ASN, Vol 3, No 1, Pages 68-73, 2016

DE GRUYTER OPEN

Acta Scientifica Naturalis

Former Annual of Konstantin Preslavsky University – Chemistry, Physics, Biology, Geography
Journal homepage: http://www.shu.bg

Received: 30.10.2015
Accepted: 11.03.2016

Effects of nitrogen and carbon sources on the production of inulinase from strain *Bacillus* sp. SG113

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Abstract: ***The effects of the carbon and nitrogen substrates on the growth of Bacillus sp. SG113 strain were studied. The use of organic nitrogen sources (peptone, beef extract, yeast extract, casein) leads to rapid cellular growth and the best results for the Bacillus strain were obtained with casein hydrolysate. From the inorganic nitrogen sources studied, the $(NH_4)_2SO_4$ proved to be the best nitrogen source. Casein hydrolysate and $(NH_4)_2SO_4$ stimulated the invertase synthesis. In the presence of Jerusalem artichoke, onion and garlic extracts as carbon sources the strain synthesized from 6 to 10 times more inulinase.***

Keywords: inulin, inulinase, Bacillus

Introduction

Inulin is found in many plants of Compositae and Gramineae. It is accumulated in the underground roots and tubers of several plants including chicory (*Cichorium intybus, Cichorium endivia*), Jerusalem artichoke (*Helianthus tuberosus*), dandelion (*Taraxacum officinale*), and dahlia (*Dahlia pinnata*) where it acts as storage polysaccharide [1]. About 15% of flowering plant species store fructans as a reserve in at least one of their organs during their life cycle [2]. Inulin type fructans are consisting of linear chains of β (2, 1)-linked fructose units attached to a terminal sucrose molecule [3].

Inulinase (2,1-β-D-fructanohydrolase EC 3.2.1.7) hydrolyses inulin into practically pure fructose, being an excellent alternative for the production of fructose syrup [3, 4]. Fructose formation from inulin offers advantage as it involves only a single enzymatic step yielding up to 95% fructose [3, 5, 6].

Inulinases can be found in higher plants [3, 7] and microorganisms as filamentous fungi, yeasts and bacteria [3, 8 - 11]. Microbial inulinases are important industrial enzymes, which are usually inducible and extracellular. The production of fructose syrup from inulin or inulin rich materials is a major area of applications of inulinases. The main sources of inulin and oligofructose that are used in the food industry are chicory and Jerusalem artichoke. Microbial inulinases play an important role in the hydrolysis of inulin for the production of fructose syrups [12] and fructo-oligosaccharides [13, 14].

Inulinases from various organisms have been reported [15 – 19]. The most common of inulinase producers are of the genera *Aspergillus* and *Kluyveromyces* together with those of the genera *Pseudomonas*, *Xanthomonas*, *Penicillium*, *Chrysosporium* and *Bacillus* [20 – 21].

Materials and Methods

Bacterial strain isolation – The strain of *Bacillus* sp. SG113 was isolated from thermal water samples from the region of Rupite (Bulgaria) with temperature 68°C and pH 7.5. Five milliliters from samples were mixed with 5 ml isolation medium and incubated at 37°C and 50°C for 48 h for enrichment. After that suspensions

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DOI: 10.1515/asn-2016-0010

were heated at 80°C for 10 min because the methods for isolation of *Bacillus* strains are based on the resistance of their spores towards elevated temperatures. After chilling, 5 ml from these suspensions were mixed again with 5 ml isolation medium and cultivated 48 h at 37°C and 50°C. Then the samples were serially diluted prior to plating 30 µl on meat agar (1.5% (w/v), Oxoid) containing inulin for isolation of single colonies. Plates were incubated at 37°C and 50°C for 3 days. Pure colonies were obtained after repetitive dilutions in peptone-yeast extract medium with additional inulin (0.2%, w/v) as carbon source which helped in the selection of colonies having inulinase enzyme activity. The cultures were incubated at 37°C and 50°C for 3 days. The active cultures were transferred several times on the same medium, and then individual colonies were isolated. Several strains of *Bacillus* were thus isolated and were screened for exo-inulinase production. The strain designated as *Bacillus* spp. SG113 achieved high enzyme activity, and it was selected for further studies and stored at 4°C.

Fermentation medium – The mediun used for strain isolation, maintenance and enzyme production had the following composition (g/L): peptone (Oxoid, Basingstoke, UK) – 2.0; yeast extract (Oxoid) – 2.0; K2HPO4 – 0.4; MgSO4 – 0.08 and inulin (from chicory, Orafti HP, Beneo GmbH, Mannheim, Germany) – 2.0. Inulin was sterilized separately for 20 min at 110°C and added to the medium before inoculation. Sterile sodium carbonate was used to adjust the medium to pH 7.5 after autoclaving. Erlenmeyer flasks (300-ml volume) were charged with 50 ml of medium, inoculated (2%) with a culture previously incubated for 18 h, and incubated at 55°C in water-bath (Julabo SW22) shaker, for 24 h, at 200 rpm.

Effect of nitrogen sources – Effect of different nitrogen sources including peptone, beef extract, yeast extract, casein (organic N-sources) and $NaNO_3$, KNO_3, $(NH_4)_2SO_4$ and $(NH_4)H_2PO_4$ (inorganic N-sources) was studied by incorporating 0.4% (w/v) of each N- source in fermentation medium. From each experimental design, 5 flasks were inoculated and the results submitted to variance analysis for verification of statistical significance (Tukey's range test).

Effect of carbon sources – For the experiments on the effect of the carbon sources, the media were formulated with 2.0% of inulin, garlic and onion extracts, extracts from topinambour (Jerusalem artichoke) flours from tubers, and 0.2% of peptone and yeast extract, as nitrogen sources. From each experimental design, 5 flasks were inoculated and the results, submitted to variance analysis for verification of statistical significance (Tukey's range test).

Inulinase assay – The culture medium was centrifuged at 4000 rpm for 15 min and the supernatant was used as the inulinase source. Inulinase activity was measured by determination of the reducing sugars released from substrate inulin by DNS-method [22]. The reaction mixture contained 100 µl substrate inulin (from chicory, Orafti HP; 20 g/l, phosphate buffer pH 7.0) and 100 µl enzyme solution. After incubation at 60°C for 20 minutes the reaction was stopped by addition of 200 µl DNS-reagent. Reducing sugars were determined by calibration curve obtained using a standard solution of fructose (Scharlab S.L., Spain). One unit of inulinase activity was defined as the amount of enzyme that liberates one µmol of fructose per minute under the assay conditions.

Invertase assay – Invertase activity was determined under the conditions described above with the difference that saccharose (sucrose) (Scharlab S.L., Spain; 20 g/l in phosphate buffer, pH 7.0) was used as a substrate. A calibration curve was obtained using an equimolar standard solution of glucose and fructose. One unit of invertase activity was defined as the amount of enzyme that hydrolyzes 1 µmol of saccharose per minute under the assay conditions.

Determination of cell growth – The culture growth was determined by the absorbance at 650 nm

Preparation of onion (*Allium cepa)*, ***garlic (****Allium sativum****) and Jerusalem artichoke extracts*** – Two kilograms of the bulbs, tubers or cloves were peeled and chopped, then heated up to 90°C with 2 liters of distilled water. The slurry obtained was allowed to cool down and to stand for sedimentation of particulate matter. Afterwards, it was filtered through muslin cloth and the filtrate was used in media formulation.

Results and discussion

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DOI: 10.1515/asn-2016-0010

Effects of nitrogen sources

The strain's development was most effective when casein hydrolysate was used as organic nitrogen source. On the other hand, growth was minimal in the presence of peptone (Figure 1).

Considering the inulinase activity (Figure 2), the best enzyme yield was observed when both peptone and casein hydrolysate were used. Inorganic nitrogen sources such as $NaNO_3$ and KNO_3 proved to inhibit the growth (Figure 3). The best growth was observed when ammonium sulfate and ammonium dihydrogen phosphate were applied as sources of mineral nitrogen. However, the lowest enzyme quantity was synthesized when ammonium dihydrogen phosphate was applied (Figure 4), thus making it not suitable for synthesis.

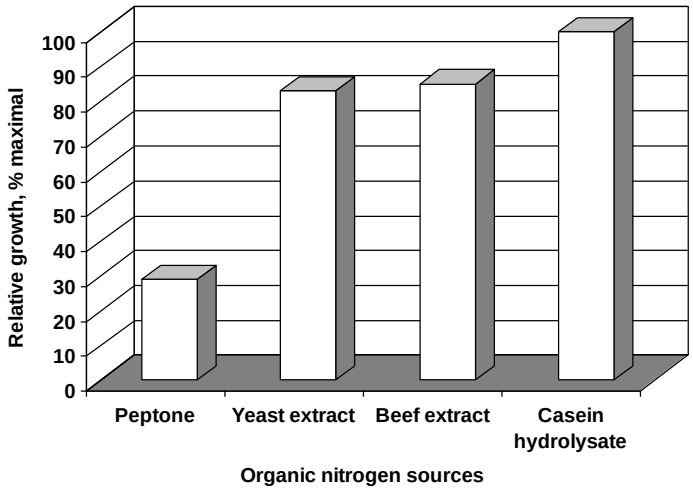

Figure 1. Effect of organic nitrogen sources on the growth of the *Bacillus* strain.

Active invertase in the presence of saccharose was not detected except for the cases when ammonium sulfate and casein hydrolysate (Figure 5) were applied, due to their ability to stimulate invertase production. Casein hudrolysate leads to up to 10 times more invertase activity in comparison with ammonium sulfate.

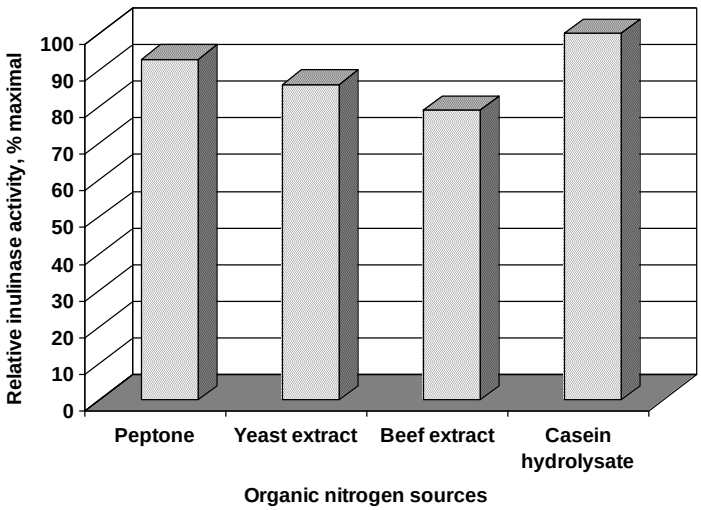

Figure 2. Effect of organic nitrogen sources on the inulinase synthesis.

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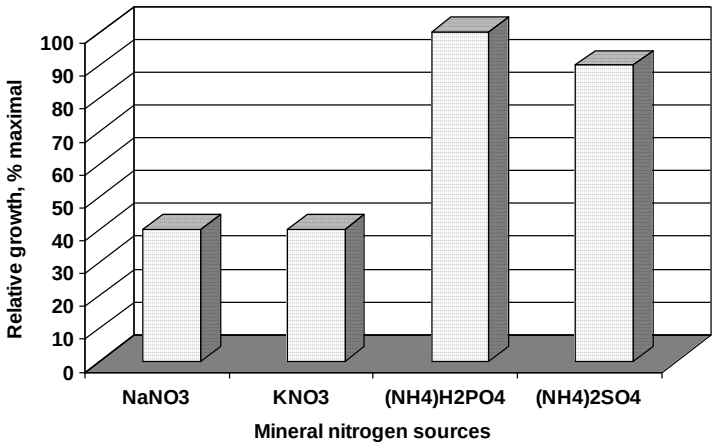

Figure 3. Effect of inorganic nitrogen sources on the growth of the *Bacillus* sp. SG113 strain.

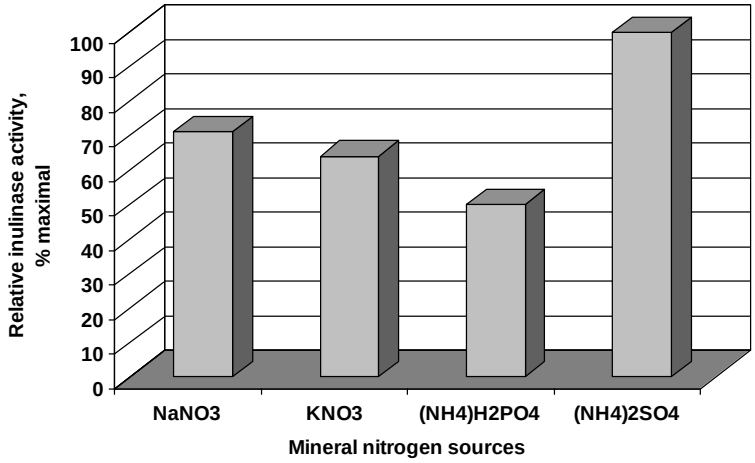

Figure 4. Effect of inorganic nitrogen sources on the inulinase synthesis.

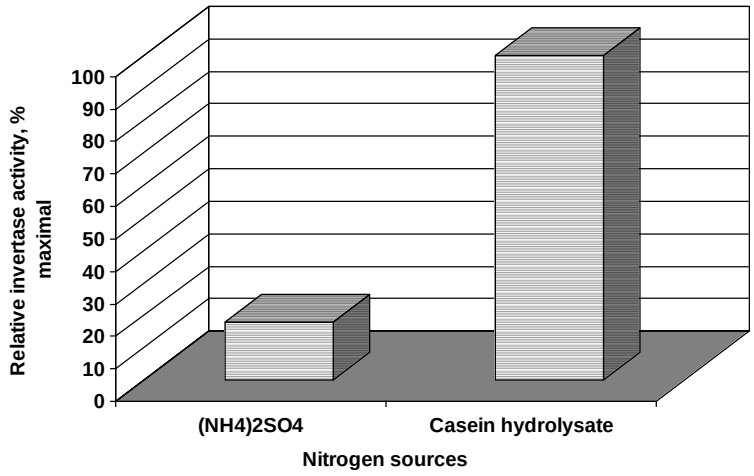

Figure 5. Effect of nitrogen sources on the invertase synthesis.

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Effects of carbon sources

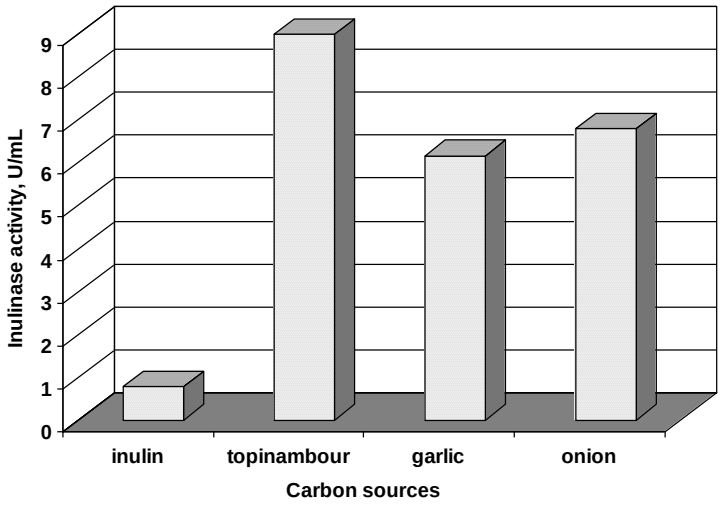

Figure 6. Effect of carbon sources on the inulinase synthesis.

Between 6 and 10 times less enzyme was synthesized in the presence of chicory innulin (Figure 6). The growth was also insignificant. Therefore, we can come to the conclusion that the onion, garlic and Jerusalem artichoke extracts contain oligofructoses and innulin with lower DP in comparison with chicory innulin Orafti HP with DP > 25 (up to 64) according to the information provided by the supplier and that these oligofructoses could stimulate both growth and inulinase synthesis.

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DOI: 10.1515/asn-2016-0010