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PRODUCTION OF CELLULASE BY IMMOBILIZED WHOLE CELLS OF *Haloarcula*

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Halophilic Archaea are adapted to a life in the extreme conditions and some of them are capable of growth on cellulosic waste as carbon and energy source by producing cellulase enzyme. The production of cellulase using free and immobilized cells of halophilic archaeal strain *Haloarcula* 2TK2 isolated from Tuzkoy Salt Mine and capable of producing cellulose was studied. The cells were cultured in a liquid medium containing 2.5 M NaCl to obtain the maximum cellulase activity and immobilized on agarose or polyacrylamide or alginate. Optimal salt dependence of free and immobilized cells of *Haloarcula* 2TK2 was established and the effects of pH and temperature were investigated. Immobilization to Na-alginate enhanced the enzymatic activity of the haloarchaeal cells when compared to free cells and other polymeric supports. From the results obtained it is reasonable to infer that decomposition of plant polymers into simpler end products does occur at high salinities and cellulase producing haloarchael cells may be potentially utilized for the treatment of hypersaline waste water to remove cellulose.

The microbial conversion of cellulose to soluble compounds requires the action of cellulase. Cellulases can be divided into 3 main groups of enzymes: endocellulase (EC 3.2.1.4), exocellulase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21) [1–3]. Cellulases have a huge economic potential in the conversion of plant biomass into fuel and chemicals, and also find applications in the food and detergent industries [4]. Although a large number of microorganisms are capable of degrading cellulose, only a few of them produce significant quantities of cellulase [5–7]. Fungi are the main cellulase producing microorganisms though a number of bacteria and *Actinomycetes* have been reported to yield cellulase activity [1, 8]. There appears to be a tendency for a broader range of carbohydrate utilization in more extreme environments such as halophilic, perhaps as consequence of cellulose input, possibly combined with the presence of fewer competing species in these habitats [7, 9]. Halophilic Archaea are a group of microorganisms that inhabit natural environments containing high salt concentrations, e.g. salt lakes and mines, crystallizer ponds of solar salterns or artificial habitats such as salted fish, vegetables, meat and hides [5, 10, 11]. Hydrocarbon-degrading isolates have been isolated from hypersaline environments and some cellulase-producing haloarchaea bacteria obtained from the Tuzkoy Salt Mine belonging to *Haloarcula* have been reported by Birbir et al. [7]. Cellulosic waste in the mine might have been utilized as carbon source by *Haloarcula* strains. *Haloarcula* is distinguished from other genera in the Halobacteriaceae family by the presence of specific derivatives of triglycosyl diether-2 (TGD-2) polar lipids [12]. Since immobilization of archeal cells and a detailed mechanism study have not been performed before and also to obviate costly and tedious purification procedures that may inactivate the enzyme and to retain the enzyme's native geometry and microenvironment [13, 14]. The aim of the study was to immobilize whole cells of *Haloarcula* 2TK2 on polyacrylamide or Na-alginate and or agarose. The effect of entrapment in different matrixes was evaluated by the determination of the cellulose activity and stability and the results obtained were compared with those of the free cells. Potential usage of the *Haloarchaeal* cells for the biological treatment of saline and hypersaline waste water to remove organic carbon was discussed.

MATERIALS AND METHODS

Chemicals. Carboxymethyl cellulose (CM-cellulose), casamino acids, yeast extract, Na-alginate, agarose and acrylamide were purchased from Merck, (Germany). All the chemicals used were analytical grade.

Microorganism. A cellulase producing strain 2TK2 was isolated in our laboratory (Marmara University, Biology Department, Turkey). Identification of the organism was achieved by microbiological and biochemical tests [12]. DNA sequences and phylogenetic analysis revealed that the isolated strain was mainly species of the genus *Haloarcula* [7].

Production. The cells of *Haloarcula* 2TK2 were grown for 7 days at 40°C in a liquid medium (g/l): CM-cellulose – 3.0, KCl – 2.0, $MgSO_4 \cdot 7H_2O$ – 20,

casamino acids – 1.0, yeast extract – 1.0 and 2.5 M NaCl in an orbital shaker incubator [12]. The resulting culture suspension was adjusted to the McFarland nephelometer standard number 3.0 [12–15]. The cells were separated by centrifugation at 10000 g and 4°C for 30 min [16]. The packed cells were suspended in sterile 2.5 M NaCl solution.

Immobilization of whole cells to polyacrylamide, alginate and agarose. The sedimented cells were immobilized on polyacrylamide or alginate or agarose.

Polyacrylamide immobilization. 750 mg of acrylamide monomer and 40 mg of metyhlenebisacrylamide monomer were mixed in 2 ml of 2.5 M NaCl. The wet *Haloarcula* 2TK2 cells (2.5 g) were added to this solution. 70 μl of TEMED were added as polymerization agent. Polymerization was completed in 20 min at 25°C. The gels formed were cut into pieces in assay buffer containing 50 mM Tris-HCl (pH 7.2) with 10 μM $MnCl_2$, 0.1% β-mercaptoethanol (w/v) and 2.5 M NaCl. Any free cells were removed by washing with 2.5 M NaCl solution [13, 14].

Alginate immobilization. Sterile 3% sodium alginate suspension (w/v) was prepared by suspending sodium alginate in assay buffer. The wet *Haloarcula* 2TK2 cells (2.5 g) were suspended in 5 ml of 2.5 M NaCl and stirred thoroughly to ensure complete mixing. 1 ml of cell paste was suspended in 50 ml of alginate solution. Alginate mixture was added dropwise to 1.0 l of 0.15 M $CaCl_2$ with constant stirring and the addition was continued until the desired number of beads was obtained. Alginate beads were washed with 2.5 M NaCl solution and filtered [13, 14].

Agarose immobilization. Agarose solution was prepared by dissolving agarose (3% w/v) in 2.5 M NaCl at 100°C. The solution was cooled to 40°C and mixed with 2.5 g of the wet *Haloarcula* 2TK2 cells. The mixture was allowed to cool at room temperature. The gel formed was shredded in a blender and free cells were removed by washing with 2.5 M NaCl solution [17].

Activation of immobilized beads. Immobilized cells were grown in the nutrient rich liquid medium containing (g/l): CM-cellulose – 10.0; casamino acids – 5.0; yeast extract – 5.0; NaCl – 250; $MgSO_4 \cdot 7H_2O$ – 20 and KCl – 2.0, at 40°C and 100 rpm for 7 days. After incubation, the immobilized cells were washed twice with 2.5 M NaCl and transferred to the nutrient poor liquid medium containing (g/l): CM-cellulose – 0.2, casamino acids – 0.1, yeast extract – 0.1, NaCl – 250, $MgSO_4 \cdot 7H_2O$ – 20 and KCl – 2. A nutrient poor medium was used as a reaction medium for improving the activity of cells [17].

Assay of the cellulase activity. The cellulase activity of *Haloarcula* 2TK2 was determined in triplicate with CM-cellulose as a substrate and glucose as the product [18, 19]. Released glucose was determined in a hexokinase/glucose-6-phosphate dehydrogenase system at 340 nm. One unit of cellulase activity was defined as 0.01 mg of the glucose formation per min from CM cellulose at 25°C.

The effect of pH on the stability of cellulose of *Haloarcula* 2TK2. The effect of different values of pH from 4.0 to 12.0 on the stability of the enzyme was measured, both for free and immobilized cells of *Haloarcula* 2TK2, at optimal salt concentration [13, 17].

The effect of salt on the activity of cellulose of *Haloarcula* 2TK2. The effect of NaCl concentration from 0.1 to 5 M on cellulase activity was determined by varying the concentration of NaCl used in the assay buffer whilst keeping all other conditions constant [20, 21].

The effect of temperature on the stability of cellulose of *Haloarcula* 2TK2. Temperature profile from 25 to 65°C on the cellulase activity both for free and immobilized cells were monitored at optimal salt concentration and pH [22].

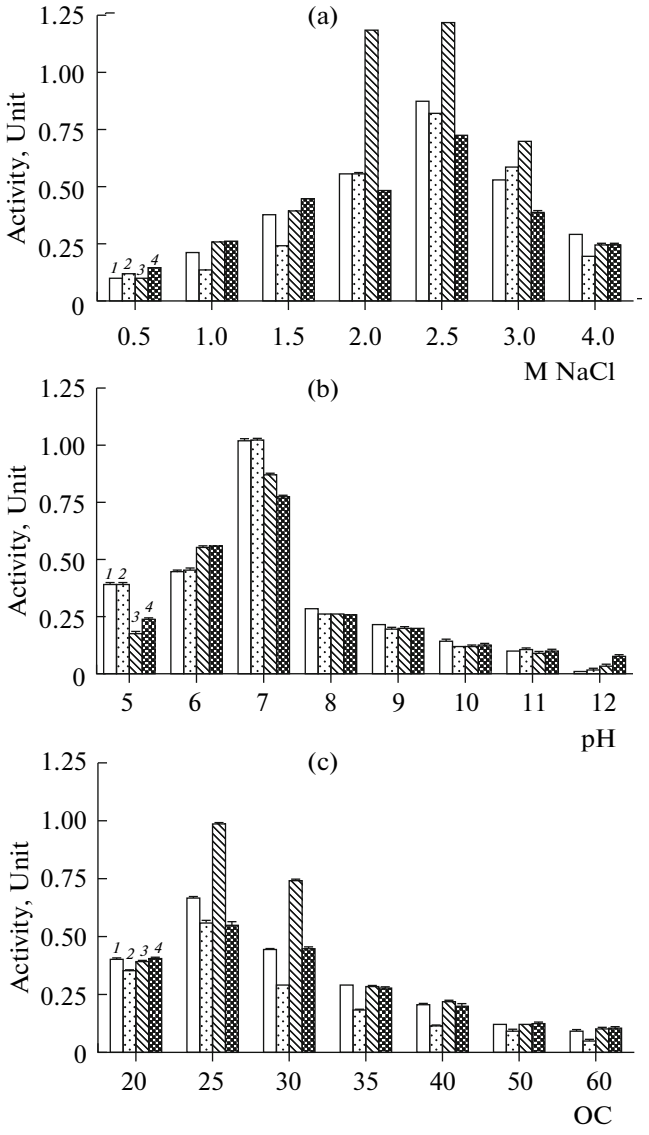
Storage stability. The storage stability of the immobilized *Haloarcula* 2TK2 cells on to alginate in nutrient poor liquid medium containing CM-cellulose as substrate (0.2 g) at 2–6°C was assessed by cellulase assays monitoring the production of glucose [13, 14].

RESULTS AND DISCUSSION

Although ability to digest cellulose is not common among halophils, some cellulase-producing microorganisms in hypersaline environments have been revealed [7, 9, 22, 23]. Birbir et al. [7] have showed the presence of cellulase-producing extremely halophilic archeael strains in Tuzkoy Salt Mine. Among them *Haloarcula* 2TK2 displayed the highest cellulase activity [24]. *Haloarcula* like other members of the halobactericeae family requires at least 1.5 M NaCl for growth but its optimal growth is obtained in 2.0–4.5 M NaCl. Enzymes of *Haloarcula* are of potential interest, for example, amylase produced by *Haloarcula* sp. functions optimally at 4.3 M salt at 50°C and is stable in benzene, toluene and chloroform [25].

The results obtained revealed that the time required for optimum cellulase formation for the *Haloarcula* 2TK2 cells was 7 days. Cellulase-producing strain preferred to hydrolyze CM-cellulose rather than avicel as substrate indicating that the strain is mainly endocellulase producer [26]. Nutrient poor medium as reaction medium has been used to improve the activity [15]. The effect of salt concentration of the enzyme working solution and pH on the cellulase activity of the free *Haloarcula* 2TK2 cells and the cells immobilized on polyacrylamide or alginate or agarose are presented in Figure a and b respectively and the effect of temperature on enzyme activity of free and immobilized cells are shown in Figure c.

Maximum cellulase activity was attained at 2.5 M NaCl both for the free and immobilized *Haloarcula* 2TK2 forms. Cellulase activity of the cells immobilized on alginate was significantly higher than that of free cells (Figure a) but for the cells immobilized to



The effect of salt concentration (a), pH (b) and temperature (c) on cellulase activity for free the *Haloarcula* 2TK2 (*1*) cells and cells immobilized on polyacrylamide (*2*) or alginate (*3*) or agarose (*4*).

polyacrylamide and agarose it was significantly lower when compared to free cells ($p < 0.001$). Cellulase that we obtained from *Haloarcula* 2TK2 is highly salt tolerant but its specific activity was lower when compared to the enzyme from halophilic bacteria *Salinivibrio* sp. strain NTU-05 described by Wang et al. [22] since they found the maximum specific activity at 0.1 M NaCl as 32.4 U/mg. The optimum pH for cellulase of *Haloarcula* 2TK2 was present over a broad range and showed maximum activity at pH 7.0 (Figure b). Cellulase activities of free cells and the cells immobilized on 3 supports were similar to each other and dramatically higher at pH 7.0 in comparison to other pH. The results in Figure c demonstrate that the optimal temperature of cellulase of *Haloarcula* 2TK2 was 25°C. Cells immobilized to alginate showed higher activity than those of free cells and the cells immobilized to polyacrylamide and agarose ($p < 0.001$). Polyacrylamide and agarose were not a good support for the cells perhaps due to the diffusional barriers set up by the gel against the transport of the substrates [27]. It is important to note that not only the isolated haloarchaeal strain was extremely salt tolerant but it was also capable of retaining stability at ambient pH and temperatures. A similar range of pH and optimal temperatures have been reported for halotolerant cellulase from *Salinivibrio* sp. strain NTU-05 by Wang et al. [22].

Since agarose and polyacrylamide were not good supports, the storage stability of *Haloarcula* 2TK2 cells was only performed for alginate in nutrient poor liquid medium containing CM-cellulose as substrate (0.2 g). The cells were stable for 3 weeks, activity could not be determined at the end of 4-th week. Nutrient poor medium had been used instead of nutrient rich to improve the activity [15]. The nutrient poor storage medium for the immobilized *Haloarcula* 2TK2 cells by itself could not ensure the viability of the cells and the cells became eventually non-viable. The results showed that calcium alginate immobilization is a promising method for the halophilic archaeal cells. We can propose that micro-environment cage created by the alginate gel seems to be beneficial for haloarchaeal cells to maintain their activity [28].

The large volumes of cellulosic waste generated from households and industry is the largest contributor of organic pollution in salt sources [29]. The presence of cellulose-consuming microorganisms in the mine, confirms the organic pollution. For the biological treatment of the industrial waste waters with salt concentrations up to 10%, such as the brines generated by the pickling industry, aerobic treatment systems have been developed. But the results were satisfactory only at salt concentrations up to around 6%, at higher salinities the systems perform less well [30]. Future application of the *Haloarcula* 2TK2 cells immobilized to alginate at higher salinities appears to be a feasible and useful method for the treatment of hypersaline waste water to remove organic carbon.

For the halobacterial cells immobilized on sodium alginate, high enzyme activities relative to the free cells were obtained. The results showed that calcium alginate immobilization is a promising method for halophilic archaeal cells. The use of whole cells instead of isolated cellulase may obviate costly and tedious purification procedures that may inactivate the enzyme. Although a detailed mechanism study was not performed on the whole cells, we can conclude that the microenvironment created by alginate cage seems to be beneficial for the archaeal cells to maintain their activity and stability.

In conclusion, biologic treatment of hypersaline waste by extremely halophilic archaeal strains immobilized to alginate may bring to reduce the environ-

mental impact and cost of waste disposal in hypersaline environments.

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