (ANOVA) and pair-wise multiple comparisons procedure (Tukey test) were carried out using the software SigmaStat (Jandel Scientific, CA, USA). For RSM, ANOVA was automatically carried out by the MODDE 5 software (Umetrics AB, Sweden).

**Results and discussion.**

Among the 19 isolates tested, strain BM17 appeared to be the best producer of chitinase both in MA and MB. In particular, on MA chitinase activity started after less than 24 h of fermentation reaching a maximum (205.8 U/l) after 96 h to decrease thereafter. On this medium the activity released by all the other strains did not exceed 100 U/l (Fig. 1). On MB all the isolates showed less activity than in MA. Strain BM17 peaked at 144 h (ca 120 U/l) and other strains activities remained below 60 U/l (Figure not shown). On MC all the strains produced chitinolytic activities well below 20 U/l (Figure not shown). Due to it rather high production of extracellular chitinolytic enzymes, strain BM17 was selected for further experiments. Prior to further investigate its chitinase production, the strain was identified by comparison of its partial rRNA sequence with the EMBL database. Strain BM17 appeared to belong to the species *Paenibacillus amylolyticus* (Firmicutes, *Paenibacillaceae, Paenibacillus amylolyticus* strain p114. Overlapping 586 bp, access n° AM062690, identity 99%)

Since the same amount of chitin was present in the 3 media tested, the different activity revealed by *P. amylolyticus* BM17 appeared related with other media components and very likely with the additional nitrogen source. YNB seems to be the most suitable nitrogen supplement to induce strong enzyme activity as already reported for other microbial chitinase producers (Fenice et al., 1999). However, in view of industrial applications, YNB is definitely to expensive, therefore further media optimisation (RSM) was carried out using cheaper nitrogen supplements such as CSL an YE.

As for the majority of chitinolytic microorganisms the *P. amylolyticus* BM17 extracellular chitinase activity appeared to be induced by chitin. However, the increase of this polysaccharide concentration did not seems to have any statistical significant affect on the enzyme activity in the range tested (data not shown). On the contrary, the nitrogen source, YE in particular, strongly affected the enzyme production. Figure 2 shows the increase of enzyme activity in relation to YE concentration. Figure 3 shows the response surface of chitinase production with constant CSL and different concentrations of YE and chitin. It is evident the strong effect of the nitrogen supplement and the little effect (not statistically significant as reported by the software) of chitin in particular with high YE concentration. However, the combination of chitin and YE led to the best activity (upper part of the curve).

Although the single effects of CSL and chitin are not very effective in pushing the bacterial enzyme activity, again, the combination of the 2 substrates leads to the maximum enzyme production (Fig. 4). However, maximum enzyme activity (155,21 U/l, Table 2) was obtained in medium containing chitin 15 g/l, YE 5 g/l and CSL 5 g/l; this was selected as optimal medium.

Figure 5 shows the time course of chitinolytic enzyme production on the optimised medium. Enzyme activity was recorded already at the 24 h of incubation and increased up to the 144 h of incubation when it peaked (181.4 U/l) to rapidly decrease thereafter. Maximal enzyme activity in the low-cost optimised medium was not statistically different from that obtained in MA (205.8) even if it was delayed (from 96 to144 h).

This work, though preliminary, confirms that the use of RSM could be of great help in medium optimisation for the production of microbial enzymes performing a

rather limited number of experiments (D'Annibale *et al.,* 2006). The use of statistic based experimental deign is common for optimisation of chitinase production also and it has been successfully tested even for marine bacteria (Gohel *et al.* 2006).

Strain BM17 of *P. amylolyticus* seems to be a promising organism for the production of chitinases. In order to better understand its potentiality, further sequencing of BM17 full rRNA amplicon and detailed characterisation of its chitinolytic system are in progress.

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**References**

**[1]** Aloise, P. A., Lumme, M., and Haynes C. A. (1996). In: Chitin Enzymology, Muzzarelli R. A. A. (Ed.) pp. 581-594.

**[2]** Altschul S.F., T.L. Madden, A.A. Schaeffer, J. Zhang, Z. Zhang, W. Miller, and D.J. Lipman. 1997. Nucleic Acid Res. **25**:3389-3402

**[3]** BioScience 1996. Marine Biotechnology Special Issue, 46.

**[4]** Christian, J.R., and Karl, D.M. (1995) Limnol Oceanogr 40: 1042-1049.

**[5]** D'Annibale, A., Brozzoli, V., Crognale, S., Gallo, A.M., Federici, F., and Petruccioli, M. (2006). J. Chem Technol Biotechnol. 81: 1586-1593.

**[6]** Deshpande, M. V. (1998). In: Proceeding of biotechnology – a challenge for 2005. New Delhi: All India Biotech. Association, pp. 1-4.

**[7]** Esti, M., Ceccantoni, B. and Fenice, M. International Workshop on the Advanced in Grapevine and Wine Research. Venosa, Italy. September 15/17 2005.

**[8]** Faulkner, D. J. (1994). Nat Prod Rep 11: 355-394.

**[9]** Fenice, M., Selbmann, L., Zucconi, L. and Onofri, S., (1997). Polar Biol 17: 275-280.

**[10]** Fenice, M., Digiambattista, R., Leuba, JL. and Federici, F. (1999). Int J Food Microbiol 52:109-113.

**[11]** Gohel, V., Vhaundhary, T., Vyas, P., and Chhaptar, H.S. (2006). Bioch Eng. J. 28: 50-56

**[12]** Keyhani, N. O., and Roseman, S. R. (1999). Biochim Biophys Acta 1473: 108-122.

**[13]** Malakoff, D. (1997). Science 277: 486-488.

**[14]** Munn, C. B. (2004). In: Marine Microbiology, ecology and applications. Garland Sciences, NY, USA. Pp. 266-267.

**[15]** Patil, R. S., Ghormade, V. and Deshpande, M. V. (2000). Enzyme Microb Tech 26: 473-483.

**[16]** Pomponi, S. A. (1999). J Biotechnol 70: 5-13.

**[17]** Ramajah, N., Hill, R.T., Chun, J., Ravel J., Matte, M.H., Straube W.L., and Colwell R.R. (2000). FEMS Microbiol Echol 34: 63-71.

**[18]** Shaikh, S. A., and Deshpande, M. V. (1993). J Microbiol Biotechnol 9: 468-475.

**[19]** Sponga, F., Cavalletti, L., Lazzaroni, A., Borghi, A., Ciliato, I., Losi, D., and Marinelli, F. (1999). J. Biotechnol. 70, 65-79.

**[20]** Tulp, M., and Bohlin, L. (2004). DDT 9: 450-458

**[21]** Vinuesa, P., J.L.W. Rademaker, F.J. de Bruijn, and D. Werner. 1998. Appl. Environ. Microbiol **64**: 2096-2104.

**[22]** Weisburgh, WG, S.M. Barn, D.A. Pelletier, and D.J. Lane**.** 1991. J. Bacteriol **173:**697-703.

**[23]** Zaccone, R., Caruso, G., and Calì, C. (2002). Mar Environ Res 54: 1-19.

**Table 1**: Factorial design used for cultural medium optimization by RSM and enzyme activity obtained with the various media tested.

| Thesis | Chitin (g/l) | CSL (g/l) | YE (g/l) | Activity (U/l) |
|---|---|---|---|---|
| T1 | 5 | 0 | 0 | 11,56 |
| T2 | 15 | 0 | 0 | 42,5 |
| T3 | 5 | 10 | 0 | 18,7 |
| T4 | 15 | 10 | 0 | 119 |
| T5 | 5 | 0 | 10 | 65,28 |
| T6 | 15 | 0 | 10 | 122,9 |
| T7 | 5 | 10 | 10 | 146,7 |
| T8 | 15 | 10 | 10 | 137,7 |
| T9 | 10 | 5 | 5 | 153 |
| T10 | 10 | 5 | 5 | 155,2 |
| T11 | 10 | 5 | 5 | 147,9 |
| T12 | 5 | 0 | 0 | 8,5 |
| T13 | 15 | 0 | 0 | 40,8 |
| T14 | 5 | 10 | 0 | 17,85 |
| T15 | 15 | 10 | 0 | 117,3 |
| T16 | 5 | 0 | 10 | 59,84 |
| T17 | 15 | 0 | 10 | 107,1 |
| T18 | 5 | 10 | 10 | 141,4 |
| T19 | 15 | 10 | 10 | 120,7 |
| T20 | 10 | 5 | 5 | 153 |
| T21 | 10 | 5 | 5 | 155,2 |
| T22 | 10 | 5 | 5 | 147,9 |

**Figure 1 :** Time course of extracellular chitinolytic activity by 19 selected marine bacterial isolates. Standard deviation less than 10%.

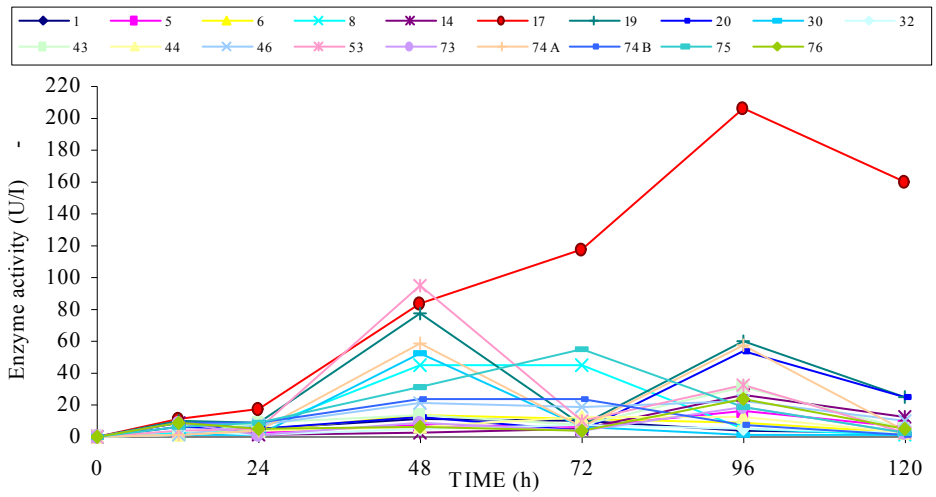


**Figure 2 :** Increase of chitinolytic enzyme activity in relation with YE concentration. Bar indicate the dispersion of real data in relation with the expected results.

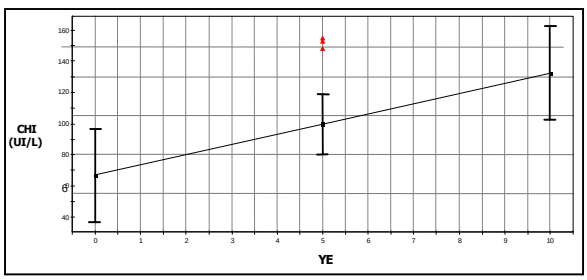

**Figure 3 :** Surface response of chitinolytic enzyme production at different concentration of Chitin and YE with constant concentration (5 g/l) of CSL.

**Figure 4 :** Surface response of chitinolytic enzyme production at different concentration of Chitin and CSL with constant concentration (5 g/l) of YE.

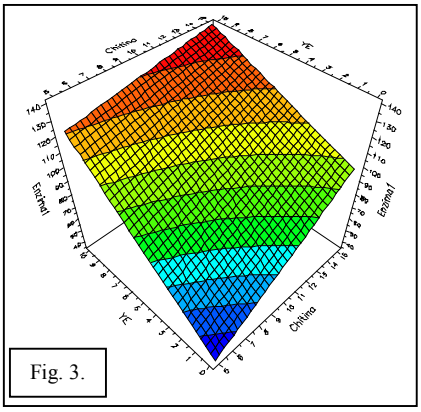


Fig. 3.

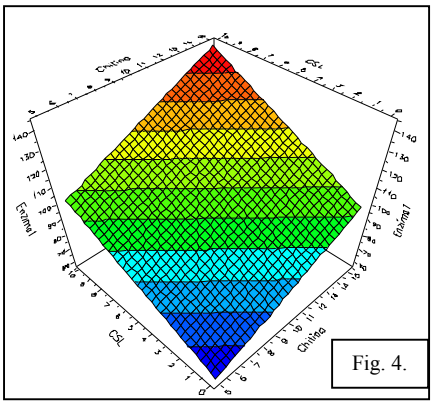


Fig. 4.

**Figure 5 :** Time course of chitinolytic enzyme activity in shaken cultures using optimized medium. Standard deviation below 10%.