Production of a bacterial β-glucanase by expression in *Escherichia coli* and simultaneous adsorption on a metal chelate affinity resin

(Received: 25.02.2003; A accepted: 21.04.200)

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ABSTRACT

The production of a chimeric β-glucanase from Bacillus species was studied in an integrated process by expression as well as excretion in E. coli and simultaneous adsorption on a metal chelate resin in shake-flask cultures. In order to establish an effective metal chelate affinity adsorption process, the binding capacity of free PDC-S and Zn^{2+}-PDC (Pentadentate Chelator) as well as its effect on bacterial growth was investigated. The results of integrated batch cultivation revealed that Zn^{2+}-PDC is superior to free PDC, because it was characterized by a higher binding capacity and faster adsorption. The results also showed that the use of Zn^{2+}-PDC resulted in a high -glucanase activity adsorbed on the resin (1697 U ml^{-1}), which is 1.3-fold higher than that obtained in the presence of metal-free PDC (1315 U ml^{-1}). The integrated process in the presence of Zn^{2+}-PDC resulted in a global volumetric activity of β-glucanase of 3590 U ml^{-1}, which was 1.5 and 3.1-fold higher than that obtained with free PDC (2500 U ml^{-1}) and in the absence of PDC (1144 U ml^{-1}), respectively. Moreover, a series of experiments were carried out in order to find out the ideal operating conditions of an integrated process for the production of β-glucanase based on the metal chelate protein purification technique.

Key words: *Escherichia coli, simultaneous fermentation and purification, β-glucanase.*

INTRODUCTION

The production of foreign proteins with the necessary modifications using a selected host is one of the key successes in modern biotechnology. This methodology allows the industrial production of proteins that were otherwise produced in small quantities only. However, the separation and purification of these proteins from the cultivation media constitutes a major bottleneck for the widespread commercialization of recombinant proteins. The major production costs (50-90%) for a

typical biological product resides in the purification step. There is a need for efficient, effective and economic large-scale bioseparation techniques to achieve high purity and high recovery, while maintaining the biological activity of the molecular entities. The key now lies in understanding downstream processing, integrating it with upstream steps, and thus providing better strategies to improve the economics of the whole process. Various separation techniques have been used in combination with fermentation to form effective integrated systems, e.g., extraction with organic solvents (Busche, 1991), extraction in aqueous two-phase systems (Larsson *et al.*, 1989), adsorption (Wang *et al.*, 1989), membrane techniques, and pervaporation (Groot *et al.*, 1991) as well as reverse micellar extraction of proteins (Poppenborg and Flaschel, 1994). Common sense dictates that a large-scale process should be designed to minimize the number of steps while maintaining high yields and product purity, quality and activity. The choice of the purification scheme depends on the location of the target protein and the desired purity of the product, which is determined by the utilization of the protein. Recombinant DNA technology allows the fusion of affinity tags to the target protein. This genetic modification can greatly increase the purification efficiency of recombinant proteins. Our particular interest is in the simplification of downstream processing schemes for the purification of proteins. Since the products are proteins, a prerequisite for integrated processes is that they have to be released into the medium - a requirement not evident especially in the case of Gram-negative bacteria. Proteins in the periplasm of *Escherichia coli* may be released into the extracellular space by means of the Kil protein (Miksch *et al.*, 1997). The aim of this work is to integrate genetic engineering with process engineering strategies for the development of integrated processes for the production of recombinant proteins with *E. coli*. Therefore, a capturing technique was applied using free and chelated Pentadentate-Sepharose (PDC) for simultaneous cultivation of bacteria and affinity adsorption of β-glucanase.

MATERIALS AND METHODS

Bacterial strains and plasmids

Recombinant *E. coli* BL21(DE3) pET-bgl-his was used throughout all experiments of this study (Wittler, 2000). A chimeric *Bacillus* β-glucanase was expressed constitutively under the control of the promoter of the β-glucanase gene of *B. amyloliquefaciens*. Secretion of the β-glucanase into the medium was achieved by expressing the *kil* gene under the control of the stationary-phase promoter of the *fic* gene (Miksch *et al.*, 1997). The expression vector pET20b-bgl-his was constructed by inserting both the hexahistidine-tagged β-glucanase and the secretion cassette into the vector pET20b (Stratagene). The secretion cassette was described elsewhere (Miksch *et al.*, 1997).

Media, antibiotics and cultivation conditions

For batch cultivation, the TB medium (Terrific Broth) containing the following ingredients (g l^{-1}): casein peptone, 12g; yeast extract, 24g; glycerol, 4g; KH_2PO_4, 2.4g; K_2HPO_4, 12.5g was used. Antibiotics were added at the following concentrations: 100 μg ml^{-1} ampicillin and 50 μg ml^{-1} Kanamycin. Induction was carried out after about 5 hr with 10 mM IPTG. Batch cultures were carried out in Erlenmeyer flasks with 50 mi bacterial suspension in 250 ml flasks at 37 °C with shaking at 175 rpm.

Adsorbent material

Pentadentate chelator-Sepharose CL-4B resin (PDC) was purchased from Affiland (Belgium). PDC resin coupled with Sepharose CL-4B is capable of forming complexes with all polyvalent metal ions including Cu^{2+}, Ni^{2+}, Zn^{2+}, and Co^{2+} to give an octahedral metal ion-chelator complex with five coordination sites occupied by the chelator. These properties lead to a high stability of the metal ion-chelator complex and no leakage of Ni^{2+}, $Zn^{2+}$and Co^{2+} during chromatography steps. PDC has one free site for interaction and selective binding of accessible cysteine or histidine residues. The resin can be regenerated for at least 10 cycles without significant loss of binding capacity. In addition, PDC-Sepharose has a high binding capacity as shown in Table (1).

Adsorbent preparation

A plastic column with a diameter of 2.5 cm and a length of 15 cm was packed with 20 ml PDC resin and equilibrated under gravity with 10 volumes of distilled water. A metal ion solution of 100 ml was loaded as 0.05 g l^{-1} $ZnCl_2$ in water. The column was washed with 5 column volumes of distilled water followed by 5 volumes K_2HPO_4 (50 mM, pH 8.0) and KH_2PO_4 (50 mM, pH 6.0) solutions. Finally, the column was re-equilibrated by washing with 5 volumes of binding phosphate buffer saline PBS (50 mM Na_2HPO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0).

Cell concentration

Samples were withdrawn during the cultivation process and their optical density was determined at a wavelength of 600 nm using a spectrophotometer. These values were converted into cell dry mass concentrations according to a standard curve. The integrated approach studied in this work aims to find an optimal strategy to combine high β-glucanase productivity and purity using metal ion affinity adsorption technique. Which one optical density unit equals 0.416 g l^{-1} cell dry mass.

Enzyme assay

For measuring the β-glucanase activity in the culture medium, 0.5 ml bacterial cells was centrifuged at 15,000g for 5 min and the supernatant was used for the assay.

Activity determination of adsorbed enzyme

For analyzing the enzyme adsorbed on the PDC resin, 20-100 μl of metal chelate supported with adsorbed β-glucanase were washed twice with distilled water, centrifuged (1000 xg^{-1}, 10 min), and the pellet was resuspended with 50 mM phosphate buffer, 0.5 M imidazol, pH 7.2 for 10 min, and centrifuged again (1000 xg^{-1}, 10 min). Finally, β-glucanase activity was determined as described by Borriss *et al.* (1989).

RESULTS AND DISCUSSION

Batch production of β-glucanase in shake-flask cultures

Batch cultivations were carried out in aflusk shaker in order to study the pattern of -glucanase production in the absence of metal chelating resins as a basis for comparison. Fig. (1) shows the time course of cell concentration and the extracellular β-glucanase activity during this cultivation. The biomass concentration reached 3 g l^{-1} after 24 hr and kept more or less constant until the end of the cultivation process. The extracellular β-glucanase activity showed a certain time lag against cell growth due to the stationary promoter being responsible for secretion into the medium. Thus, the maximal β-glucanase concentration (1144 U ml^{-1}) was obtained at the end of the cultivation process after 45 hr.

Integrated batch process for the production of β-glucanase

Metal ions used in metal chelate protein binding may have a significant influence on process performance, especially if in integrated processes. Therefore, the integration of bacterial cultivation and capturing of the enzyme was studied with PDC-resins, which were either uncharged or charged with Zn-ions. Results of these processes are gathered in Fig. (2). Obviously, even the uncharged resin did adsorb β-glucanase. In fact, the resin was saturated rapidly in both cases and the capacities were not sufficient to take up more than the half of the final enzyme activity. In both cases, 1 ml of PDC resin was applied in 50 ml bacterial suspension culture. Under these conditions, only 53% of the enzyme was adsorbed after 26 hr on the uncharged resin, as shown in Fig. (2A). Results obtained in a similar experiment with PDC resin charged with Zn-ions are shown in Fig. (2B). These data revealed that the Zn^{2+}-PDC resin resulted in a higher β-glucanase binding (1697 U ml^{-1}), about 1.3 times higher than that obtained in the presence of uncharged PDC (1315 U ml^{-1}). It was not astonishing that the charged resin did adsorb the His_6-tagged enzyme, but that the uncharged resin showed an appreciable capacity for the enzyme, too. The final global concentrations observed were 3590 U ml^{-1} in the case of Zn^{2+}-PDC resin and 2500 U ml^{-1} with uncharged resin - much more than that obtained in the absence of PDC (1144 U ml^{-1}) as shown above.

Optimization of the amount of PDC for β-glucanase purification

The amount of Zn^{2+}-PDC added and its effect on both cell growth and β-glucanase activity in the medium, on the affinity resin, and the sum of both activities are given in Fig. (3). Unexpectedly, the process performance deteriorated by increasing the amount of resin. Both, growth rate and enzyme productivity were affected. Thus, other factors than simple adsorption of the His_6-tagged enzyme governed the over-all process performance. These results agree with previous results about the influence of the binding capacity of PDC resins (Wittler, 2000). A similar negative effect of the amount of activated charcoal was observed during the purification of a protease (Tunga *et al.*, 1999). In these cases, there may be problems with the adsorption of medium components, but this has to be studied in details, before any final conclusion may be drawn.

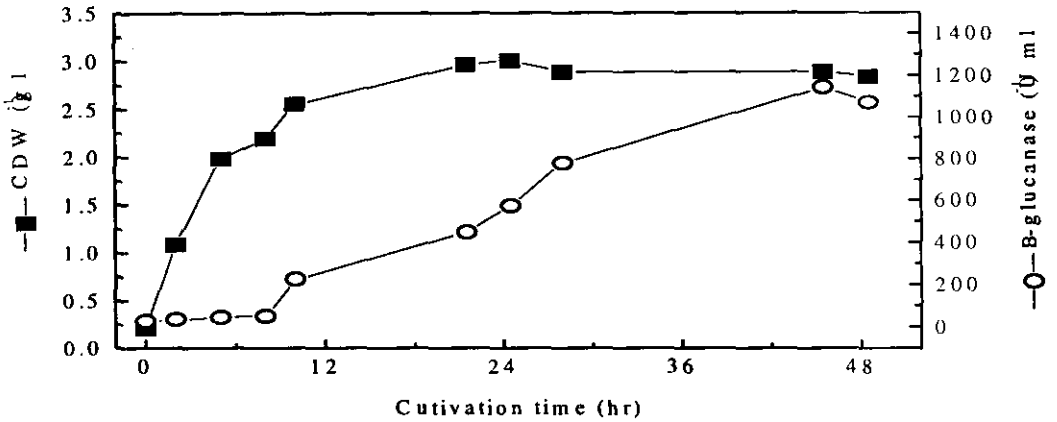

Fig. (1): Batch cultivation of E. coli secreting β-glucanase into the medium.

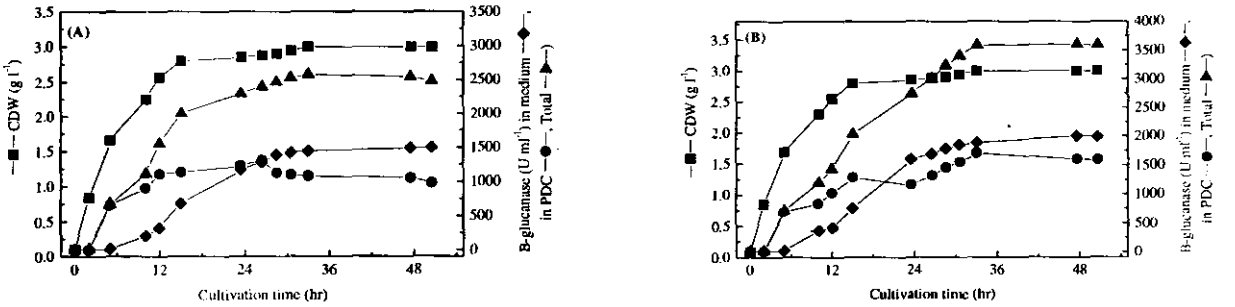

Fig. (2): Integrated batch production and purification of β-glucanase on PDC uncharged (A) and charged with Zn^{2+}-ions (B) in shake flasks.

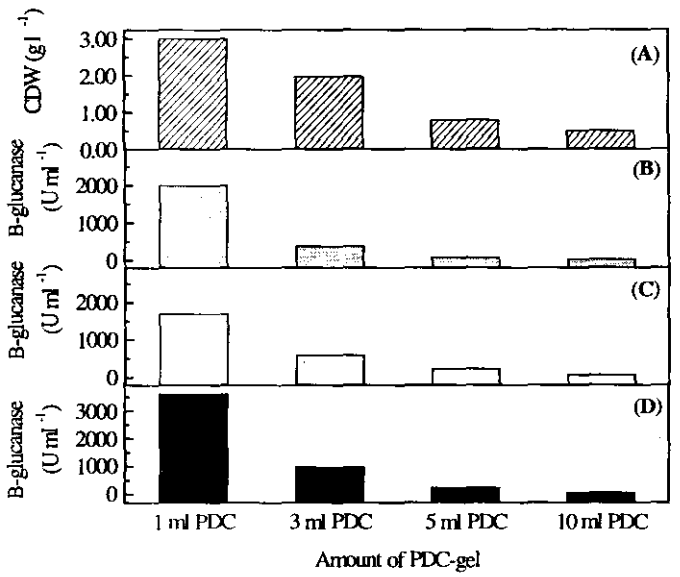

Fig. (3): Effect of the amount of Zn^{2+}-PDC on cell growth (A) and β-glucanase in the medium (B), adsorbed on Zn^{2+}-PDC (C), and the total activity (D).

Table (1): Binding capacity and stability of the metal chelating resin PDC.

Binding capacity	35-40 mol ml^{-1} of M^{2+} on wet gel (M= Cu, Ni, Zn, Co) 15-20 mg ml^{-1} of His_6-tagged HSP60 on wet Zn^{2+}-PDC
Stability	pH range 2-12 Several years at physiological pH (4 °C)
Particle size	45-165 μm

ACKNOWLEDGEMENTS

This work was supported by a fellowship from the "*Deutscher Akademischer Austauschdienst*" (DAAD) to Dr. U. Beshay.

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الملخص العربي

إنتاج وتنقية إنزيم البيتاجلوكاناز البكتيرى من سلالة الإيشيريشيا كولاى باستخدام طريقة الإدمصاص على الراتينجات المشحونة بأيونات المعدن

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تم فى هذه الدراسة انتاج انزيم البيتاجلوكاناز من سلالة الايشيريشيا كولاى التى تحتوى على جين الأنزيم من باسيلس مع تنقية الإنزيم وإنتاجه فى عملية واحدة باستخدام تقنية الادمصاص على سطح بعض الراتينجات الصناعية فى الدوارق المعملية. فى البداية تم عمل مقارنة معملية بين كفاءة الراتينج الحر (بى دى سى إس) والراتينج المحمل بأيونات الزنك (زنك بى دى سى) فى إدمصاص وربط إنزيم البيتاجلوكاناز وتأثيره على نمو البكتيريا. ولقد أثبتت النتائج أن قدرة الراتينج المحمل بأيونات الزنك (زنك - بى دى سى) أعلى من قدرة الراتينج الحر (بى دى سى إس) فى ربط وامساك الإنزيم. كما أوضحت النتائج أن وجود الراتينج المحمل بأيونات الزنك أدى إلى زيادة كمية الإنزيم داخل الراتينج لتصل إلى (١٦٩٧ وحدة/مل) وهى تعادل ١,٣ ضعف كمية الإنزيم فى حالة استخدام الراتينج الحر (١٣١٥ وحدة/مل). كما أوضحت الدراسة أن استخدام الراتينج المحمل بأيونات الزنك (زنك-بى دى سى) قد أدت إلى زيادة الكمية الكلية لإنزيم البيتاجلوكاناز لتصل إلى (٣٥٩٠ وحدة/مل) التى تعادل ١,٥ و ٣,١ ضعف إذا ما قورنت مع كمية الإنزيم المنتجة باستخدام الراتينج الحر (٢٥٠٠ وحدة/مل) أو فى حالة عدم وجود الراتينج نهائياً (١١٤٤ وحدة/مل) على الترتيب . هذا بالإضافة إلى عمل سلسلة من التجارب لمعرفة الظروف الملائمة لإنتاج وتنقية إنزيم البيتاجلوكاناز باستخدام هذه التقنية.