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EXPERIMENTAL ARTICLES

A Plate Method to Screen for Microorganisms Producing Xylose Isomerase

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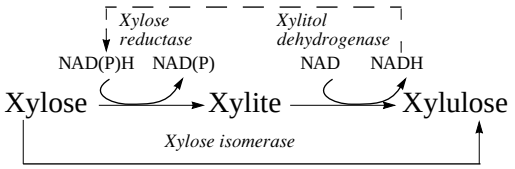
Abstract—A plate method was developed to screen for xylose isomerase–producing microorganisms based on the use of 2,3,5-triphenyltetrazolium as an indicator of D-xylulose, the D-xylose isomerization product. The use of this method allows microorganisms to be differentiated by the character of the enzyme synthesis (inducible or constitutive).

Key words: xylose isomerase, constitutive and inducible synthesis, microorganisms, screening, the plate method, 2,3,5-triphenyltetrazolium.

Xylose isomerase (D-xylose ketol-isomerase, EC 5.3.1.5) catalyzes reversible conversion of D-xylose and D-glucose to D-xylulose and D-fructose, respectively. The enzyme is used for the large-scale production of glucose–fructose syrups [1], and the possibility of its use for transforming plant hemicelluloses into ethanol and other organic compounds is currently under investigation [2].

The effectiveness of the existing technologies for xylose isomerase production is based on the development of mutant and genetic engineered strains having an enzyme activity that is higher than the enzyme activity of known producers. However, the performance of such works is connected with assaying a large number of clones and is significantly hampered by the absence of sensitive, inexpensive, rapid, and not arduous methods of screening.

At present, there are various methods for qualitative and quantitative determination of xylose isomerase [3–8]. Primary selection of xylose isomerase–producing microorganisms is usually carried out on solid diagnostic media including D-xylose or containing its natural polymer xylan (hemicellulose) [9–12]. However, considering only the microorganism growth on agarized media with D-xylose, it is not possible to unequivocally judge the key enzyme involved in the utilization of this substrate. This is due to the fact that D-xylose utilization by microorganisms is possible both by its direct conversion to D-xylulose under the action of xylose isomerase (EC 5.3.1.5) and by reduction, with the involvement of xylose reductase (EC 1.1.1.21), to xylitol, whose further oxidation to D-xylulose occurs by means of xylitol dehydrogenase (EC 1.1.1.9):



The method most frequently used to detect xylose isomerase activity in a microorganism is thin-layer chromatography of the products formed upon the substrate exposition to intact cells or cellular extracts. This method allows identification of the substrate and the end product of the enzymatic reaction, aldo- and ketosaccharides, which stain differently when the chromatograms are developed. The modifications of this highly sensitive but time-consuming and laborious method differ in the specific substrates used, the conditions of isomerization, separation, staining, and development of the initial and end products [7, 13–15].

The "sandwich" solid-phase immunoenzymatic assay is another method proposed for the primary assessment of the ability of microorganisms to synthesize xylose isomerase [5]. A reliable qualitative estimation of the enzyme activity is performed visually using an indicator scale based on the existing relationship between the intensity of coloration of the reaction mixture and the concentration of the enzyme determined. This proposed rapid screening method makes it possible to reveal the antigen specific for the enzyme protein in a large number of strains of a certain species and, thus, to find the most productive variants. It is evident that this costly and laborious method is inapplicable for selecting xylose isomerase producers among microorganisms belonging to different taxonomic groups.

The qualitative reaction of benzidine with D-glucose, the product of D-fructose isomerization, forms the basis of the method used to screen for transgenic

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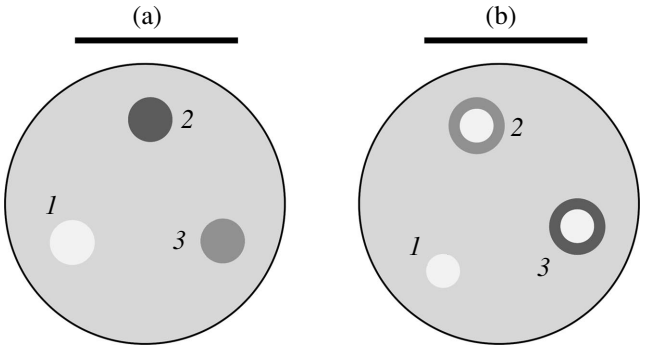

Fig. 1. Screening for xylose isomerase producers among microorganisms grown on the peptone–yeast agar containing 0.01 M D-xylose. Plates (a) not subjected to the reaction mixture and (b) incubated with the reaction mixture at 70°C for 10 min were developed with 2,3,5-triphenyltetrazolium. (*1*) *Arthrobacter citreus*; (*2*) *A. ureafaciens*; (*3*) *Arthrobacter* sp.

Escherichia coli clones producing xylose isomerase [16]. Positive clones first grown on xylose-containing agar and then covered with a layer of agarized medium with D-fructose and maintained at 50°C form a dark brown halo around the light colonies. One of the shortcomings of this method is the use of the carcinogenic substance benzidine as the indicator.

To reveal xylose isomerase among other microbial proteins after their isoelectric focusing and native electrophoresis in PAAG, the reaction of 2,3,5-triphenyltetrazolium oxidation by D-xylulose with the formation of formazan is used [4, 17]. This redox reaction has never previously been used to screen for enzyme producers with the plating method; it is used for quantitative determination of D-xylulose by thin-layer chromatography [13]. 2,3,5-triphenyltetrazolium, which is readily metabolized in the respiratory chain and does not affect the viability of microorganisms [18], appears to be an appropriate indicator to be used to screen for xylose isomerase producers on agarized media.

Table 1. Influence of the sources of carbon on the synthesis of xylose isomerase during submerged cultivation of *Arthrobacter* sp., *A. ureafaciens*, and *A. citreus*

Carbon source, 1%	Xylose isomerase, OD_{540}		
	Arthrobacter sp.	*A. ureafaciens*	*A. citreus*
Xylose	1.88	3.08	0
Xylan	2.88	0	0
Xylitol	2.84	3.16	0
Glucose	2.22	0	0
Fructose	1.50	0	0
Glycerol	1.78	0	0
Lactose	1.25	0	0
Citric acid	1.20	0	0
Apple pectin	1.52	0	0

The aim of this investigation was to develop a plate method of screening for xylose isomerase–producing microorganisms based on the use of 2,3,5-triphenyltetrazolium as the indicator of D-xylulose, the product of D-xylose isomerization.

MATERIALS AND METHODS

When developing the plate method of screening for xylose isomerase–producing microorganisms, the following bacteria of the genus *Arthrobacter*, differing in both the activity and the character of xylose isomerase production, were used: *Arthrobacter* sp. and *A. ureafaciens* were characterized by constitutive and inducible enzyme synthesis, respectively, and *A. citreus* did not produce the enzyme [19]. These bacteria are stored in the collection of microorganisms of the Institute of Microbiology, National Academy of Sciences of Belarus.

Unless otherwise indicated, peptone–yeast agar with 0.01 M of different carbon sources was inoculated with the test bacteria. After cultivation at 28–29°C for three days, the plates, either untreated or treated with the reaction mixture, were developed with a 0.1% 2,3,5-triphenyltetrazolium solution in 1 M NaOH at 30°C for 1 min in the dark.

The reaction mixture for treating the plates with the test cultures was composed of distilled water, 0.2 M K,Na-phosphate buffer (pH 7.8), 0.1 M solution of $MgSO_4 \cdot 7H_2O$, and 0.1 M solution of D-xylose in a 12 : 5 : 2 : 1 ratio.

The xylose isomerase activity of microorganisms grown on peptone–yeast agar with different carbon sources was judged by the rose-red coloration of the surface of the colonies or the nutrient medium around them.

When studying the effect of 2,3,5-triphenyltetrazolium on viability and xylose isomerase activity, test bacteria treated with this compound were plated onto peptone–yeast agar, grown at 28–29°C for three days, and then used for inoculating liquid nutrient medium in an amount of $0.5 \times 10^7 – 1.0 \times 10^7$ cells/ml.

The nutrient medium for submerged cultivation of the bacteria included the following (%): peptone, 1.0; yeast extract, 0.5; K_2HPO_4, 0.3; $MgSO_4 \cdot 7H_2O$, 0.1; pH 6.8. Mono- and disaccharides, sugar alcohols, and organic acids at a concentration of 1% by carbon and polysaccharides in an amount of 1% by weight were used as sources of carbon.

Submerged cultivation of the bacteria was carried out in 250-ml Erlenmeyer flasks on a shaker (180–200 rpm) at 28 to 30°C for 72 h.

Upon completion of the cultivation, bacterial cells were separated from the culture fluid by centrifugation (8000 *g*, 15 min), washed with distilled water, and used for determination of the xylose isomerase activity.

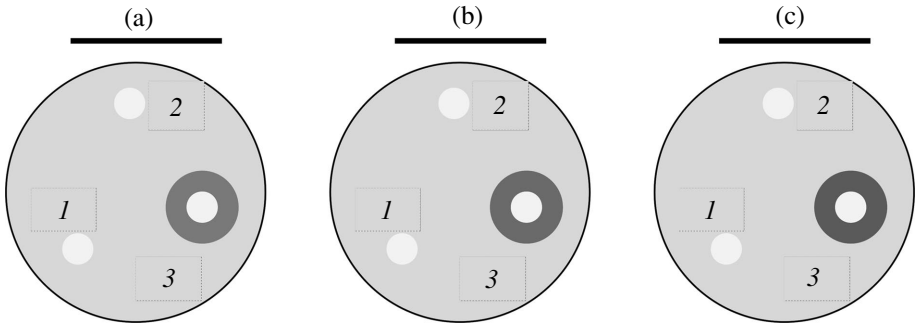

Fig. 2. Screening for xylose isomerase producers among microorganisms grown on peptone–yeast agar containing (a) 0.01, (b) 0.05, and (c) 0.1 M D-glucose. After incubation with the reaction mixture at 70°C for 10 min, the plates were developed with 2,3,5-triphenyltetrazolium. (*1*) *Arthrobacter citreus*; (*2*) *A. ureafaciens*; (*3*) *Arthrobacter* sp.

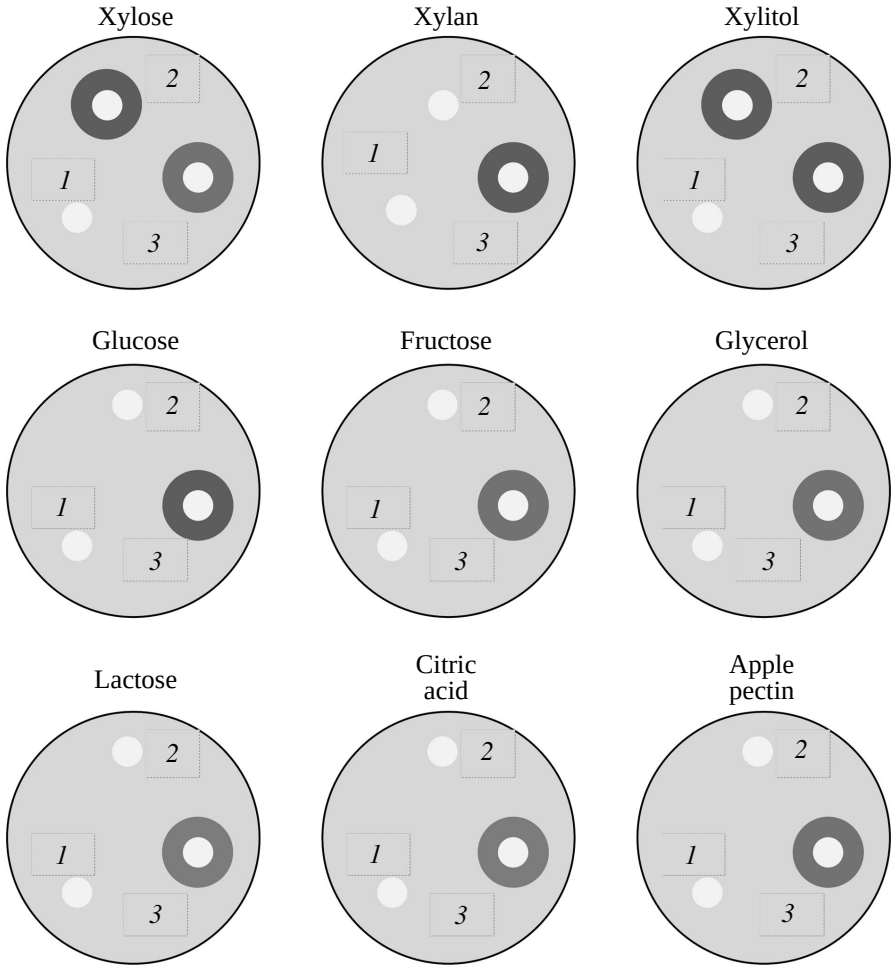

Fig. 3. Differentiation of microorganisms grown on peptone–yeast agar with various carbon sources (0.01 M) by the character of xylose isomerase synthesis. After incubation with the reaction mixture at 70°C for 10 min, the plates were developed with 2,3,5-triphenyltetrazolium. (*1*) *A. citreus*; (*2*) *A. ureafaciens*; (*3*) *Arthrobacter* sp.

The reaction mixture for the quantitative determination of the xylose isomerase activity contained 1 M D-xylose solution, 0.2 ml; 0.2 M K,Na-phosphate buffer (pH 7.8), 0.5 ml; 0.1 M $MgSO_4 \cdot 7H_2O$, 0.1 ml; bacterial cells, 20 mg (dry cell mass); and distilled water to adjust the volume to 2 ml. The duration of the isomerization reaction was 1 h at 70°C.

The enzyme activity was expressed in units of optical density at a wavelength (OD_{540}) corresponding to the absorption maximum of hydroxymethyl furfurol

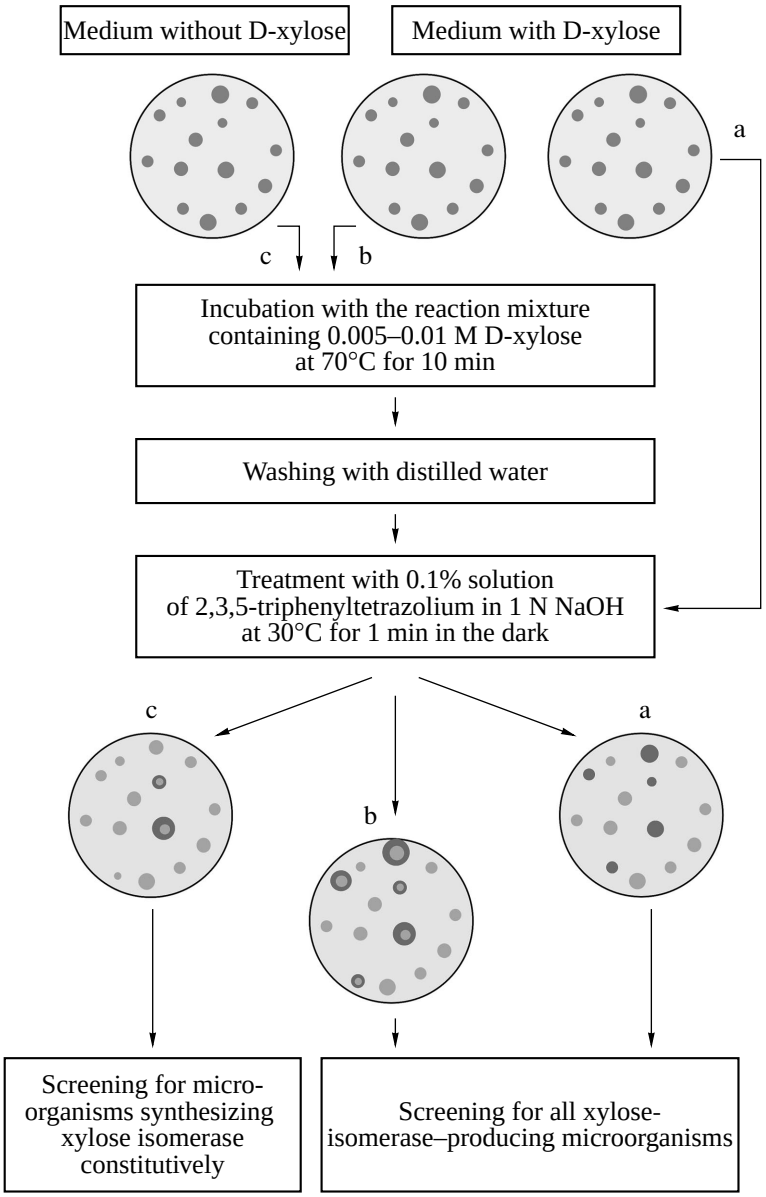

Fig. 4. The scheme of screening for xylose isomerase–producing microorganisms and their differentiation by the character of enzyme synthesis.

formed when D-xylulose is determined by the cysteine–carbazole method [3].

Biomass accumulation by the bacteria was expressed in mg of dry cell mass in 1 ml of medium.

The work presents average data of three to five experiments performed in three replicates.

RESULTS AND DISCUSSION

The basis of the proposed plate method for determining the xylose isomerase activity of microorganisms on agarized media is the capacity of D-xylulose to oxidize colorless 2,3,5-triphenyltetrazolium chloride in an alkaline medium with the formation of formazan having a dark pink color.

Xylose isomerase is a thermostable enzyme whose activity optimum is in the 70–80°C range [1]. At this temperature, in addition to enzymatic isomerization, spontaneous isomerization of D-xylose to D-xylulose also occurs, and this, after development with 2,3,5-triphenyltetrazolium, results in strong background staining of the agarized diagnostic media. This, along with the difference between the temperature optimal for growth of most of the mesophilic producer microorganisms (28–30°C) and the temperature optimal for the activity of the xylose isomerase synthesized by them (70 to 80°C), as well as the instability of 2,3,5-triphe-

Table 2. Effect of treatment with 2,3,5-triphenyltetrazolium on the growth and xylose isomerase synthesis by *Arthrobacter* sp., *A. ureafaciens*, and *A. citreus*

Bacterial species	Bacteria untreated with 2,3,5-triphenyltetrazolium		Bacteria treated with 2,3,5-triphenyltetrazolium	
	Biomass, mg/ml	Xylose isomerase, OD_{540}	Biomass, mg/ml	Xylose isomerase, OD_{540}
Arthrobacter sp.	5.30	1.85	5.20	1.89
A. ureafaciens	4.35	1.95	4.51	1.90
A. citreus	5.00	0	5.15	0

nyltetrazolium in the light, poses formidable obstacles to direct screening for the enzyme producers on solid media with D-xylose.

As was established earlier, the first of the problems specified above can be solved by decreasing the D-xylose content in solid media to 0.005–0.01 M and shortening to 10 min the duration of the enzymatic reaction performed at 70°C [20]. Other difficulties may be overcome if the plates with test microorganisms grown on agarized media with the specific substrate are first treated with a reaction mixture containing D-xylose and then with 2,3,5-triphenyltetrazolium as an indicator revealing the end product, D-xylulose.

Figure 1 shows the results of quantitative determination of the xylose isomerase activity of the *Arthrobacter* sp., *A. ureafaciens*, and *A. citreus* test bacteria grown on peptone–yeast agar with 0.01 M D-xylose for three days. When the plates were developed with 2,3,5-triphenyltetrazolium without preliminarily treating them with a substrate solution, the colonies of *Arthrobacter* sp. and *A. ureafaciens*, synthesizing xylose isomerase, stained rose-red (Fig. 1a). The colonies of *A. ureafaciens*, which differed from *Arthrobacter* sp. by a higher level of the enzyme synthesis also during submerged cultivation on medium with D-xylose, stained more intensely (Table 1). The color of the colonies of the xylose isomerase–negative bacterium *A. citrus* remained unchanged (Fig. 1a).

In the experiments that involved pretreatment of the test bacteria with a reaction mixture containing D-xylose prior to staining with 2,3,5-triphenyltetrazolium, a pink halo of different tints formed around the *Arthrobacter* sp. and *A. ureafaciens* colonies, which are positive in the capacity for producing xylose isomerase. At the same time, no characteristic agar staining was observed around the colonies of *A. citreus*, a bacterium lacking the xylose isomerase activity (Fig. 1b).

It is especially noteworthy that not only did the bacteria stained with 2,3,5-triphenyltetrazolium retain viability; they also did not differ in xylose isomerase activity from the initial cultures (Table 2).

We looked into the possibility of using our method to identify microorganisms synthesizing xylose isomerase constitutively. For this purpose, the cultures of test bacteria were grown on peptone–yeast agar with different D-glucose concentrations at 28–30°C for three days, and then the plates pretreated with a reaction mixture containing D-xylose were developed with 2,3,5-triphenyltetrazolium. The data presented in Fig. 2 show that it was only the *Arthrobacter* sp. bacteria, synthesizing the enzyme constitutively, that yielded a positive reaction; the test results did not depend on the amount of D-glucose contained in the medium.

Identification of xylose isomerase producers by the method proposed and their differentiation according to the mechanism of enzyme formation—constitutive or inducible—proved to be possible after growth of the test cultures on agarized media with monosaccharides (aldo- or keto-), disaccharides, polysaccharides, sugar alcohols, or organic acids. As evidenced by the results shown in Fig. 3, in all the experimental variants, the test for the formation of D-xylulose was always negative in the xylose isomerase–negative *A. citreus* and always positive in *Arthrobacter* sp., which produces the enzyme constitutively. The xylose isomerase activity of *A. ureafaciens* was revealed only after growth on media with D-xylose and xylitol, compounds that induce the enzyme formation under submerged cultivation conditions as well. Note that the results of the screening performed by the plate method are consistent with the results of quantitative determination of the xylose isomerase activity of the microorganisms studied grown in submerged cultures (Table 1).

The scheme of screening for xylose isomerase–producing microorganisms and their differentiation by the character of the enzyme synthesis—inducible or constitutive—is shown in Fig. 4.

Thus, the proposed plate method of screening for microorganisms producing xylose isomerase is not only nontoxic, sensitive, rapid, and simple but also allows differentiation of microorganisms by the character of enzyme synthesis. The method is indispensable in direct screening for mutant or gene-engineered strains producing xylose isomerase constitutively.

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