Comparative Study for immobilization enzyme Inulinase by using different methods and carriers

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ABSTRACT

Enzyme Inulinase from yeast Kluyveromyces marxianus and the fungi Aspergillus awamori, had been immobilized by Adsorption, Glutraldehyde, Modified glutraldehyde methods using four carriers AV-16-GS , VION KN-1,AV-17-2P and AM-21-A.The degree of saving catalytic activity immobilized inulinase for free enzyme activity were 30 ,60 ,85 % for the three methods respectively .The result of degree maintain specific activity of native enzyme were 75.5%, 27.5%, 17.8%, and 14.5% for the carriers respectively.

INTRODUCTION

Application of hydrolytic enzymes in the food industry and medicine has helped to significantly improve many existing means food and drugs. Getting fructose from inulin-containing plants cultivated by Enzymatic hydrolysis of poly fructosides is an integral part of modern pharmaceutical and food industry. Research in the field of chemical engineering and enzymology are the most important areas of modern biotechnology. Immobilized enzymes are bound to carriers by chemical or physical interactions become important for industrial technology feature - they are easily separated from the products catalyzed reaction, which allows their use in continuous or periodic processes. Heterogeneous enzyme samples can be regarded as a very Find model bioactive systems operating in vivo within the membrane or on the surface thereof. In medicine, along with immobilization increased stability facilitates the fixation of certain enzymes in the parts and promotes prolonged local act medications.

Special attention researchers paid to the problem of selection and modification of carriers the development of methods immobilization, the study of kinetic aspects of heterogeneous catalysis enzyme preparations. It is now one of the most common products functional food is fructose. Fructose syrups are be used for the prevention of diabetes, caries and obesity. Currently, the glucose-fructose syrups produced three ways: 1) hydrolysis of sucrose and inulin 2) isomerisation of glucose, 3) a three-step enzymatic from starch. Hydrolysis of sucrose fructose and glucose carried out under the action of β-fructofuranosidase, ensures the formation of invert sugars, fructose and glucose, which are in an equimolar ratio [1]. Getting fructose from inulin possible by acid or enzymatic hydrolysis. In acid hydrolysis as catalyst include mineral or organic acids: sulfuric acid,hydrochloric acid, oxalic acid, citric acid, etc., which may enter into the final products. Moreover, with a need to apply a special acid-resistant equipment. [2] showed that hydrolysis inulin-containing materials with phosphoric acid of side The product is isolated coloring compounds that worsen marketability of fructose [3]. Inulinase (2,1-β-D-fruktanfruktanogidrolaza, EC 3.2.1.7) specifically hydrolyzes the β-2,1-bonds and inulin to fructose fructooligosaccharides under milder conditions compared to the acid hydrolysis, which requires considerable concentration of hydrogen ions, high temperature and the use of special acid-equipment. At the end of XX century has just started accumulating data on the nature inulinase synthesis of various microorganisms [4-8], the search for the most promising its producers [9,10], depending on the establishment catalytic activity of the enzyme on the temperature, concentration, substrate, pH environment present in the reaction mixtures of different activators and inhibitors [11-14]. Were held research hydrolysis process chemically pure inulin and fructo oligosaccharides contained in Jerusalem artichoke extracts [15] chicory [16] and asparagus [17].

Analysis of literature data shows that :

- In comparison with the usual sugar, fructose has a more pleasant taste, "honey" instead of "cloying." It is 60-70% sweeter than sucrose, which helps to reduce calorie product, and therefore, it is important to in terms of nutrition supply [18, 19].
- Fructose, not like glucose, or sugar can be consumed by patients diabetes
- It is much less harmful to teeth than sugar
- In a mixture with glucose, fructose does not crystallize (not candy), which important in the manufacture of ice cream, pastry, etc.;
- It has good solubility and low viscosity;
- Fructose has on the human body toning effect enhances the activity of bifidobacteria
- It has the property underscores flavors and can form new aromatic substances
- Fructose contributes to the removal of ethanol from the blood of patients alcoholism
- Improves iron absorption in children
- Lowers blood cholesterol levels [20];
- Fructose is more stable than many other fructo oligosaccharides [18];
- Fructose syrups at 40% less sugar [21].

In this regard, the purpose of research was to develop, based on inulinase immobilized heterogeneous biocatalyst reaction hydrolysis contained in the extract of inulin and pick Optimal conditions of its operation. To achieve this purpose it was necessary to solve the following problems Identify the optimal synthetic polymer carrier for immobilization of inulinase and the most promising method for enzyme immobilization.

MATERIAL AND METHODS

The enzyme inulinase isolated from yeast Kluyveromyces marxianus and micro mycetomas Aspergillus awamori. Features culture producers, as well as methods isolation and purification of enzymes described in detail [22, 23]. The preparation of ion-exchange resins to immobilize performed by ion exchangers, air-conditioning and converting them into the desired ion exchange form [23, 24]. For immobilization sorptive carrier 5g allowed to overnight at room temperature in 50 ml of acetate buffer (pH 4.5). Go to resin slurry was added 5 ml of enzyme in a flask and stirred with using an electric stirrer for 1.5hours at 25 º C, more centrifuged for 5 minutes at250 g.

For covalent immobilization of glutaraldehyde using 2.5 g of resin was left for over night at room temperature 20% -solution of glutaraldehyde at a 1:1 ratio with a closed stopper. Thereafter, the carrier was washed with distilled water, was added 2.5 ml enzyme solution (pH 4.5) and incubated for days in a closed vessel. The immobilized preparation obtained in the course of the sorption and covalent immobilization was washed with acetate buffer until no in lavage protein. Monitoring was carried out on an SF- 46 at λ = 280 nm.

In order to enhance the catalytic activity of inulinase we used a modified glutaraldehyde method. The experiment was conducted according to the following procedure: To 2 g of anion exchanger was added 45 ml of a 4% solution of succinic anhydride in chloroform and refluxed for 4.5 hours, the mixture was further incubated for another 16 hours at room temperature. The resin was washed chloroform and air dried, and then 10 ml of thionyl chloride and refluxed for 30 minutes. To the washed with toluene and dried carrier was added dropwise 20 ml of ethylenediamine, maintaining the temperature at 20 ° C, and the mixture stood 20 hours, it was washed 10 times with distilled water, 5 times - ammonia solution (3%) and then - water. To 10 ml of ion exchanger solution of glutaraldehyde (2%) and stirred

with a magnetic stirrer for 3 hours at 50 ° C. The resin was separated from the solution and washed.

To determine the catalytic activity of free and immobilized inulinase inulin hydrolysis reaction was performed on a setup consisting of a thermostat connected with two fermenters placed on a magnetic stirrer. One fermenter was incubated with inulinase substrate for 20 min at pH 4.7 and 50 ° C, the other - instead of the enzyme solution was placed the same amount of acetate buffer, pH 4.7. When analyzing the catalytic activity of the immobilized enzyme preparation of substrate hydrolysis temperature was 70 ° C and pH 4.5.

RESULTS AND DISCUSSION

Currently under active development related issues inulinase immobilization on different media. The enzyme was successfully immobilized on a porous glass, bentonite, included alginate hydrogels [25]. There is evidence enough effective adsorption This enzyme is isolated from Escherichia coli, an anion exchange resin Duolite A 568 and Amberlite 94 S [26]. A.K. Gupta et. al. showed that inulinase Included in the polyacrylamide gel retains more than 45% of the initial Activity at 45 ° C [27]. It is proved that the use of pure crosslinked inulin and fully methylated inulin not are substrates for the inulinase as a carrier when it is immobilization is impractical due to low activity resulting biocatalysts [3]. Carried inulinase adsorption of Kluyveromyces marxianus on the anion exchanger Streamline DEAE [28]. Based on immobilized fructose dehydrogenase and inulinase was created amperometric dvufermentny biosensor for determination of inulin in food [29].

However, despite the fact that a large number of described immobilized on different carriers enzyme preparations, the choice polyelectrolyte and a method of protein binding to the matrix ion exchanger remains largely empirical. Therefore, the first series of experiments choosing the best medium for heterogeneous inulinase

preparation. It is shown that the adsorptive immobilization inulinase of Kluyveromyces marxianus on synthetic ion-exchangers AV-17-2P, VION KN-1, AB-16-GS, AM-21A and degree maintain specific activity of native enzyme were75.5%, 27.5%, and14.5% respectively .(Fig. 1) [22,30, 31].

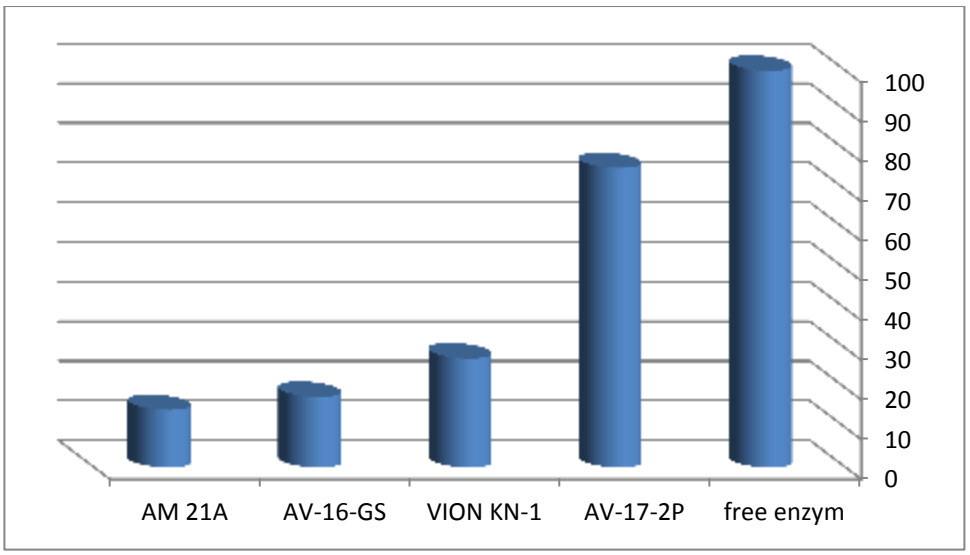

Fig. 1. Percentage retention of activity of the immobilized inulinase Kluyveromyces marxianus

In order to determine a more promising method of producing heterogeneous enzyme immobilization was carried out for Inulinase of Aspergillus awamori on a macroporous anion exchange resin AB-16-GS adsorption, glutaraldehyde and the modified glutaraldehyde method (Table 1) [32].

The method of immobilization	The degree of saving catalytic activity immobilized inulinase % for free enzyme activity
Adsorption	30
Glutraldehyde	60
Modified glutraldehyde	85

Table(1) Characteristics of enzyme inulinase immobilized on AB-16-GS various methods

This result, mean that is in spite of the fact that the modified glutaraldehyde method of immobilization of Inulinase by ion exchanger AB-16-GS is the most difficult and time consuming, it also seems to be the most promising method for the industry, as it allows to receive enzymes that retain up to 85% of the catalytic ability free enzyme and having optimum temperature operation 75 ° C. With the conversion curves of V S in the coordinates Lineweaver-Burk, Hanes and Idi-Hofstee were determined Km and Vmax hydrolysis of inulin and free enzyme preparation apparent Vmax 'and Km' for immobilized samples. It is shown that Immobilization leads to an increase in the values of the Michaelis constant and reducing the maximum rate of reaction compared to the native enzyme (Table 2).

The enzyme preparation	Km (Km'), 10-4 mol / l	Vmax (Vmax '), mol · mg / min
Free inulinase	2.2	102
Inulinase immobilized on AB-16-GS	5.4	19
Inulinase immobilized an AM-21-A	6.1	15
Inulinase immobilized by VION KN-1	3.3	35
Inulinase immobilized by AV-17-2P	3.2	59

Table(2) Values of Km '**and** Vmax' for inulin hydrolysis inulinase immobilized on different carriers

Km and Vmax values differ from enzymes isolated from various sources. In inulinase of Penicillium janczemskii Km towards inulin has a value of 4.3 x 10-4 mol / l of Kluyveromyces marxianus and Bacillus licheniformis - 3,1 · 10-4 mol / L and 4.4 x 10-4 mol / L, respectively [11, 33]. Km value for inulinase of Aspergillus awamori is 1.5 x 10-4 mol / l, the maximum reaction rate of hydrolysis of the inulin is 172 mol · mg / min [6].

Comparative analysis of the values of basic kinetic parameters the hydrolysis of inulin immobilized preparations of inulinase Kluyveromyces marxianus allows us to conclude that the greatest catalytic activity is shown by inulinase adsorbed on macroporous anion exchange resin AV-17-2P. Apparently, the enzyme sufficiently tightly bound to the matrix of the carrier significantly without changing its catalytically active conformation that allows us to consider AV-17-2P promising for further research in order to obtain stable and highly heterogeneous biocatalysts based on immobilized Inulinase.

By increasing the flow velocity of the substrate catalytic activity immobilized Inulinase reduced. This phenomenon can be explained by follows. It is known that in the catalysis reaction If necessary the immobilized enzyme to the substrate molecule came up to the surface of the support matrix and diffuse into it. At low speeds the flow diffusion difficulties are overcome by increasing the reaction time of the enzyme with a substrate and creates favorable conditions for the emergence of an enzyme-substrate complex. Accordingly, by increasing the flow velocity of the substrate the contact time decreases inulin molecules with molecule Inulinase and hence minimal amount of fructose formed [34].

CONCLUSION

It is shown that the adsorptive immobilization of inulinase Kluyveromyces marxianus on synthetic ion-exchangers AV-17-2P, , VION KN-1, AB-16-GS, AM, 21A degree of conservation the specific activity of the native enzyme is respectively 75.5%, 27.5%, 17.8%, and 14.5%. Proved that despite the fact that the modified glutaraldehyde inulinase immobilization method is the most difficult and time consuming, it also seems to be the most promising method for the industry, as it allows you to receive enzymes that retain up to 85% of the catalytic ability free enzyme and having optimum temperature operation 75 ° C.Comparative analysis of the values of basic kinetic parameters the hydrolysis of inulin immobilized preparations of inulinase Kluyveromyces marxianus allows us to conclude that the greatest catalytic activity is shown by inulinase adsorbed on macroporous anion exchange resin AV-17-2P. With a further extension of this research on the topic and scale experiments to pilot plant level and experienced enterprises, may be able to offer innovative heterogeneous inulinase preparation for fructose as one of the most important functional food products for deterministic groups, as well as preventive medicines for diseases such as diabetes, obesity and tooth decay.

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ملخص البحث

تم تثبيت (ربط) انزيم الانيليز (**Inulinase**) المستخلص من الخميرة **Kluyveromyces marxianus** والفطر **Aspergillus awamori** بثلاثة طرق هي الادمصاص ، الكلوترالديهايد وطريقة الكلوترالديهايد المعدلة بأستخدام اربعة انواع من الحوامل البوليميرية **AM-21-A , AV-17-2P, VION KN-1, AV-16-GS** وكانت فعالية الانزيم المثبت (المرتبط) مقارنة بالانزيم الحر **30,60,85%** للطرق الثلاث على التوالي في حين كانت نسبة احتفاظ الانزيم المثبت بفعاليته بعد تثبيته على الحوامل المستخدمة مقارنة بالانزيم الحر **75.5%, 27.5%, 17.8%, 14.5%** وعلى التوالي .