Bull. Food Res. (Bratislava) Special issue, 45—53, 1984

Conversion of D-glucose to D-fructose catalyzed by yeasts and yeast-like organisms

JIŘÍ ZEMEK — BEATRICA KADLEČÍKOVÁ — ĽUDOVÍT KUNIAK — ŠTEFAN KUČÁR — ANNA KOCKOVÁ-KRATOCHVÍLOVÁ

Summary. Isomerization between certain aldo- and keto-sugars due to presence of D-xylose isomerase in some yeast-like organisms is described in this paper together with methods of selection of the producing strains, immobilization of the yeast-like organisms and subsequent manufacture of D-fructose.

D-Xylose isomerase (D(+)-xyloseketolisomerase, EC 5.3.15) is an enzyme predominantly present in microbial cells, which catalyzes reversibly isomerization between certain aldoses and ketoses. The physiological role of the enzyme is probably mediating the formation of D-xylulose from D-xylose. D-Xylulose is phosphorylated then to enter the pentose phosphate pathway [1—3]. However, since its unspecificity in the D-glucose isomerization was reported, the enzyme has been the subject of intensified research [4]. The enzyme is used now on industrial scale to manufacture fructose syrups for food industry and to prepare special medical formulations. Due to the extention of its production and industrial application it is at present the third important enzyme preparation, following the proteolytic and amylolytic enzymes [5—7]. Xylose-isomerase activity has been demonstrated in more than 100 different microbial species, mainly bacteria. On the other hand, xylose isomerase has not been described to occur in fungi so far. Therefore, it is not too surprising that among yeasts the only strains reported so far to produce this enzyme are *Candida utilis* [8] and *Rhodotorula gracilis* [9]. The aim of this work was to study the ability of yeasts and yeast-like organisms to catalyze the aldo-ketose transformation and in the same time to replace expensive D-xylose through its cheap sources provided that the strains produce xylanase.

Ing. Jiří Zemek, CSc., RNDr. Beatrica Kadlečíková, Ing. Ľudovít Kuniak, RNDr. Štefan Kučár, CSc., RNDr. Anna Kocková-Kratochvílová, DrSc., Institute of Chemistry, Centre of Chemical Research, Slovak Academy of Sciences, Dúbravská cesta 9, 842 38 Bratislava.

Experimental part

Microorganisms. The strains to be studied in the experiment (i.e. 465) originated from the Czechoslovak Collection of Yeasts and Yeast-like Organisms, Institute of Chemistry, Centre of Chemical Research of the Slovak Academy of Sciences, Bratislava. In experiments the same strains were used as described previously [10].

Selection of the xylanase-producing strains. The gel method based on gel prepared in crosslinking reaction from xylan (ex *Fagus silvatica)* using 2-chloromethyloxyrane as a crosslinking agent. The gel assuming dry weight 5% forming agar-like hydrophilic and transparent medium preserves its substrate properties for the endo-acting xylanase (β-1,4-xylan xylanohydrolase, EC 3.2.1.8) whereas its substrate properties for exo-acting beta-xylosidase are highly suppressed due to crosslinking. Modification of the polysaccharide by crosslinking was carried out in alkali [11, 12]. The folia of gel prepared was washed out from alkali in tap water and in distilled water. Gel layers 4.5 mm thick were cut into equilateral triangular pieces with side length of 15 cm, placed into Petri dishes, immersed into Yeast Nitrogen Base (Oxid) growth medium reaching to a half of the gel thickness, and sterilized at 100°C with streaming steam for 1 h (three times). The producing strains of beta-xylanase are capable to liquefy the gel.

Selection of D-*xylose isomerase producers.* A synthetic selection medium for yeast-like microorganisms was used according to [13]. D-Xylose as a component in the medium was sterilized separately.

Positive strains and stock cultures have been maintained on Yeast Malt Agar slants, containing additional 0.1% D-xylose. For biomass production, a complex medium was composed, containing D-xylose or beta-xylan (6 g), D-glucose (4 g), amino acid mixture obtained through total hydrolysis of egg white (10 g), $CoCl_2.6H_2O$ (0.3 g), $MgSO_4.7H_2O$ (0.5 g), KH_2PO_4 (1 g). Sugars were autoclaved separately. Distilled water was added to 1 l volume and the medium obtained was adjusted to pH 7.0. The yeast biomass was produced in 500 ml Erlenmayer flasks, containing 100 ml medium, incubated for 120 h on a reciprocal shaker at 28°C.

Enzyme assay. Incubation mixture (1 ml) for xylose isomerase assay contained 122 mmol/l D-glucose or another sugar, 0.1 mol/l $MgSO_4.7H_2O$ and 1 mmol/l $CoCl_2.6H_2O$. The enzyme reaction was carried out at 70°C (pH 8.0). At appropriate time intervals the isomerization reaction was stopped by adding 10 ml 0.1 mol/l $HClO_4$. Cells or debris were removed by centrifugation at 10,000 g and formed ketose was quantitatively measured, following the modified cystein-carbazol colorimetric method [14, 15]. Qualitative detection of D-fruc-

tose and its separation from D-glucose were performed by descending paper chromatography on Whatman No. 1 paper, in solvent aceton—butanol—water system (7 : 2 : 1, by volume) and detected with benzidine [16]. D-Fructose and D-glucose were determined radiochemically in a separate experiment using [U-^{14}C] D-glucose or D-fructose (Institute for Research, Production and Application of Radioisotopes, Prague) (10 mg; radioactivity 248 MBq). Separation of both hexoses was carried out as described previously.

Cell immobilization

The biomass obtained from complex medium was concentrated by centrifugation (2,500 g). The sediment was twice washed in distilled water and freez-dried. The dried biomass was washed with ethanol and toluene and the cells were permeabilized by action of toluene (70°C, 1 h) and vacuum sicced then. The cells' immobilization was carried out in polyethylenimine (Fluka, Buchs, Switzerland). Polyethylenimine (molecular weight 45,000) as a 10% (w/v) aqueous solution (19 g) was mixed with 0.095 g of dried permeabilized cells and 0.4 ml of 2-chloromethyloxyrane prepared for the suspension. The crosslinking reaction lasted 10 min at 30°C, and the gel prepared in this way was disintegrated in a mixer, washed with water (100 ml), dried and sieved [17]. Similarly, as in the case of enzyme immobilization [18].

The biological purity of the experiment was checked by microscopic and cultivation tests. Growth of the cells was followed nephelometrically and/or by counting the cells in the Bürker chamber.

Results and discussion

Almost 20% of the tested strains (i.e. 75 strains) of yeast-like microorganisms gave positive reaction to the synthetic selection medium with D-xylose, as follows: *Bullera alba* 12-1-1, *Oospora lactis* 16-1-1, *Cryptococcus albidus* 17-4-1, *Dioszegia hungarica* 18-1-1, *Sporobolomyces salmonicolor* 19-4-1, *Rhodotorula glutinis* 20-2-1, -13, *Torulopsis kruisii* 26-19-7, *Aureobasidium pullulans* 27-1-1, -6, -7, -8, -14, 16, -20, -22, -23, -25, -27, -28, -30, -32, -33, -34, -36, -37, -38, -41, *Metschnikowia pulcherrima alfa* 29-2-115, *Candida albicans* 29-3-19, *C. tropicalis* 29-7-23, *C. humicola* 29-11-2, *C. intermedia* 29-12-1, *C. curvata* 29-18-1, *C. muscorum* 29-19-1, *C. parapsilopsis* 29-20-9, -13, *C. solani* 29-23-1, *C. utilis* 29-38-1, -18, *C. oregonensis* 29-42-1, *C. natalensis* 29-44-1, *C. lusitaniae* 29-59-1, *C. obtusa*

29-60-1, *C. stellatoidea* 29-64-1, *C. mogii* 29-66-1, *C. shehatae* 29-68-1, *C. stearolytica* 29-75-1, *Trichosporon cutaneum* 30-5-4, *T. fermentans* 30-3-1, *T. brassic* 30-8-1 *T. aculeatum* 30-11-1, *T. terrestre* 30-12-1, *T. lutetiae* 30-14-1 *Lipomyces lipofer* 33-2-1, *Nadsonia fulvescens* 36-2-2, *Hormoascus platypodis* 38-8-1, -4, *Pichia membranaefaciens* 39-1-4, *Citeromyces matritensis* 40-1-1, *Hansenula phaffii* 38-7-2, *Endomyces magnusii* 42-1-1, *E. selenospora* 42-10-1, *Fabospora fragilis* 51-1-1, *Pachysolen tannophilus* 53-1-1, *Nematospora coryli* 56-1-1, *Dekkera bruxelensis* 59-2-1, *Wickerhamia fluorescens* 61-1-1, *Rhodosporidium toruloides* 62-2-3, *Leucosporidium scottii* 64-1-1, -2, *L. capsuligenum* 64-2-1, -2, -4, *Lodderomyces elongasporus* 65-1-1, *Wingea robertsii* 68-1-1, *Filobasidium capsuligenum* 71-1-1.

On the other hand, only 9 strains were proved as producers of endo-acting xylanolytic activity (i.e. *Bullera alba* 12-1-1, *Cryptococcus laurentii* 17-3-3, *Cryptococcus albidus* 17-4-1, *Trichosporon cutaneum* 30-5-4, *Hansenula phaffii* 41-7-2, *Ambrosiozyma ambrosiae* 60-1-1, *Leucosporidium scottii* 64-1-2, *Lodderomyces elongasporus* 65-1-1, *Wingea robertsii* 68-1-1. All the strains of *Aureobasidium pullulans* tested (i.e. 21) have been found beta-xylanase producers [19].

With respect to the price of D-xylose as an inducer for xylose isomerase, we considered in further experiments just those producers of xylose isomerase which were capable of splitting xylan. Already Takasaki et al. [20] shown that cheap D-xylose sources, such as wheat bran, corncob and chaff, containing xylan, could replace pure D-xylose as an inducer, provided that the strains involved produce beta-xylanase. Xylan-utilizing strains will obviously grow satisfactorily on xylose and xylose-oligosaccharides present in assorted wood liquors and spent sulphite liquors now beeing used as a single cell proteing substrate and are thus potentional xylose-isomerase producers.

Considering the morphology and the semiquantitavely evaluated extent of the isomerization reaction (paper chromatography) and beta-xylanase reaction (monitored with the substrate — 21), *Wingea robertsii* was found to be the most suitable xylose-isomerase producer.

The strain of *Wingea robertsii* CCY 68-1-1 was inoculated on selected medium and grown for 96 h at 28°C. For biomass production a complex medium (2 l) was used. In Table 1 cultivation of the strain on medium with D-xylose and corresponding activities of beta-xylanase and xylose isomerase are demonstrated. Similarly, in Table 2 growth on the complex medium with xylan and in Table 3 on the medium with tamarind (ex *Tamarindus indica)* is shown. According to the results obtained in absence of xylan, a very low xylanolytic activity was observed; presence of D-xylose in the medium maintained, however, a sufficiently high xylose-isomerase activity. Replacement of D-xylose in the medium for xylan resulted in increasing xylanolytic activity in the medium; the xylose-isomerase activity, however, remained unchanged. When tamarind,

Table 1. Formation of xylose isomerase and beta-xylanase with *Wingea robertsii* cells on medium with D-xylose

Cultivation time [h]	Dry cells' weight [g/l]	Xylose-isomerase activity [μkat/l]	Beta-xylanase activity [μkat/l]
2	0.01	0.	0.
6	0.015	0.	0.
12	0.02	0.	0.
24	0.038	0.	0.
30	0.059	0.	0.
36	0.1	0.3	0.15
42	0.34	0.6	0.15
48	0.6	0.6	0.15
60	0.9	0.7	0.15
72	1.5	1.4	1.15
84	1.5	1.6	0.15
96	1.55	1.6	0.1
108	1.55	1.6	0.1
120	1.55	1.8	0.05
132	1.55	1.8	0.05
144	1.55	1.8	0.05

Table 2. Formation of xylose isomerase and beta-xylanase with *Wingea robertsii* cells on medium with xylan

Cultivation time [h]	Dry cells' weight [g/l]	Xylose-isomerase activity [μkat/l]	Bety-xylanase activity [μkat/l]
2	0.01	0.	0.01
6	0.015	0.	0.015
12	0.02	0.	0.03
24	0.036	0.	0.05
30	0.055	0.34	0.08
36	0.095	0.4	0.09
42	0.10	0.4	0.09
48	0.34	0.5	0.1
60	0.6	0.7	0.3
72	1.6	1.3	0.4
84	1.65	1.4	0.6
96	1.65	1.5	0.7
108	1.65	1.7	0.9
120	1.7	1.8	1.0
132	1.7	1.8	1.0
144	1.7	1.8	1.0

a native derivative of cellulose containing D-xylose was used in the complex medium instead of xylan, production of both xylanase and xylose isomerase was retarded.

Activity of xylose isomerase reached its maximum after 120 h of cultivation.

Table 3. Formation of xylose isomerase and beta-xylanase with *Winga robertsii* cells on medium with tamarind

Cultivation time [h]	Dry cells' weight [g/l]	Xylose-isomerase activity [μkat/l]	Beta-xylanase activity [μkat/l]
2	0.1	0.	0.
6	0.015	0.	0.
12	0.02	0.	0.
24	0.03	0.	0.02
30	0.04	0.1	0.05
36	0.05	0.2	0.06
42	0.07	0.3	0.07
48	0.1	0.4	0.08
60	0.34	0.5	0.09
72	0.8	0.7	0.2
84	0.96	0.8	0.25
96	1.1	0.8	0.23
108	1.1	0.9	0.24
120	1.1	0.9	0.25
132	1.0	0.7	0.2
144	1.0	0.6	0.15

Table 4. Conversion of D-glucose to D-fructose catalyzed with immobilized *Wingea robertsii* cells

Time	Formation of D-fructose [%]	
	from D-glucose	from starch hydrolysate
1	4	2
2	8	4
3	16	6
4	20	12
5	29	18
6	36	27
7	45	35
8	45	42
10	45	42

The *Wingea robertsii* cells were harvested by centrifugation, washed with water and permeabilized as described in Experimental.

Permeabilized cells were used for immobilization through encapsulation and covalent bonding in the gel of polyethylenimine crosslinked with 2-chloromethyloxyrane. Activity of xylose isomerase in the immobilized *Wingea robertsii* cells was followed as described in Experimental. When starting with [U-^{14}C] D-glucose as a substrate in the incubation mixture (1 ml) under conditions stated in Enzyme assay, and immobilized *Wingea robertsii* (5 mg), the optimal yield of

D-fructose was gained after 8 h at 70°C (pH 8.0). In equilibrium it reached 45% (Table 4).

In a separate experiment instead of [U-^{14}C] D-glucose 10 mg soluble starch (ex potato) labelled with [U-^{14}] starch (ex *Chlorella* sp.) 248 MBq was used. Before adding the immobilized cells, alpha-amylase (ex *Bacillus subtilis*, Bolamylase, LIKO, CSSR; 5 nkat) and glucoamylase (3 nkat, ex *Endomycopsis bispora*, Prowiko, GDR) were applied. After 20 min preincubation immobilized cells of *Wingea robertsii* (5 mg) were added. In equilibrium demonstrated after 10 h, concentration of D-fructose reached 42% of the total sugars in the mixture (Table 4).

Two types of glucose-isomerase activities have been established experimentally. Numerous microorganisms were screened for D-glucose-isomerizing activity, but a genuine, specific D-glucose isomerase apparently does not exist. Instead, most of the enzymes in this category turned out to be either D-xylose isomerase or D-glucose-6-phosphate isomerase. The latter activity, however, requires arsenate for isomerization. As *Wingea* cells are capable to catalyze aldo-ketose isomerization in absence of arsenate, it may be considered a proof for presence of xylose isomerase in the cells. Because the primary market for glucose/fructose syrups is in food application, it makes the isomerization reaction catalyzed with these yeast-like organisms more attractive. With respect to the bacterial producers of xylose isomerase [7], when using *Wingea robertsii* cells there is no danger in pathogenesis of the organism or need to separate toxic substances. These cells seem to be more advantageous from the technological point of view than bacteria or the alkaline, non-enzymic isomerization of glucose, because of colour, flavour and composition problems [22, 23]. It is evident that D-fructose can be separated from D-glucose in the reaction mixture similarly as proposed by the German firm C. F. Boehringer for the production of fructose from sucrose [24]. In the Boehringer process, sucrose is hydrolyzed to glucose and fructose in a column packed with a sulphonated polystyrene resin which is partially in a calcium form. The glucose and fructose are eluciated separately after chromatographic separation on the same column, and fructose may be crystallized then. The concentration step, crystallization of D-glucose and purification of D-fructose from the mother liquors, is appreciated especially when fructose received should find its application in parenteral solutions for intravenous infusions [25] or as an alcohol de-intoxicant [26].

Regarding the enzyme make-up of yeasts and yeast-like organisms, according to literary data known, it is obviously not of marginal importance that these organisms are able to catalyze the aldo-ketose transformation in such great extent. Beside the technological aspect, their ability to isomerize aldoses and ketoses can find its application in taxonomy, as well.

Selected permeabilized and immobilized cells of *Wingea robertsii* have already

been made use of at the Institute for Research, Production and Application of Radioisotopes (Prague, Czechoslovakia) for preparation of [U-^{14}C] D-fructose from the amylolytic hydrolysate of [U-^{14}C] starch (ex *Chlorella vulgaris)* [27]. According to our preliminary results, unspecificity of xylose isomerase of *Wingea robertsii* can result in preparation of such medical formulations as maltoheptulose and lactulose.

References

1. MORTLOCK, R. P. — WOOD, W. A.: J. Bacteriol., *88*, 1964, p. 838.
2. BISSON, T. M. — MORTLOCK, R. P.: J. Bacteriol., *95*, 1968, p. 925.
3. DAVID, J. D. — WIESMEYER, H.: Biochim. Biophys. Acta, *201*, 1970, p. 497.
4. MARSHALL, R. O. — KOOI, E. R.: Science, *125*, 1957, p. 648.
5. GEYER, H. U.: Die Stärke, *26*, 1974, p. 225.
6. GRAMERA, R. E.: Starch/Stärke, *30*, 1978, p. 20.
7. HAMILTON, B. K. — COLTON, C. K. — COONEY, C. L.: In: Immobilized Enzymes in Food and Microbial Processes. Eds. N. F. Olson, C. L. Cooney. New York—London, Plenum Press 1974, p. 85.
8. TOMOYEDA, M. — HORITSU, H.: Agr. Biol. Chem., *28*, 1964, p. 139.
9. HOFER, M. — BETZ, A. — KOTYK, A.: Biochim. Biophys. Acta, *252*, 1971, p. 1.
10. ZEMEK, J. — AUGUSTÍN, J. — KOCKOVÁ-KRATOCHVÍLOVÁ, A. — KUNIAK, Ľ.: Biológia, *36*, 1981, p. 439.
11. ZEMEK, J. — KUNIAK, Ľ.: Czechoslov. Patent No. 186059, 1978.
12. ZEMEK, J. — KUNIAK, Ľ. — AUGUSTÍN, J.: Czechoslov. Patent No. 200711, 1983.
13. VANDAMME, E. J. — DE LAPORTE, M. — DE VOCHT, M. — VAN HOE, M.: Mikrobielle Enzymproduktion. Abh. Akad. Wiss. der DDR. Berlin, Akademie-Verlag 1981, p. 193.
14. DISCHE, Z. — BORENFEREUND, E.: J. Biol. Chem., *192*, 1951, p. 583.
15. NAKAMARA, M.: Agr. Biol. Chem., *32*, 1968, p. 701.
16. CIFONELLI, J. A. — SMITH, F.: Analyt. Chem., *26*, 1954, p. 1132.
17. ZEMEK, J. — KUNIAK, Ľ.: Czechoslov. Patent No. 5541, 1983.
18. ZEMEK, J. — KUNIAK, Ľ. — GEMEINER, P. — ZÁMOCKÝ, J. — KUČÁR, Š.: Enzyme Microb. Technol., *4*, 1982, p. 233.
19. ČERNÁKOVÁ, M. — KOCKOVÁ-KRATOCHVÍLOVÁ, A. — ŠUTÝ, L. — ZEMEK, J. — KUNIAK, Ľ.: Folia Microbiol., *25*, 1980, p. 68.
20. TAKASAKI, Y. — KOSUGI, Y. — KAMBAYASHI, A.: In: Streptomyces Glucose Isomerase. Fermentation Advances. Ed. D. Perlman. New York, Academic Press 1969, p. 561.
21. KUNIAK, Ľ. — ZEMEK, J.: Czechoslov. Patent No. 192202, 1979.
22. LLOYD, N. E. — LEWIS, L. T. — LOGAN, R. M. — PATEL, D. N.: U.S. Patent No. 3694314, 1972.
23. KUČÁR, Š. — ZEMEK, J. — FARKAŠ, J.: Czechoslov. Patent No. 187229, 1981.
24. BOEHRINGER, C. F. and Sons: British Patent No. 1117903, 1968.

25. WARD, C. E.: In: Microbial Technology. Ed. H. J. Peppler. Reinhold 1976, p. 200.
26. GORDON, H. W.: U.S. Patent No. 3584122, 1971.
27. ZEMEK, J. — KUČÁR, Š. — KOLINA. J.: Czechoslov. Patent No. 5254, 1983.

Способ подготовки D-фруктозы из D-глюкозы с использованием дрожжевых микроорганизмов

Резюме

В работе описывается изомеризация определенных альдо- и кетосахаридов D-ксилоза изомеразой, присутствующей в некоторых дрожжевых микроорганизмах. Приводится способ селекции продуктивных штаммов, иммобилизации дрожжевых микроорганизмов, а также приготовления D-фруктозного сиропа.

Spôsob prípravy D-fruktózy z D-glukózy pri použití kvasinkových mikroorganizmov

Súhrn

V práci sa opisuje izomerizácia určitých aldo- a keto-sacharidov D-xylóza izomerázou prítomnou v niektorých kvasinkovitých organizmoch. Uvedený je spôsob selekcie produkčných kmeňov, imobilizácie kvasinkovitých organizmov, ako aj príprava D-fruktózového sirupu.