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Inulinase Synthesis from a Mesophilic Culture in Submerged Cultivation

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Abstract

A newly isolated mesophilic bacterial strain from dahlia rhizosphere, identified as *Staphylococcus* sp. and designated as RRL-M-5, was evaluated for inulinase synthesis in submerged cultivation using different carbon sources individually or in combination with inulin as substrate. Inulin appeared as the most favorable substrate at a 0.5–1.0% concentration. Media pH influenced the enzyme synthesis by the bacterial strain, which showed an optimum pH at 7.0–7.5. Supplementation of fermentation medium with external nitrogen (organic and inorganic) showed a mixed impact on bacterial activity of enzyme synthesis. The addition of soybean meal and corn steep solid resulted in about an 11% increase in enzyme titers. Among inorganic nitrogen sources, ammonium sulfate was found to be the most suitable. Maximum enzyme activities (446 U/L) were obtained when fermentation was carried out at 30°C for 24 h with a medium containing 0.5% inulin as a sole carbon source and 0.5% soybean meal as the nitrogen source. Bacterial inulinase could be a good source for the hydrolysis of inulin for the production of D-fructose.

Index Entries: Inulinase; mesophilic bacteria; inulin; submerged fermentation; carbon and nitrogen sources.

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Introduction

Inulin, a polyfructan, present as a reserve carbohydrate in the roots and tubers of plants, such as Jerusalem artichoke, chicory and dahlia, consists of linear chains of β-2,1 linked polyfructose, terminated by a glucose residue attached through a sucrose type linkage *(1,2)*. Recently, interest in the use of inulin sources as the potential feedstock for the production of value-added chemicals such as fuel ethanol has grown *(3–6)*.

Microbial inulinases (2,1-β-D-fructan fructanohydrolase, EC.3.2.1.7) are an important class of industrial enzymes that have recently gained much attention *(2)*. These are usually inducible and exoacting enzymes, which catalyze the hydrolysis of inulin by splitting off terminal fructosyl units (D-fructose) *(1,2)*. Hydrolysis of inulin using inulinases leads to the formation of D-fructose, which has gained an important place in human diets today.

Microbial inulinases could be produced from a host of organisms, including fungi, yeast, and bacteria. Several species of yeast, such as *Kluyveromyces fragilis (7)*, *Kluyveromyces marxianus (8,9)*, *Candida kefyr (10)*, and *Debaryomyces cantarellii (11)*, have generally been considered as the preferred choice for inulinase production because enzyme complex from them can hydrolyze both inulin and sucrose. However, fungi such as *Penicillium* sp. *(12)*, *Aspergillus niger (13)*, *Aspergillus ficuum (14)*, *Fusarium oxysporum (15)*, and *Chrysoporium pannorum (16)*, and bacterial species such as *Clostridium acetobutylicum (17)*, *Pseudomonas* sp. *(18)*, and *Staphylococcus* sp. *(19)* have also been used. A bacterial strain, YLW, isolated from rotting dahlia tubers showed inulinolytic activity in association with the formation of difructofuranose anhydride *(19)*. Bacterial inulinase could also be promising for industrial applications.

In this article, we describe the inulinase activity from a newly isolated bacterial strain of *Staphylococcus* sp. RRL-M-5, grown on inulin in submerged fermentation.

Materials and Methods

Microorganism

A newly isolated bacterial culture of *Staphylococcus* sp., designated as RRL-M-5, was used. The culture was maintained on yeast extract-peptone-inulin agar, subcultured fortnightly, and stored at 4°C for routine use. For long-term preservation, it was stored in 50% glycerol solution at –70°C.

Inoculum

Inoculum was prepared in a medium containing the following: 10 g/L of yeast extract, 20 g/L of casein peptone, and 10 g/L of inulin. A loopful of cells from a stock culture was transferred into 20 mL of sterile medium held in a 250-mL Erlenmeyer flask. The flask was incubated on a rotary shaker (250 rpm) at 30°C for 24 h.

Fermentation

The medium used for fermentation was as follows: 10 g/L of casein peptone, 5 g/L of yeast extract, 5 g/L of inulin, and 5 g/L of NaCl in distilled water. The pH was adjusted between 7.2 and 7.4 with 1 *N* NaOH. Twenty milliliters of medium were placed in each Erlenmeyer flask (250 mL) and autoclaved at 121°C for 20 min. Inoculum was used at a 5% (v/v) level, which resulted from a 24-h-old culture. The flasks were incubated on a rotary shaker (250 rpm) at 30°C for the desired length of time. Samples, as whole flasks in duplicate, were withdrawn at stipulated intervals.

Enzyme Assay

Enzyme assay was carried out by the following procedure. Fermented broth was centrifuged at 10,000 rpm for 10 min at room temperature. The supernatant obtained was used as the source of crude enzyme. To 2 mL of 0.2% inulin in 0.01 mol/L of acetate buffer (pH 4.6) were added, 0.5 mL of supernatant culture, diluted, if necessary, and the mixture was incubated at 50°C for 20 min. After incubation, the tubes were kept in a boiling water bath for 10 min to inactivate the enzyme and were then cooled to room temperature. The reaction mixture was assayed for reducing sugars as fructose by the DNS method *(20)* by reading the absorbance at 575 nm on a spectrophotometer (Shimadzu 160A, Japan). A calibration curve was prepared with fructose solution of known strength. Blanks were run simultaneously with the enzyme and substrate solutions. One unit of inulinase activity was defined as the amount of enzyme that produced 1 µmol of fructose under the assay conditions.

Assay Methods

Cell growth of the culture was measured by determining the optical density of fermenting broth at 540 nm using a spectrophotometer (Shimadzu 160A). Soluble protein was determined in the culture supernatant by the method of Lowry et al. *(21)* using bovine serum albumin (Sigma, St. Louis, MO) as the standard. All the results reported are the average of three sets of the experiments.

Results and Discussion

Fermentation Profile of the Strain RRL-M-5

Fermentation was carried out for 72 h; the results are shown in Fig. 1. Figure 1 shows that cell growth continued until 18 h when maximum biomass concentration was observed. Inulinase synthesis, however, continued beyond this period, and maximum extracellular inulinase accumulation (400 U/L) in the medium was observed after 24 h of fermentation.

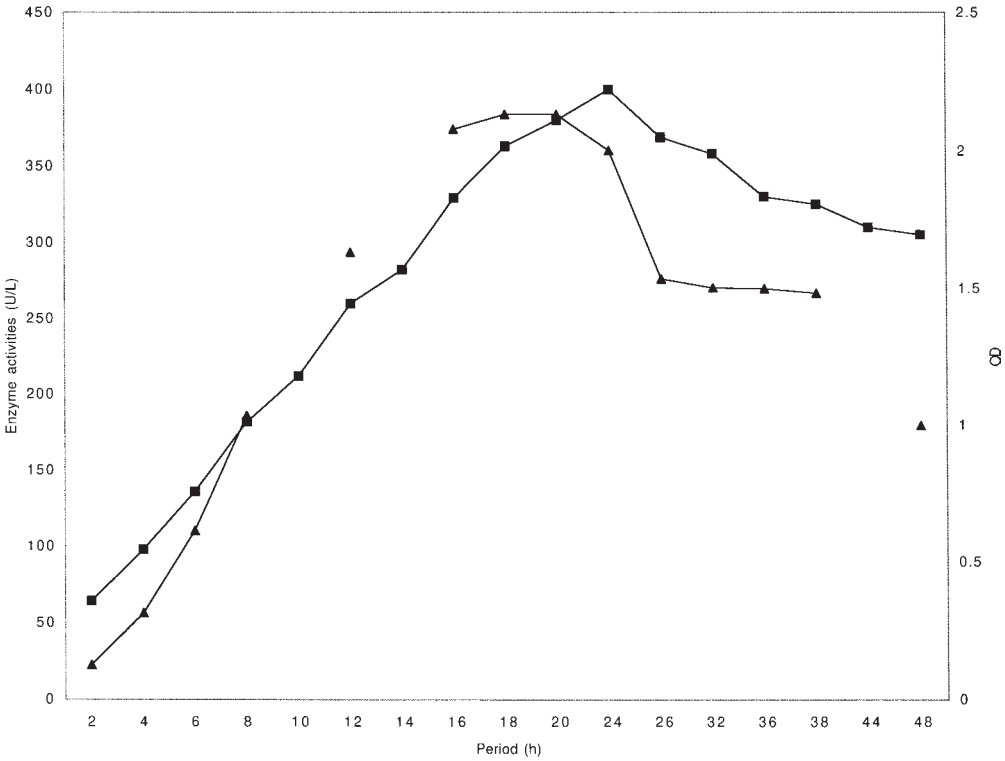

Fig. 1. Growth and fermentation profile of the bacterial strain *Staphylococcus* sp. RRL-M-5. (—■—), Inulinase; (—▲—), biomass.

Effect of Media pH

When fermentation was carried out with media having different initial pH values, namely, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0, there were variations in the microbial activity of inulinase synthesis. Media pH has a profound impact on the optimal activity of a microbial strain, and it is necessary to optimize the best conditions for each strain. In the present study, there was an ascending order of enzyme activity with an increase of pH from 5.0 to 7.0, when it attained its peak (412 U/L inulinase after 24 h; Fig. 2). Subsequently, enzyme titers decreased with an increase of media pH. By contrast, Nakamura et al. *(12)* found higher inulinase synthesis from a fungal strain of *Penicillium* sp. TN88 when the pH of fermentation medium was highly acidic, i.e., 3.0–4.0. For a yeast strain of *K. marxianus* NCYC 587, the best pH for highest inulinase activity was 5.0 *(8)*.

During the course of fermentation, there was a fall in the pH, which was 4.7 as the lowest (in case of medium with an initial pH of 5.0) and 7.6 as the highest final pH (with medium having an initial pH of 8.0). For media with an initial pH of 6.5–7.5, the final pH was 5.9–6.6 (data not shown).

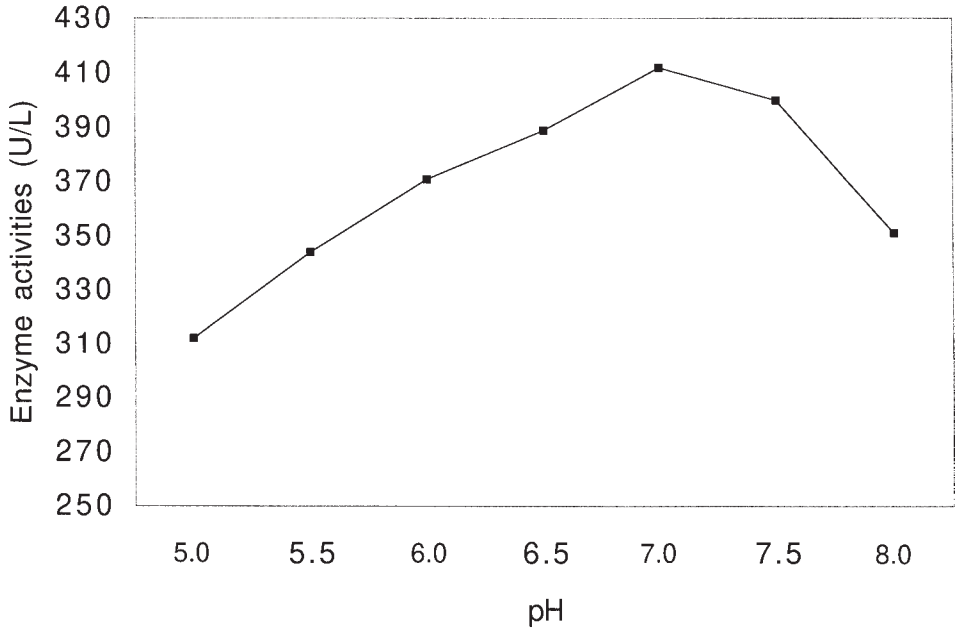

Fig. 2. Effect of initial pH of the medium on inulinase synthesis by *Staphylococcus* sp. RRL-M-5 in 24 h.

Effect of Different Carbon Sources

Six different carbon sources were tested as substrate for the production of inulinase: sucrose, fructose, glucose, maltose, inulin, and starch at 0.5% (w/v) concentration. Fermentation was carried out for 72 h and samples were withdrawn each 12 or 24 h. For all the carbon sources tested, maximum inulinase activity was observed after 24 h of fermentation (Fig. 3). Inulin was found to be the best carbon source for inulinase production, which after 24 h of fermentation resulted in 402 U/L of enzyme activity, but glucose and fructose also supported the bacterial activity of inulinase synthesis reasonably well. The enzyme activities obtained were 381 and 350 U/L for glucose and fructose, respectively. Thus, apparently the enzyme synthesis by the bacterial strain is constitutive and is not depressed. Table 1 shows the soluble protein contents during the course of 72 h of fermentation when different carbon sources were used as the substrate.

Subsequently, studies were performed to determine the effect of inulin concentration on inulinase production by the bacterial strain. Media were prepared as we previously described with the only difference being that different concentrations (0.5, 1.0, 1.5, 2.0, 5.0% [w/v]) of inulin were used. Fermentation was carried out for 24 h; the results are shown in Fig. 4. Whereas there was a marginal increase in enzyme production with a 1% inulin concentration in comparison with 0.5%, higher concentrations were apparently not useful. With a 5% inulin concentration, the rate of enzyme synthesis was much slower than that with lower concentrations

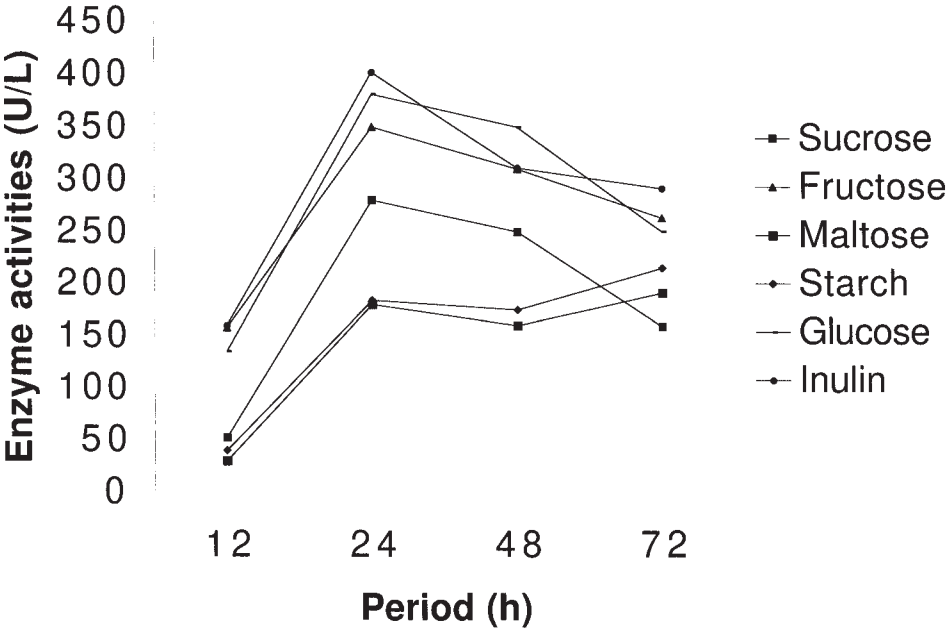

Fig. 3. Effect of different carbon sources (0.5%) as substrate on inulinase synthesis by *Staphylococcus* sp. RRL-M-5.

Table 1
Concentration of Soluble Proteins
in Fermented Broth with Different Carbon Sources (mg/mL)

Carbon source (0.5%)	Fermentation period (h)			
	12	24	48	72
Sucrose	5.84	8.71	8.98	10.26
Fructose	8.11	12.60	13.40	12.91
Maltose	5.21	7.72	8.62	9.63
Glucose	6.13	11.40	12.90	12.80
Inulin	6.21	13.70	13.40	13.00
Starch	5.92	8.64	8.40	7.90

(0.5 and 1.5%), and it took 48 h in this case to reach the level of enzyme produced in 24 h with lower inulin concentrations (data not shown).

Another experiment was carried out to analyze the effect of different carbon sources in combination with inulin for the production of inulinase. The carbon sources tested were sucrose, fructose, maltose, starch, and glucose at a concentration of 0.5% each of inulin and experimental compound. None of the tested combinations showed any stimulating effect on enzyme production in comparison to that when inulin was used alone (Fig. 5). As is apparent in Fig. 5, in most of the cases, the enzyme production pattern was more or less similar to the individual performance (cf. Fig. 3). Thus, in

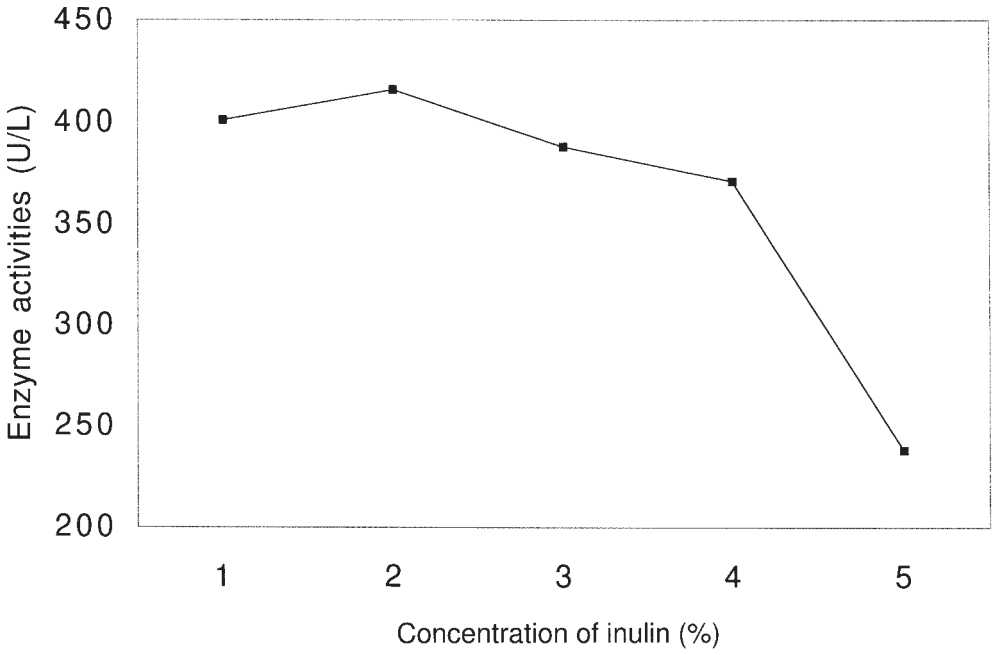

Fig. 4. Effect of concentration of inulin on inulinase synthesis by *Staphylococcus* sp. RRL-M-5 in 24 h.

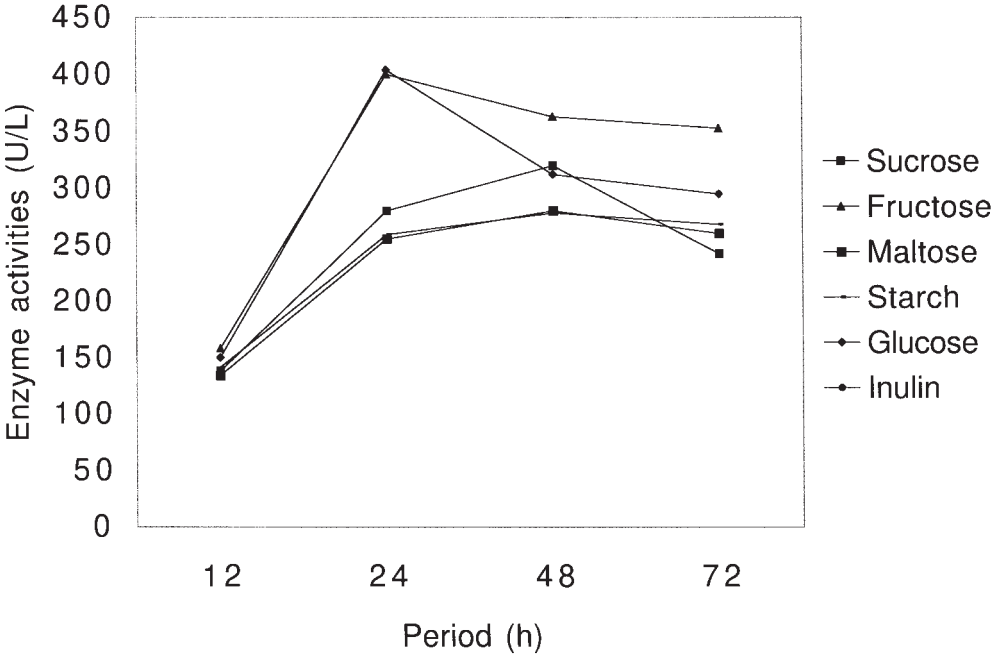

Fig. 5. Effect of different carbon sources (0.5%) in combination with inulin (0.5%) on inulinase synthesis by *Staphylococcus* sp. RRL-M-5.

Table 2
Concentration of Soluble Proteins
in Fermented Broth with Different Combined Substrates (mg/mL)

Carbon source (0.5%) (+0.5% inulin)	Fermentation period (h)			
	12	24	48	72
Sucrose	6.11	9.76	12.06	12.98
Fructose	7.70	11.70	13.90	13.30
Maltose	5.86	7.55	9.21	12.80
Glucose	7.10	11.20	13.70	12.00
Inulin	7.40	12.30	14.30	14.00
Starch	6.20	7.80	12.90	13.70

this experiment too, the enzyme synthesis appeared constitutive. Table 2 shows the soluble protein contents during the course of 72 h of fermentation with different substrate combinations.

Inulinase production using different substrates has been reported by several investigators. Passador-Gurgel et al. *(22)* reported varying amounts of inulinase synthesis (0.9–2.6 U/mL) by several strains of yeasts on different carbon sources including inulin. A patent was obtained for the development of a medium for inulinase synthesis in which two soil isolates produced 3.7 and 2.5 U/mL of inulinase when cultivated on inulin *(23)*. Kim et al. *(18)* reported 97 U/g of inulinase from a bacterial strain of *Pseudomonas* sp. Poorna and Kulkarni *(24)* conducted a study on the use of various carbon sources, singly or in combination, for inulinase production. Results suggested that inulin was the most favored carbon source. Many other investigators have also observed higher inulinase activity with the medium containing inulin as the sole carbon source *(16)*. Fontana et al. *(25)* reported that chemically modified inulins could also be used as the substrate for inulin production.

Effect of Nitrogen Sources

To determine the effect of supplementation of external nitrogen (organic and inorganic nature), different substances, such as corn steep liquor, corn steep solid, and soybean meal (organic nitrogen), and sodium nitrite, ammonium chloride, ammonium sulfate, ammonium dihydrogen phosphate, and diammonium hydrogen phosphate (inorganic nitrogen), were added at 0.5% (w/v) concentrations in the culture medium. Fermentation was carried out for 72 h and samples were withdrawn each 12 or 24 h.

As seen in Fig. 6, all the organic nitrogenous substances resulted in an increase in inulinase production. Maximum enzyme production in all cases was reached in 24 h. The order of effectiveness was soybean meal > corn steep solid > corn steep liquid > control (401 U/L; data not shown). Soybean meal and corn steep solid exerted a similar impact, resulting in 446 and 445 U/L of enzyme activities, respectively (Fig. 6). As seen in Table 3, the

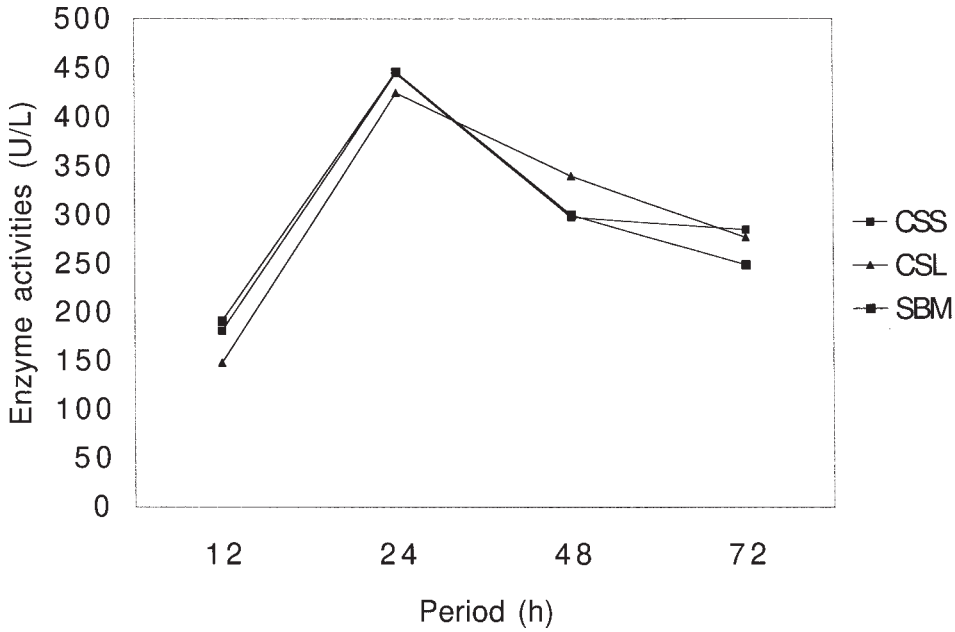

Fig. 6. Effect of different organic nitrogen sources (0.5%) on inulinase synthesis by *Staphylococcus* sp. RRL-M-5.

Table 3
Concentration of Soluble Proteins
in Fermented Broth with Different Organic Nitrogen Sources (mg/mL)

Organic nitrogen source (0.5%)	Fermentation period (h)			
	12	24	48	72
Corn steep solid	3.98	6.80	10.10	6.70
Corn steep liquor	3.91	5.46	7.60	5.10
Soybean meal	3.96	6.39	7.98	4.85

contents of soluble protein in the samples with organic nitrogen greatly differed from that of the control.

Figure 7 shows the results of the effect of supplementation of inorganic N on inulinase synthesis by the bacterial strain RRL-M-5. In these studies also, maximum enzyme production in all cases was reached in 24 h. While sodium nitrate showed a retarding impact, resulting in decreased enzyme activities, compounds with ammonical nitrogen had either a marginal impact or increased the enzyme titers. Interestingly, diammonium phosphate resulted in higher enzyme activities (432 U/L) than monoammonium phosphate (400 U/L). In the latter case, the activities were equivalent to the control. Also, in these studies, the soluble protein concentrations greatly differed from that of the control (Table 4).

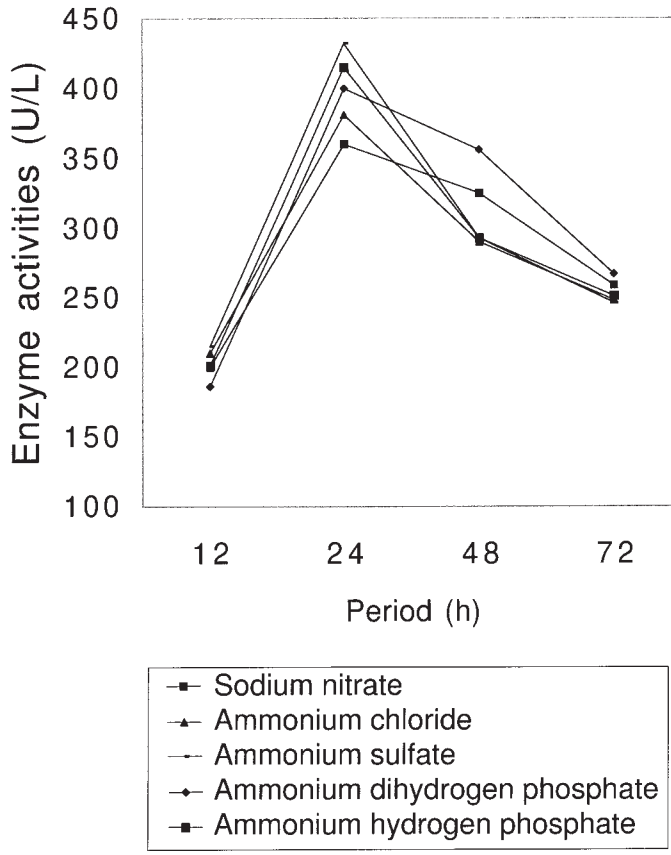

Fig. 7. Effect of different inorganic nitrogen sources (0.5%) on inulinase synthesis by *Staphylococcus* sp. RRL-M-5.

Table 4
Concentration of Soluble Proteins
in Fermented Broth with Different Inorganic Nitrogen Sources (mg/mL)

Inorganic nitrogen source (0.5%)	Fermentation period (h)			
	12	24	48	72
$NaNO_3$	2.38	3.18	4.18	3.25
NH_4Cl	2.29	3.09	4.60	2.88
$(NH_4)_2SO_4$	3.93	6.16	4.31	2.50
$(NH_4)_2HPO_4$	2.54	2.91	3.96	2.99
$NH_4H_2PO_4$	2.30	2.83	4.25	2.11

Supplementation of fermentation medium with ammonium compounds has also been found useful for inulinase synthesis by other investigators. Nakamura et al. *(12)* reported reasonably high production of inulinase by *Penicillium* sp. TN88 with ammonium chloride. Xiao et al. *(16)*

reported that the addition of ammonium sulfate, ammonium hydrogen phosphate, and ammonium chloride gave higher inulinase yields with a 0.02 *M* concentration. Our findings are similar.

Conclusions

In this study, the bacterial strain of *Staphylococcus* sp. was found to be capable of utilizing a wide range of carbon sources for inulinase synthesis. The enzyme synthesis by the strain appeared constitutive. The production of normally inducible catabolic enzymes in microorganisms limits the choice of fermentation substrates to those that are capable of regulating the enzyme formation in the strain or that at least play a role in this regulation. From an industrial point of view, it would be desirable to use strains without inducer requirements and to develop them to use a large number of substrates including lower-priced materials. Thus, this strain could be a useful bacterial source of inulinase and should be explored further for possible commercial exploitation with some genetic improvements to enhance yields.

Acknowledgments

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