Journal of Biotechnology 242 (2017) 55–63

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

In situ affinity purification of his-tagged protein A from *Bacillus megaterium* cultivation using recyclable superparamagnetic iron oxide nanoparticles

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ARTICLE INFO

Article history:
Received 13 October 2016
Received in revised form
16 November 2016
Accepted 22 November 2016
Available online 23 November 2016

Keywords:
In situ purification
Particle regeneration
Protein purification
SPION
Bacillus megaterium
Magnetic separation

ABSTRACT

This paper discusses the use of recyclable functionalized nanoparticles for an improved downstream processing of recombinant products. The Gram-positive bacterium *Bacillus megaterium* was used to secrete recombinant protein A fused to a histidine tag into the culture supernatant in shaker flasks. Superparamagnetic iron oxide nanoparticles functionalized with 3-glycidoxypropyl-trimethoxysilane-coupled-nitrilotriacetic-acid groups (GNTA-SPION) were synthesized and added directly to the growing culture. After 10 min incubation time, > 85% of the product was adsorbed onto the particles. The particles were magnetically separated using handheld neodymium magnets and the product was eluted. The GNTA-SPION were successfully regenerated and reused in five consecutive cycles. In the one-step purification, the purity of the product reached > 99.9% regarding protein A. A very low particle concentration of 0.5 g/L was sufficient for effective product separation. Bacterial growth was not influenced negatively by this concentration. Particle analysis showed similar properties between freshly synthesized and regenerated GNTA-SPION. The overall process efficiency was however influenced by partial disintegration of particle agglomerates and thus loss of particles. The demonstration of very fast *in situ* product removal from growing bacterial culture combined with a very high product purity within one step shows possibilities for automated large scale purification combined with recycling of biomass.

1. Introduction

Any biotechnological manufacturing process must be designed and optimized with respect to the product yield and costs. Critical in concentration and purification of cultivation broths is the fact that any changes in the cultivation, in particular the apparent optimization to higher product titre, entail a complicated adjustment of the subsequent processes and an increase in the costs of the downstream part. In this area, there is a considerable need for improvement. Furthermore, downstream processing contributes to the major part of the overall process price and therefore to the product costs (Gagnon, 2012). The cost of downstream processing can range up to 80% of the overall process costs in medical applications (Roque et al., 2004). Thus, it is necessary not only to research improvements in upscaling, e.g. improved strains and cultivation processes, but also in downstream processing. Depending on the application of the product, multiple purification steps have to be performed. For the downstream processing of therapeutic products, according to U.S. Department of Health and Human Services (1997) a final purity of > 99.99 % has to be reached. As each purification step has an efficiency < 100%, every reduction in the number of

Abbreviations: CFU, colony forming unit; DLS, dynamic light scattering; ELISA, enzyme linked immunosorbent assay; GNTA-SPION, functionalized SPION analyzed in this article; OD, optical density; SPION, superparamagnetic iron oxide nanoparticles; TGA, thermogravimetric analysis.

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http://dx.doi.org/10.1016/j.jbiotec.2016.11.018
0168-1656/

steps, while maintaining the same purity, is correlated to a higher yield.

The first step in the downstream processing of almost every biotech product is a solid-liquid separation, i.e. the separation of the supernatant from the cells either by means of filtration or centrifugation. Depending on the location of the product within the cells or in the medium, the cells or the supernatant are further processed (Gronemeyer et al., 2014). With continuous centrifugation steps, biomass can be reused after the separation from the supernatant, if the product is located in the latter. However, the centrifugal stress induces changes in the metabolic state of the cells due to shear stress and can therefore lower the productivity of recycled biomass (Hutchinson et al., 2006).

In order to bypass the need of centrifugation and other solid-liquid separation steps, several continuous purification systems are presented in literature. These systems reduce the time consuming separation steps. Moreover, they reduce the downtime between several batch-processes (Henzler, 2012). However, using chromatographic processes for continuous processing has drawbacks in the up-scaling, as a mere enlargement of the columns or an increased flow rate does not necessarily result in better efficiencies (Susanto et al., 2009). They furthermore readily show fouling effects due to the biomass being partially retained within the chromatographic material. A good overview of this phenomenon is given by Carstensen et al. (2012).

These problems can mostly be avoided by an *in situ* purification method. In this concept, the product binds either reversibly or irreversibly to a matrix which can easily be separated from the media and regenerated (Schügerl and Hubbuch, 2005). One possibility is the use of functionalized magnetic nanoparticles which bind to the product, using affinity effects, and can be separated by magnetic manipulation (Dunnill and Lilly, 1974; Fish and Lilly, 1984). The particles are added to the growing and producing cell culture and separated shortly after. Scale-up of the separation can easily be performed using higher volumetric flow rates combined with sequential or parallel aligned magnets entailing appropriately increased magnetic field intensities and gradients.

For this investigation as model protein for magnetic separation the recombinant protein A is used. Protein A is a protein abundant in 99% of the *Staphylococcus aureus* strains (Forsgren, 1970) with 8 to 30% being secreted in *S. aureus* during exponential growth (Movitz, 1976). It has a very high stability against heat and denaturating agents (Sjoholm, 1975) and is mostly used in purification of antibodies due to its ability to very selectively bind to the constant region of the heavy chain region (Holschuh and Schwämmle, 2005).

In this paper nanoparticles according to Masthoff et al. (2014) were used. These functionalized Superparamagnetic Iron Oxide Nanoparticles (SPION) allow *in situ* adsorption mediated by Ni^{2+}-chelating with recombinant protein A fused to a 6 x histidine tag and are easily separated from the media and regenerated in the following, in order to provide a fast and reusable purification method with the need of neither filtration nor centrifugation steps to separate the product from cells and cultivation supernatant.

Previous studies have shown the feasibility of *ex situ* product purification from the cell-free cultivation supernatant (Masthoff et al., 2014) and cell lysate (Fraga García et al., 2015) using SPION. The present investigation enquires into the possibility of using SPION as an agent for the *in situ* product recovery, i.e. recovery of the product directly from the growing cell cultivation without disrupting the cellular integrity. Furthermore, the SPION are regenerated and used in subsequent and multiple purification cycles. Particle and purification properties were evaluated. The particles were compared with commercial particles in microtiter plates. Purification and regeneration cycles were performed in shake flasks. Hereby, protein A fused to a 6 x His tag was used as model protein, due to the high protein stability and high product yield reached in the course of the research. The protein was recombinantly produced and secreted in the Gram-positive *Bacillus megaterium* MS941.

2. Materials and methods

If not stated otherwise, chemicals were used in HPLC grade from Roth (Carl Roth GmbH & Co. KG, Karlsruhe, Germany).

2.1. Strain construction

B. megaterium MS941 was transformed with the plasmid pRBBm181 encoding a truncated protein A (GI:1480566). For this, the gene *spA* was amplified using the forward primer 5′ tatcaagatctccgcgcaacacgatgaagctc 3′ and the reverse primer (5′ tatcagcatgccctttggtgcttgagcatcgtttagc 3′) from genomic DNA of *S. aureus*. After clarifying, the PCR product was cut with BglII and SphI and ligated in the equally cut pSSBm110 (Stammen et al., 2010). Doing so, the gene was fused to the coding sequence of the signal peptide of the lipase A and a 6 x histidine tag was introduced at the C-terminal end of the protein A. Screening on correct plasmids was done using ampicillin during cloning in *Escherichia coli* DH10 B (Invitrogen, Carlsbad, USA) and tetracycline during the transformation of *B. megaterium*. The transformation was done using protoplast transformation as described previously (Biedendieck et al., 2011).

2.2. Cultivation procedure

The obtained strain was cultivated in defined medium (169.6 µg/L $MnCl_2 \cdot 4\,H_2O$, 271.36 µg/L $CaCl_2 \cdot 2\,H_2O$, 6.72 µg/L $FeSO_4 \cdot 7\,H_2O$, 1.04 µg/L $(NH_4)_6Mo_7O_{24} \cdot 4\,H_2O$, 8 µg/L $CuSO_4 \cdot 5H_2O$, 124 µg/L H_3BO_3, 25 µg/L $ZnSO_4 \cdot 4\,H_2O$, 560 ng/L $CoCl_2$, 300 mg/L $MgSO_4 \cdot 7\,H_2O$, 2.5 g/L $(NH_4)_2SO_4$, 5 g/L Fructose, 3.52 g/L KH_2PO_4, 5.3 g/L Na_2HPO_4) in purified water, pH 7.0, (Gradient A10, Merck Millipore, Darmstadt, Germany)) at 37 °C and 130 min^{-1} in (Multitron, Infors AG, Bottmingen, Switzerland, 25 mm shaking radius). 10 mg/L tetracycline was used as antibiotic.

An overnight culture of 100 mL was inoculated with 500 µL bacteria from cryogenic stocks in 500 mL baffled Erlenmeyer flasks. For the main culture, the optical density (OD) of the overnight culture was measured at 600 nm (Libra S11, Biochrom GmbH, Berlin, Germany). Inoculum volume resulting in a starting OD of 0.1 was taken and centrifuged at room temperature and 2,000 g for 5 min (Biofuge Stratos, Heraeus Holding GmbH, Hanau, Germany). The supernatant was discarded and bacteria were suspended in freshly made minimal medium before addition to the media reservoir. Afterwards, 75 mL of the inoculated culture was transferred to 500 mL baffled shaker flasks. At an $OD_{600} > 0.3$, the production was induced by adding D-xylose to a final concentration of 5 g/L, including the dilution of the media by the addition of the xylose solution itself. The particle dispersion was added five hours after the induction with xylose. Samples were taken in regular intervals and analyzed according to their OD_{600}, carbon source concentration and product concentration. The latter was analyzed using enzyme linked immunosorbent assay (ELISA). Carbon source concentration was measured using high performance liquid chromatography (HPLC). Each shaker flask experiment was performed in triplicates with additional flasks as control blank.

2.3. Preparation of the particles

The particles were synthesized according to Masthoff et al. (2014) with a modification of the silane coupling step in which the quantities of deionized water, silane and lysine derivate were