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Immobilization of Alkaliphilic *Bacillus* sp. Cells for Xylanase Production Using Batch and Continuous Culture

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Abstract

Agar-immobilized alkaliphilic *Bacillus* sp. AR-009 cells were used for xylanase production using batch and continuous culture. In a batch culture, maximum enzyme production was observed after 48 h and remained high up to 72 h. In repeated batch cultivation, immobilized cells produced an appreciable level of xylanase activity in seven consecutive batches without any significant decline in productivity. For continuous xylanase production, immobilized cells were packed in a jacketed glass column and sterile medium was continuously pumped. A stable continuous production of xylanase was observed over a period of 1 mo. The volumetric productivity of the continuous culture was 17-fold higher than the batch culture using free cells.

Index Entries: Alkaline xylanase; thermostable xylanase; immobilized cell; hemicellulase; *Bacillus*; alkaliphile.

Introduction

The use of immobilized cells for biotechnological processes offers a number of advantages over conventional free-cell fermentation, such as enhanced cell stability, thus allowing prolonged use; reduced downstream processing; lower risk of contamination; and the ability to use less sophisticated reactors *(1–4)*. To date, immobilized cells have been used for a variety of applications *(1,2)*. Although different types of microorganisms producing extracellular enzymes have been immobilized in many laboratories in the past, almost all are organisms that optimally grow around the neutral

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pH range. In recent years, many interesting alkaline enzymes of potential biotechnological importance have been described from different alkaliphilic bacterial strains *(5)*. One such enzyme is alkaline active xylanases from different groups of alkaliphiles, which may be important for a wide range of applications *(6–8)*. Earlier we isolated a xylanase producing alkaliphilic *Bacillus* sp. AR-009 in our laboratory *(7)*. This organism produces thermostable xylanases having optimum activity and good stability in the alkaline pH range and at elevated temperature *(9)*, properties that are very attractive for many applications. In this article, we report the results of our study on the use of agar-immobilized cells of *Bacillus* sp. AR-009 for xylanase production for an extended period of time.

Materials and Methods

Organism and Growth Conditions

The organism used was *Bacillus* sp. AR-009, an alkaliphile earlier isolated in our laboratory *(7)*. The medium used for growth and enzyme production (XYP medium) was composed of 5 g/L of xylan, 5 g/L of peptone, 1 g/L of yeast extract, 5 g/L of NaCl, 1 g/L of K_2HPO_4, 0.2 g/L of $MgSO_4$, 0.1 g/L of $CaCl_2$, and 10 g/L of Na_2CO_3. For continuous cultivation, a 1:4 diluted XYP medium (except that the concentration of Na_2CO_3 was kept at 1%) was used. In all cases Na_2CO_3 was sterilized separately and added to the rest of the medium after cooling. One hundred milliliters of XYP medium in 500-mL baffled flasks was inoculated with an overnight culture. The flasks were incubated at 37°C with rotary shaking.

Cell Immobilization

Cells were immobilized in agar beads following the methods of Nilsson et al. *(10)*. In brief, 4 g (wet weight) of cells was suspended in 40 mL of 3% agar solution, and beads were formed by dropping the agar-cell slurry into a layer of vegetable oil. After thorough washing with sterile saline solution, beads were transferred to fresh XYP medium and incubated at 37°C with rotary shaking.

Continuous Xylanase Production

Continuous xylanase production was carried out in a jacketed glass column with a height of 20 cm and internal diameter of 4 cm. Figure 1 depicts the experimental setup used for continuous cultivation. The temperature was kept constant at 37°C by circulating water through the jacketed column from a thermostat-controlled water bath. Diluted XYP medium (1:4 diluted with the concentration of Na_2CO_3 maintained at 1%) was continuously pumped at a dilution rate of 0.39/h. The reactor was aerated with filtered air at a rate of 80 cc/min.

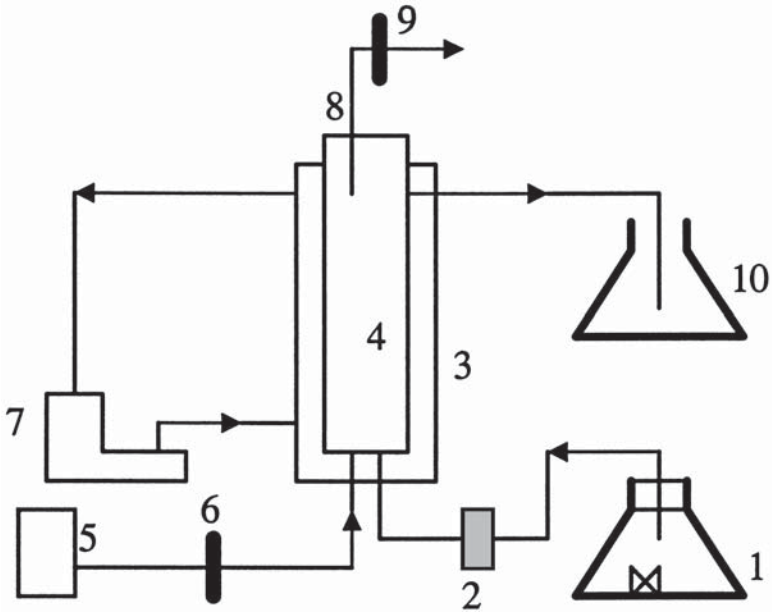

Fig. 1. Experimental setup used for the continuous production of xylanase by immobilized cells of *Bacillus* sp. AR-009. 1, Feed tank with continuous stirring; 2, peristaltic pump; 3, jacketed column; 4, reactor column with immobilized cells; 5, aerator; 6, filter; 7, thermostated water bath; 8, outlet for exhaust gases; 9, filter; 10, effluent.

Enzyme Assay

Xylanase activity was assayed as described previously *(7)* at 50°C using 1% birch wood xylan in 50 m*M* glycine NaOH buffer, pH 9.0, as a substrate. One unit of xylanase activity was defined as the amount of enzyme that released 1 μmol of reducing sugar equivalent to xylose per minute under the assay conditions.

Results

Time Course of Enzyme Production by Immobilized Cells

Xylanase production by immobilized cells of *Bacillus* sp. AR-009 was followed for 72 h. Enzyme production started after 12 h and reached a maximum after 48 h. Up to 72 h xylanase production remained high (Fig. 2).

Xylanase Production by Repeated-Batch Cultivation of Immobilized Cells

Xylanase production by immobilized cells was studied for seven consecutive batches. Every 48 h (the time for peak enzyme production), the original culture was removed and the beads were washed and resuspended with fresh XYP medium. As shown in Fig. 3, enzyme production was more

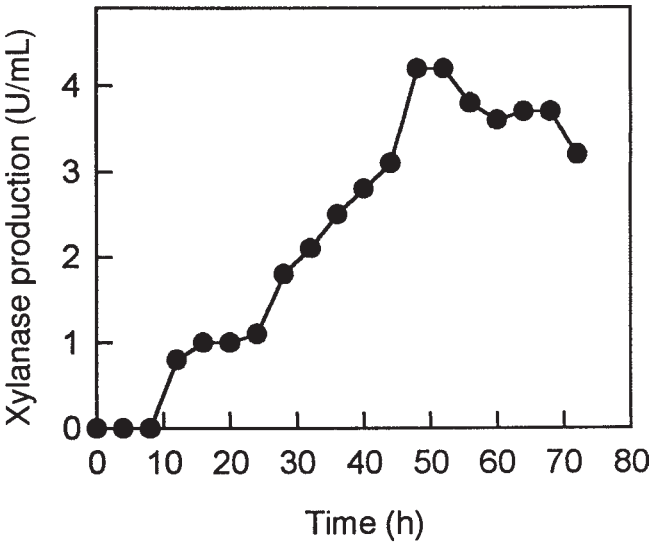

Fig. 2. Time course of xylanase production by agar-immobilized cells of *Bacillus* sp. AR-009 cells.

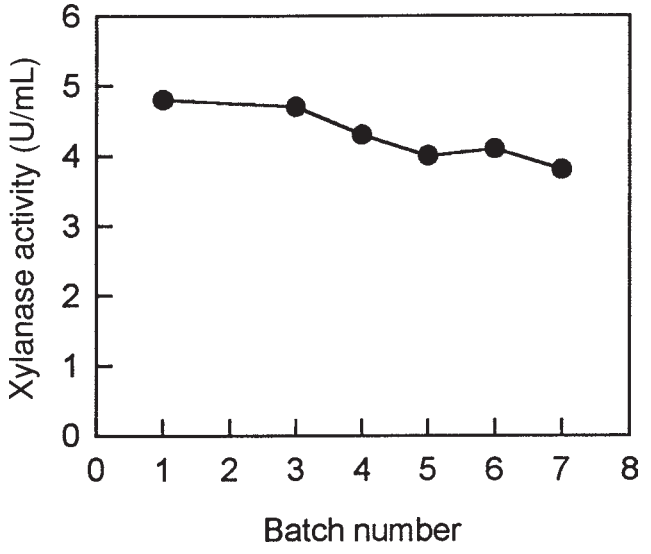

Fig. 3. Xylanase production by immobilized cells of *Bacillus* sp. AR-009 cells using repeated-batch culture. After reaching peak production, the original culture was removed and the beads were washed and resuspended with fresh XYP medium.

or less stable in seven consecutive batches. Throughout the whole cultivation cycle, the agar beads were stable without any sign of rupture.

Continuous Xylanase Production Using Immobilized Cells

Continuous xylanase production by agar-immobilized cells of *Bacillus* sp. AR-009 was followed for a period of 1 mo. During this time a constant

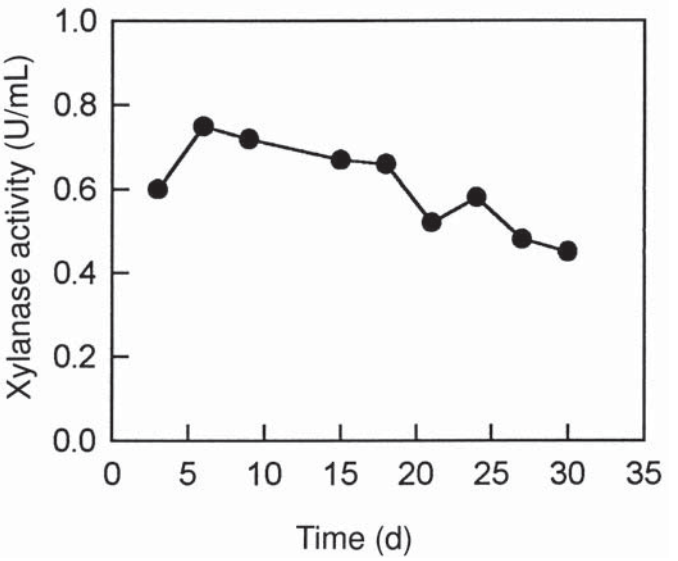

Fig. 4. Xylanase production by immobilized cells of *Bacillus* sp. AR-009 cells using continuous culture.

Table 1
Comparison of Xylanase Production by Free Cells and Immobilized Cells of *Bacillus* sp. AR-009 Under Different Cultivation Conditions

System	Productivity (U/[L·h])	Relative productivity
Free cells—batch	186.4	1.0
Immobilized cells—batch	87.5	0.5
Immobilized cells—repeated batch	64.5	0.4
Immobilized cells—continuous	3200	17.2

level of xylanase production was observed throughout the cultivation period (Fig. 4). The volumetric productivity of immobilized cells grown using continuous culture was much higher than for a batch culture using free cells, and batch and repeated-batch culture of immobilized cells (Table 1). The relative productivity of the continuous culture was 17-fold higher than for the batch culture using free cells and about 34-fold higher than batch and repeated-batch culture using immobilized cells (Table 1).

Discussion

Xylanases, which are optimally active and stable at alkaline pH and elevated temperature, have important applications in different industrial processes. To date, very few organisms are known to produce thermostable alkaline xylanases *(6,7)*. *Bacillus* sp. AR-009 produces two thermostable alkaline xylanases having optimum activity and good stability in the alka-

line pH range and elevated temperature *(9)*. In the present study, immobilized cells were used for the production of the enzyme for an extended period of time. *Bacillus* sp. AR-009 cells immobilized using agar produced an appreciable level of xylanase activity in batch and continuous culture. Because xylan is soluble at alkaline pH, and because some applications of xylanases need to be carried out at alkaline pH and high temperature (e.g., kraft pulp bleaching), development and optimization of methods for the production of alkaline active thermostable xylanases by immobilized cells of *Bacillus* sp. AR-009 could have significant economic and technical advantages.

In a batch culture of immobilized cells, xylanase activity was detected after 12 h and reached a maximum after 48 h. In our earlier study using free cells *(7)*, measurable xylanase activity was detected starting from 4 h. One possibility for the delay in xylanase production by immobilized cells may be the result of retarded growth of cells on immobilization. A similar observation was also reported for other organisms *(11–14)*. Another difference observed was that the level of enzyme production was lower in the case of immobilized cells than free cells. One explanation may be diffusional limitation of oxygen access to cells at the interior of the beads. Diffusional limitation of oxygen is known to affect growth and productivity of immobilized cells *(15)*. In the case of immobilized *Bacillus* sp. AR-009 cells, the low solubility of xylan may further complicate the situation. Because xylan cannot easily diffuse into the interior of the agar beads, only cells exposed at the surface of the beads may be induced and produce the enzyme. Cells deep in the interior of the agar bead may remain idle, being nourished by the other soluble medium components. In the future a search for low molecular weight soluble substrates that can induce xylanase production by *Bacillus* sp. AR-009 cells may help improve the level of xylanase production by immobilized cells. For example, one possibility may be the use of xylooligosaccharides after partial or complete enzymatic saccharification.

The agar beads showed remarkable stability for an extended period of time. Xylanase production was more or less constant in seven cycles of repeated-batch cultivation. This indicates the possibility of using agar-immobilized cells of *Bacillus* sp. AR-009 for repeated xylanase production. Immobilized *Bacillus* sp. AR-009 cells were also used for continuous production of xylanase for a period of 1 mo without any decline in productivity. One advantage of using immobilized cells for extracellular enzyme production is the possibility of using these cells for continuous or repeated enzyme production for an extended period of time, which will have a significant economic advantage. Under such conditions the risk of contamination will be lower, and the cost of downstream processing will be reduced. Further understanding of the physiology of immobilized *Bacillus* sp. AR-009 cells and improvement in medium composition for better enzyme induction may lead to an even higher level of xylanase production.

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