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Impact of process parameters on L-glutaminase production by marine *Vibrio costicola* in solid state fermentation using polystyrene as an inert support

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Abstract

Process parameters influencing L-glutaminase production by marine *Vibrio costicola* in solid state fermentation (SSF) using polystyrene as an inert support were optimised. Maximal enzyme yield (157 U/g dry substrate) was obtained at 2% (w/w) L-glutamine, 35°C and pH 7·0 after 24 h. Maltose and potassium dihydrogen phosphate at 1% (w/w) concentration enhanced enzyme yield by 23 and 18%, respectively, while nitrogen sources had an inhibitory effect. Leachate with high specific activity for glutaminase (4·2 U/mg protein) and low viscosity (0·966 Ns/m^2) was recovered from the polystyrene SSF system. © 1997 Elsevier Science Ltd. All rights reserved

Keywords: L-glutaminase, inert support, marine *Vibrio costicola*, polystyrene, solid state fermentation, process parameters.

Introduction

L-Glutamine amidohydrolase (E.C. 3·5·1·2), commonly referred to as L-glutaminase, has received much attention with respect to its therapeutic and industrial applications. It is a potent antileukaemic drug [1] and a flavour-enhancing additive in the production of fermented foods [2]. Its commercial importance demands not only the search for new and better yielding microbial strains, but also economically viable bioprocesses for its large scale production [3].

The use of nutritionally inert materials as supports for solid state fermentation (SSF) facilitates accurate designing of media, monitoring of process parameters, scaling-up strategies and various engineering aspects, which are either impossible or difficult with conventional SSF using organic solid substrates such as wheat bran [4]. The inert materials, when impregnated with a suitable medium, will provide a homogeneous aerobic condition throughout the fermentor, will not contribute impurities to the fermentation product [5] and will facilitate maximal recovery of the leachate with low viscosity and high specific activity for the target product [6].

Marine microorganisms, which are salt tolerant and have the potential to produce novel metabolites, are highly suitable for use in SSF by virtue of their rare ability to adsorb onto solid particles [7]. We have recently reported that polystyrene, a commercially available insulating and packaging material, could be used as an inert solid support for the production of L-glutaminase by a marine *Vibrio costicola* under SSF [6], while ion exchange resins [8], polyurethane foam [4, 9] and computer cards [10] have been used as inert carriers for SSF with fungi. In this paper we deal with the design of a suitable medium and the optimisation of process parameters towards maximal glutaminase production by a marine *V. costicola* in SSF using polystyrene as an inert support.

Materials and Methods

Microorganism

Vibrio costicola ACMR 267 was isolated from the marine environment at Cochin, India [11]. It was maintained on ZoBell's marine agar slants and subcultured

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every month. Inoculum, prepared by growing the cells in a medium the composition of which has been described previously [6], consisted of 24 h cells, washed twice and resuspended in sterile saline.

Solid substrate

Expanded polystyrene (poly(1-phenylethylene)) beads of 2–3 mm diameter were pretreated by autoclaving at 121°C for 15 min, which caused the beads to collapse and reduce to about one third of their original size [6, 12]. The reduced beads of uniform size were selected for fermentation studies.

Solid state fermentation

5 g of polystyrene beads (1–1·5 mm diameter), prepared as described earlier, were placed in 250 ml Erlenmeyer flasks, moistened with sea water containing L-glutamine at the desired concentrations at different (w/v) ratios, autoclaved for 1 h, cooled to room temperature and inoculated with 2 ml of the prepared inoculum [6]. The flasks were then incubated at the desired temperature for varying periods under 75–80% relative humidity. Enzyme was extracted using phosphate buffer (0·1 M, pH 7·0) at a 1:5 (w/v) ratio at room temperature (28±2°C) with a contact time of 30 min and agitation speed of 150 rpm on a rotary shaker. Dampened cheese-cloth was used to filter the extractant. After extracting twice, the extracts were pooled, centrifuged at 10000 rpm for 30 min at 4°C and the supernatant was subjected to various assays.

Optimisation of process parameters influencing enzyme yield

The strategy adopted for optimisation of various process parameters influencing glutaminase yield included consecutive evaluation of parameters [13]. Initially one parameter was evaluated and it was then incorporated at its optimised level in the subsequent optimisation experiment. The parameters optimised were (in order): solid to media ratio (moisture content) of the medium (1:0·2–1:1·5 w/v), initial pH (5–10, adjusted with 1 N HCl or NaOH), incubation temperature (25–50°C), L-glutamine concentration (0·5–5·0% w/w), inoculum concentration (0·125–1·250 mg dry weight/10 g dry substrate) and fermentation time (12–72 h). The effect of additional carbon and nitrogen sources, amino acids and mineral salts on enzyme production were tested by incorporation at 1% (w/w) in the sea water — glutamine medium before fermentation at the optimised conditions.

Analytical methods

Protein was estimated by Lowry's method [14] and glutaminase was assayed according to Imada *et al.* [15]. One international unit (IU) of glutaminase was defined as the amount of enzyme that liberates 1 μmol of ammonia under optimal assay conditions. Enzyme yield was expressed as units/g dry substrate (U/gds). Viscosity was measured using an Ostwald viscometer and expressed in Newtons/m^2 (Ns/m^2).

All experiments were carried out in triplicate and mean values are reported.

Results and Discussion

Effect of substrate:media ratio, pH and temperature

The data presented in Fig. 1 clearly indicate the strong influence of these process parameters on glutaminase yield from marine *V. costicola*. The enzyme yield increased with an increase in solid to media ratio from 1:0·2 (15 U/gds) to 1:1 (w/v) with a maximum at 1:1 (83 U/gds). Any further increase in the ratio resulted in the existence of free water and consequent reduction in enzyme yield. The optimum pH and temperature that promoted maximal glutaminase yield were 7·0 (98 U/gds) and 35°C (116 U/gds), respectively (Fig. 1). Nevertheless, significant enzyme yield was also recorded over a range of pH and temperature. Similar results were obtained with the same organism when nutritionally rich organic substrates such as wheat bran were used for SSF [3]. These factors are largely characteristic of the organism and were similar irrespective of the type of solid support used [3, 16].

Influence of substrate concentration, inoculum concentration and incubation time

Results presented in Fig. 2 suggest that 2% (w/w) L-glutamine concentration is optimal for maximal glutaminase yield (129 U/gds). The inoculum concen-

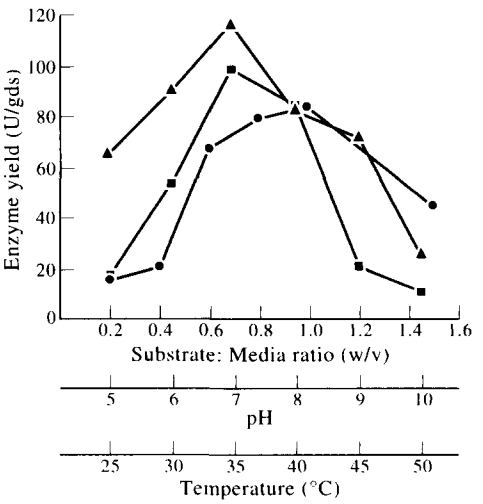

Fig. 1. Effect of substrate:media ratio, initial pH and temperature on L-glutaminase production. ●, Substrate:media ratio; ■, pH; ▲, temperature.

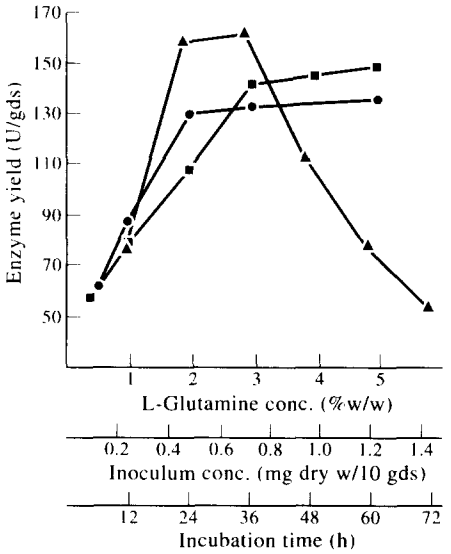

Fig. 2. Effect of L-glutamine concentration, inoculum concentration and incubation time on L-glutaminase production. ●, L-Glutamine concentration; ■, inoculum concentration; ▲, incubation time.

tration also showed an increasing linear trend with respect to glutaminase yield along with increasing concentration. Maximal glutaminase yield was obtained with 0·750 mg dry wt/10 gds (140 U/gds). Further increase in inoculum concentration did not promote additional enzyme yield. Results presented in Fig. 2 also suggest that 24 h incubation is the optimal time for maximal glutaminase yield (157 U/gds), in spite of recording 160 U/gds after 36 h of incubation. It was also noted that fermentation beyond 48 h led to a decrease in enzyme yield (52 U/gds after 72 h), which might be attributed to the inactivation of glutaminase by the proteases secreted by the bacteria during SSF [3].

Influence of additional nutrients

Incorporation of additional carbon sources enhanced the enzyme yield from 160 to 196 U/gds (Fig. 3). Among the carbon sources tested, maltose (1% w/w) promoted maximal enzyme yield (196 U/gds; 23% increase) compared to the others. This could be attributed to the rapid growth accomplished by the easy availability of additional carbon sources along with glutamine [6]. On the other hand, it was observed that nitrogen sources, including amino acids, had a negative impact on glutaminase yield (Figs 4 and 5). This could be due to the preferential utilisation of these nutrients in place of glutamine.

All the mineral salts tested enhanced glutaminase yield significantly. Potassium dihydrogen phosphate affected the maximum yield (232 U/gds; 18% increase) when compared to the control (196 U/gds; Fig. 6). Sodium dihydrogen phosphate followed by magnesium sulphate could also enhance enzyme yield significantly

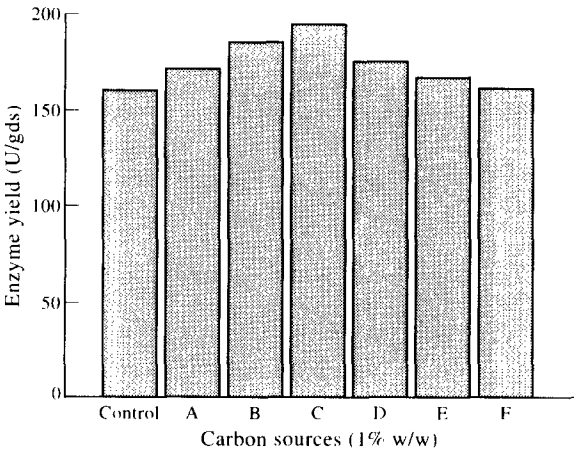

Fig. 3. Effect of additional carbon sources on L-glutaminase production. A, sucrose; B, glucose; C, maltose; D, galactose; E, lactose; F, trisodium citrate.

(14 and 12%, respectively). These results emphasise the critical role of phosphates and magnesium in the enhanced secretion of glutaminase. Zhu *et al.* [4] have achieved increased yields of nuclease P1 with polyurethane foam impregnated with a media that simulated the chemical composition of wheat bran. In the present study, the maximal yield of the target product was obtained with the use of a sea water media containing L-glutamine, maltose and KH_2PO_4.

The nutritionally inert polystyrene beads have facilitated the design of an economic medium for enhanced product yield with minimal presence of undesired proteins. The leachate from the polystyrene SSF system was less viscous (mean viscosity 0·966 Ns/m^2) and showed high specific activity for glutaminase (4·2 U/mg protein) and was free of undesired proteins, unlike that from wheat bran which was highly viscous (mean viscosity 2·072 Ns/m^2) and contained amylase and cellulase in addition to glutaminase [6]. These features

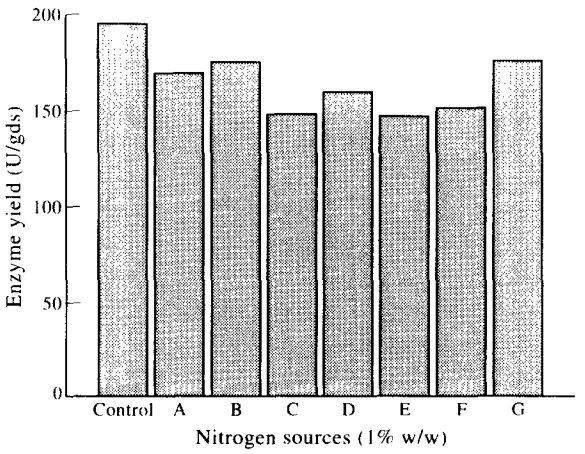

Fig. 4. Effect of additional nitrogen sources on L-glutaminase production. A, yeast extract; B, peptone; C, urea; D, caesin; E, ammonium sulphate; F, ammonium nitrate; G, sodium nitrate.

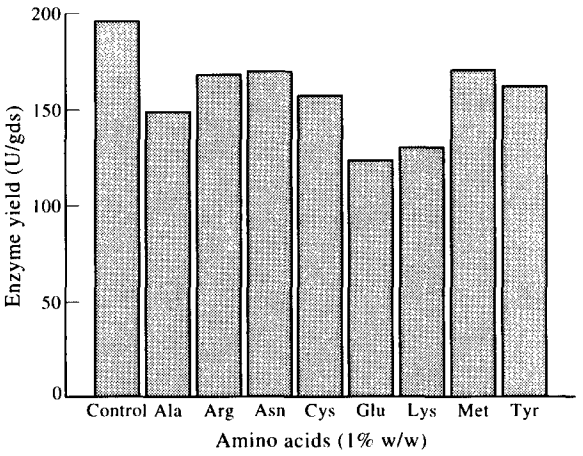

Fig. 5. Effect of amino acids on L-glutaminase production. Ala, alanine; Arg, arginine; Asn, asparagine; Cys, cysteine; Glu, glutamic acid; Lys, lysine; Met, methionine; Tyr, tyrosine.

are highly beneficial for the enzyme industry since this eliminates the problems associated with the viscous nature of the leachate from natural substrates [17]. Furthermore, 92–96% of the leachate could be recovered from this system, whereas with the conventional wheat bran system only 82–85% recovery is normally possible. The other major advantage of this new SSF system is the ability to measure biomass easily and accurately, which is almost impossible with conventional SSF systems [6].

In order to sustain interest in SSF processes and to utilise its advantages over conventional submerged fermentation, the inherent problems of SSF must be overcome. In this context, our results clearly suggest that use of nutritionally inert solid support materials in SSF could lead to further improvement of this already advantageous process for obtaining useful microbial products such as enzymes, organic acids, antibiotics and other fine chemicals of industrial importance.

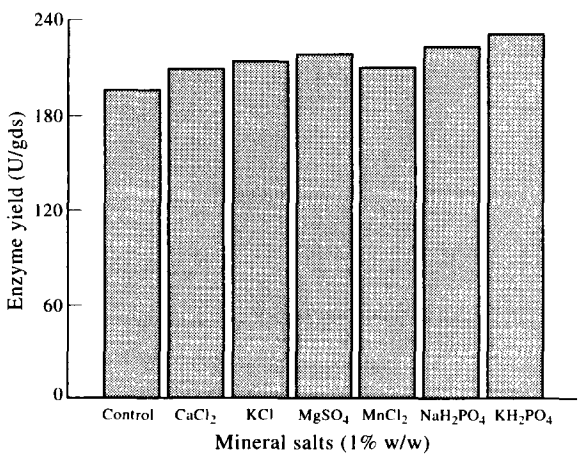

Fig. 6. Effect of mineral salts on L-glutaminase production.

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