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Article

Bioconversion of plant waste as a tool for regulating the techno-ecosystem of the pulp and paper industry in the North-Western region

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Abstract. The prospects of introducing a circular economic model for optimizing the techno-ecosystems of the pulp and paper industry (PPI) in the North-West of the Russian Federation are investigated. The potential of biorefining PPI waste (sulfite liquor and lignocellulose hydrolysates) into bioethanol is considered. A comparative analysis of the effectiveness of alcoholic fermentation of D-xylose, the main pentatomic sugar of PPI waste, and various types of xylose-assimilating yeast from domestic collections of industrial microorganisms is presented. The best rate and efficiency of alcohol formation were noted for *C. shehatae* Y-1632 (0.83 g/l×h and 0.40 g/g of consumed D-xylose), *P. stipitis* Y-1483 (0.76 g/l×h and 0.39 g/g), and *P. tannophilus* Y-1533 (0.65 g/l×h and 0.27 g/g). The features of xylose reductase (XR) and xylitol dehydrogenase (XD), key enzymes of D-xylose catabolism, which affect the level of bioethanol production, have been studied. The XR of alcohol-forming yeast had a double NADPH/NADH had coenzyme specificity, whereas XD was characterized by high affinity for NAD+. The highest XR activity was detected for *P. stipitis* Y-2160 (15.21 mmol/mg×min). *C. shehatae* Y-1632 (13.95 mmol/mg×min) had the highest XD activity. The maximum permissible values for alcohol-forming activity have been established. *C. shehatae* Y-1632 and *P. tannophilus* Y-1533 alcohol concentrations in the medium: 45.5 g/l and 46 g/l, respectively. *P. tannophilus* Y-1533 has been shown to be highly resistant to toxic impurities from PPI waste: furfural (F), oxymethylfurfural (OF), volatile organic acids (VOA), and substances of the lignofuran complex (SLC). The maximum permissible concentrations of inhibitors for the growth of these yeasts were (g/l): 0.1 (F); 0.27 (OF), 0.50 (VOA) and 1.74 (SLC). For the first time, the biotechnological potential of *Pachysolen tannophilus* Y-1533 yeast for optimizing the techno-ecosystems of the PPI region of presence is discussed.

Keywords: biorefining, negative feedback, sulfite liquor, lignocellulose hydrolysate, xylose assimilating yeast, bioethanol

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Научная статья

Биоконверсия растительных отходов как инструмент регулирования техноэкосистемы целлюлозно-бумажной промышленности Северо-Западного региона

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Аннотация. Исследованы перспективы внедрения циркулярной экономической модели для оптимизации техноэкосистем целлюлозно-бумажной промышленности (ЦБП) Северо-Запада РФ. Рассмотрен потенциал биорефайнинга отходов ЦБП (сульфитные щелока и гидролизаты лигноцеллюлозы) в биоэтанол. Представлен сравнительный анализ эффективности спиртовой ферментации D-ксилозы, основного пятиатомного сахара отходов ЦБП, различными видами ксилозоассимилирующих дрожжей из отечественных коллекций промышленных микроорганизмов. Лучшие скорость и эффективность образования спирта отмечены для *C. shehatae* Y-1632 (0.83 г/л×ч и 0.40 г/г потребленной D-ксилозы), *P. stipitis* Y-1483 (0.76 г/л×ч и 0.39 г/г), а также *P tannophilus* Y-1533 (0.65 г/л×ч и 0.27 г/г). Изучены особенности ксилозоредуктазы (XR) и ксилитдегидрогеназы (XD), ключевых ферментов катаболизма D-ксилозы, которые оказывают влияние на уровень продукции биоэтанола. XR спиртообразующих дрожжей имела двойную НАДФН/НАДН-коферментную специфичность, тогда как XD характеризовалась высоким сродством к $НАД^+$. Наибольшая активность XR выявлена для *P. stipitis* Y-2160 (15.21 мкМоль/мг×мин). Наибольшей активностью XD обладали C*. shehatae* Y-1632 (13.95 мкМоль/мг×мин). Установлены предельно допустимые для спиртообразующей активности *C. shehatae* Y-1632 и *P. tannophilus* Y-1533 концентрации спирта в среде: 45.5 г/л и 46 г/л соответственно. Продемонстрирована большая устойчивость *P. tannophilus* Y-1533 к токсическим примесям отходов ЦБП: фурфуролу (Ф), оксиметилфурфуролу (ОФ), летучим органическим кислотам (ЛОК) и веществам лигнофуранового комплекса (ВЛК). Предельно допустимые концентрации ингибиторов для роста этих дрожжей составили (г/л): 0.1 (Ф); 0.27 (ОФ), 0.50 (ЛОК) и 1.74 (ВЛК). Впервые обсуждается биотехнологический потенциал дрожжей *Pachysolen tannophilus Y-1533* для оптимизации техноэкосистем ЦБП региона присутствия.

Ключевые слова: биорефайнинг, отрицательные обратные связи, сульфитный щелок, гидролизат лигноцеллюлозы, ксилозоассимилирующие дрожжи, биоэтанол

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Introduction

It is known that modern models of economic development are characterized by loops of positive feedback loops and at the same time very weak negative feedback loops with natural ecosystems, which causes ecological instability of the formed techno-ecosystems. The subject of the study is the anthropogenic impact on natural ecosystems and the human environment of the pulp and paper industry (PPI), since, in addition to the variety of useful products, the industry generates a large amount of waste, including toxic waste.

As we have repeatedly shown, one of the ways to solve the problem of forming regulatory negative feedback in techno-ecosystems is to implement a cyclical model with a focus on a circular economy (closed-loop economy) (Kvasha and Malevskaya-Malevich, 2022, Kvasha et al., 2023). At the same time, in accordance with the specifics of the resource-consumer flows of the IPP, the basis of circular

models are biotechnological approaches aimed at obtaining products from biomass. In other words, within the framework of the pulp and paper industry, the processes of cycling resource and product flows are implemented mainly by methods of biorefining (circular bioeconomics), that is, any recycling processes based on biotechnologies and focused on the use of recycled raw materials and products.

Problems of bioconversion focus on sustainable methods of utilization of plant biomass, which helps to reduce the volume of non-recyclable waste and improve the state of ecosystems. The typology of IPP waste by its origin was previously studied by our team of authors (Kvasha et al., 2023). It has been shown that the main part of the waste is formed in the form of polymer organic residues (cellulose, hemicellulose, lignin), which, depending on the method of cooking cellulose (sulfate or sulfite), are presented either as black liquor or as sulfite liquor, respectively. The toxic effect of IPP organic waste on the lithosphere and hydrosphere serves as a prerequisite for the search for tools for the formation of regulatory negative feedback in IPP techno-ecosystems.

The pulp and paper complex in the Northwestern region of Russia is represented by large sulfite production facilities (Kondopoga Pulp and Paper Mill (Republic of Karelia) and Svetogorsky Pulp and Paper Mill (Leningrad Region)), which causes the presence of sulfite liquors and lignocellulose hydrolysates in the waste. At the same time, the high value of bioethanol as a secondary raw material and energy resource determines the focus of research on the development of technologies for its alcohol bioconversion. The substitution of coniferous species with deciduous and secondary cellulose-containing raw materials leads to the fact that pentoses dominate among the sugars obtained during cooking (Chetvertneva et al., 2021; Pereira et al., 2013). At the same time, the ability to actively utilize pentose sugars along with hexoses has been revealed mainly for microorganisms that do not have biotechnological significance today (Prakash et al., 2022).

The purpose of this study was to find effective tools for regulating the techno-ecosystems of the pulp and paper industry in the Northwestern region of Russia, in particular, the biotransformation of sulfite alkalis and lignocellulose hydrolysates. In this regard, the task is to form criteria regulating the expediency of biotechnological use of xylose assimilating yeast, non-traditional biocatalysts, for the production of ethanol from PPI waste.

Materials and methods

The object of the study was 21 strains of thirteen different types of xylose-assimilating yeast from domestic collections of microorganisms: All-Union Research Institute of Hydrolysis and Plant Materials (VNIIгidroliz, St. Petersburg); State Research Institute of Genetics and Selection of Industrial Microorganisms (GosNIIgenetika, Moscow), Russian Scientific Research Institute of Food Biotechnology (Moscow), G.K. Scriabin Institute of Biochemistry and Physiology of Microorganisms (IBPM RAS, Pushchino-on-Oka). Among them are representatives of the genera *Candida* (*C. didensiae*, *C. scottii*, *C. intermediae*, *C. parapsilosis*, *C. shehatae*, *C. silvanorum*, *C. tropicalis*), *Pichia* (*P. guillermondii*, *P. stipitis*) and *Kluyveromyces* (*K. fragilis*, *K. marxianus*), as well as *Pachysolene tannophilus* and *Torulopsis molishiama*. Their physiological and biochemical features were studied by us in the period from 2002 to 2019. The main criteria for selecting strains for this experiment were belonging to the group of facultative anaerobes and the ability to assimilate D-xylose (Kreger-Van Rij, 1982).

Saccharomyces cerevisiae LV-7 yeast, which does not assimilate D-xylose, but has a pronounced fermentative type of sugar catabolism, was used as a control for D-xylose and D-glucose fermentations (Kudryavtsev, 1981). Fermentation of D-xylose and D-glucose was performed under microaerobic conditions optimal for alcohol production from pentoses (Yablochkova et al., 2003). Standard methods were used to prepare cell-free yeast extracts, as well as to study the activity of xylose reductase (CF 1.1.1.21) (XR) and xylitol dehydrogenase (CF 1.1.1.9) (XD), enzymes of the initial stages of D-xylose catabolism (Bolotnikova et al., 2020).

The growth dynamics of xylose assimilating yeast was studied at the Biostat M laboratory fermentation plant (Braun, Germany), according to the conditions described by O.I. Bolotnikova et al. (2013a). The seed material was grown in accordance with O.I. Bolotnikova et al. (2012), and inoculation was carried out with a culture in the middle of an exponential growth phase. The specific growth rate (μ) was determined during microkinetic studies (Ogorodnikova et al., 1995). Quantitative characteristics of yeast growth with changes in incubation temperature, pH, D-xylose and ethyl alcohol concentrations were established similarly (Knorre and Emmanuel, 1974).

To analyze the sensitivity of yeast to ethanol, the constant α of the Luong equation was calculated (Luong, 1985). The effect of inhibitors (furfural, oxymethylfurfural, volatile organic acids and substances

of the lignofuran complex) on yeast growth was evaluated according to the methods described in detail in the work of O.I. Shapovalov et al. (2008). The concentration of D-xylose was determined by Felling (Kostenko, 1977). Yeast biomass was calculated spectrophotometrically at a wavelength of 620 nm (Lowry, 1951). The amount of ethyl alcohol was determined by gas-liquid chromatography in the distillate obtained after distillation of the test sample (Yablochkova et al., 2003). The amount of ethanol was evaluated on a Vista 600 gas chromatograph (Varian, 600). The concentrations of furfural, oxymethylfurfural, volatile organic acids, and substances of the lignofuran complex were determined by standard methods (Emelyanova, 1976).

The analysis of the parameters of alcoholic fermentation of industrial waste of plant biomass was carried out using a sample of sulfite liquor, a by-product of sulfate cooking of mixed wood pulp (Svetlogorsk PPM). Sulfite liquor was subjected to preliminary detoxification to reduce the concentrations of toxic impurities to finite values that did not inhibit alcohol formation in the yeast *P. tannophilus* Y-1533 (Shapovalov et al., 2008). The qualitative composition of the sugars of the sulfite liquor sample corresponded to the following scheme (g/l): 17.85 (D-xylose); 1.2 (L-Arabinose); 7.95 (D-mannose); 2.8 (D-glucose); 2.45 (D-galactose). Alcoholic fermentation of sulfite liquor was carried out for 76 hours on a Biostat M pilot plant (Braun, Germany) under the conditions described in detail in the work of O.I. Shapovalov et al. (2008). The initial biomass concentration of *P. tannophilus* Y-1533 was 15 g/l (75% humidity), and the forced aeration regime favored the highest ethanol yield (Bolotnikova et al., 2013a). Statistical processing of the experimental results was carried out using standard methods for determining the Student's criteria and χ^2 (Glotov et al., 1982).

Results and discussion

The method of selecting collection strains of xylose-assimilating yeast for alcohol conversion of substrates from plant biomass waste is shown in Fig. 1. At the first stage of selection, a comparative analysis of the microaerobic fermentation of D-xylose and D-glucose, the main sugars of substrates from plant biomass waste were performed (Bolotnikova et al., 2019; Kvasha et al., 2023). It was found that collection strains of xylose-assimilating yeast fermented D-xylose at different rates and efficiencies. The increase in yeast biomass in all xylose assimilating yeasts was insignificant. The minimum ethanol yield did not exceed 0.01 g per gram of consumed D-xylose. The best characteristics of alcohol formation were distinguished by *C. shehatae* Y-1632. These yeasts completely assimilated 2.0% D-xylose within 24 hours at a rate of 0.83 g/l×h and an ethanol productivity of 0.40 g/g (78% of the theoretically calculated maximum (Marinchenko et al., 1981)). In addition, a sufficiently high rate of D-xylose consumption and the economic coefficient of alcohol formation were noted for *P. stipitis* Y-1483 strains (0.76 g/l×h and 0.39 g/g, respectively), as well as *P. tannophilus* Y-1533 (0.65 g/l×h and 0.27 g/g, respectively).

Then, microaerobic fermentation of D-glucose, the direct substrate of glycolysis reactions, was carried out. Her results indirectly confirmed the rather high fermentation activity of the above-mentioned xylose assimilating strains. The volume rate and efficiency of alcohol production reached the following values: 0.80 g/l×h and 0.40 g/g (*C. shehatae* Y-1632); 0.78 g/l×h and 0.37 g/g (*P. stipitis* Y-1483); 0.79 g/l×h and 0.36 g/g (*P. tannophilus* Y-1533). Nevertheless, in all cases, alcohol production by xylose assimilating yeast was inferior to that of the control strain *S. cerevisiae* LV-7 (0.42 g/g). This confirmed the relationship between the level of ethanol production and the activity of the enzymes of the initial stages of D-xylose catabolism that catalyze the sequential reactions of conversion of D-xylose to D-xylulose: xylose reductase (XR) and xylitol dehydrogenase (XD) (Bolotnikova et al., 2020).

The next stage of selection included an assessment of the activity and coenzyme specificity of XR and XD enzymes in xylose assimilating yeast strains with the best ethanol production. It was found that the XR of *P. tannophilus*, *P. stipitis*, and *C. shehatae* in the microaerobic regime had double NADPH/NADH had coenzyme specificity, whereas XD was characterized by high affinity for NAD^+. At the same time, the total specific activity of XR of *P. tannophilus* Y-1533 (4.16 mmol/mg×min) was lower than the similar value of XD (5.26 mmol/mg×min). In *P.stipitis* Y-2160, the total enzyme activity of the initial stages of D-xylose catabolism was significantly higher: 15.21 mmol/mg×min (XR) and 8.64 mmol/mg×min (XD), although their ratio did not change. The *C. shehatae* strain Y-1632, on the contrary, isolated a lower activity of XR C. (8.33 mmol/mg×min) compared to XD (13.95 mmol/mg×min).

The expediency of practical use of a particular strain is determined not only by its catalytic potential, but also by its physiological plasticity. Ethanol is the so-called primary metabolite of the yeast cell (Bolotnikova et al., 2013a). At the same time, the accumulation of alcohol in the fermentation medium inhibits metabolic processes and the growth of yeast culture (Bolotnikova et al., 2013b). Toxic

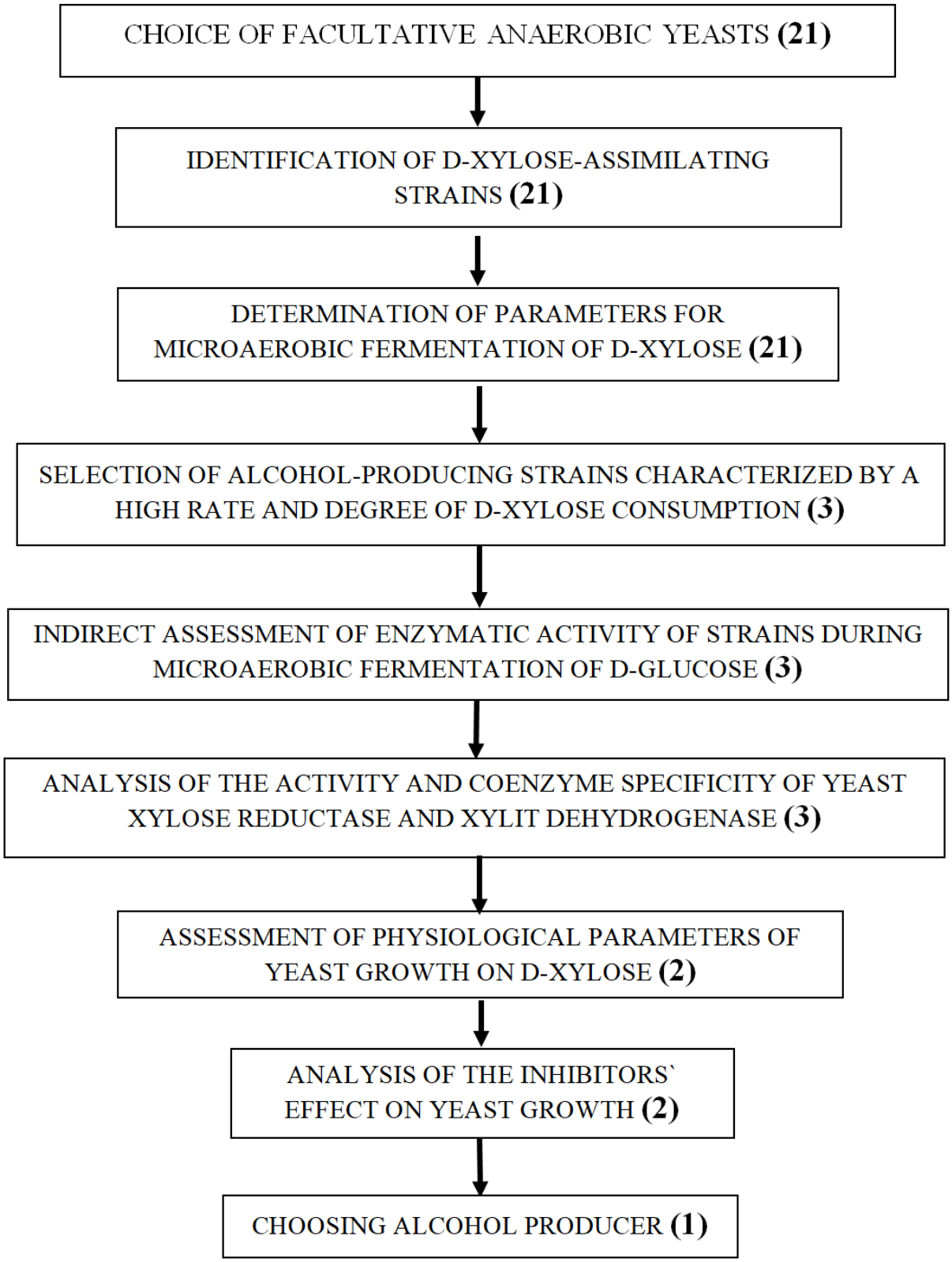

Fig. 1. Screening of an ethanol producer from D-xylose. The number of yeast strains selected at each screening stage is indicated in parentheses.

components of industrial waste from plant biomass, sulfite liquors, have a similar effect: furfural (F), oxymethylfurfural (OF), volatile organic acids (VOA) and substances of the lignofuran complex (SLC) (Shapovalov et al., 2008). Therefore, at the final stages of selection, the kinetics of growth of yeast *C. shehatae* (a probableteleomorph of *P. stipitis* (Delweg et al., 1984)) and *P. tannophilus* on D-xylose, as well as their sensitivity to the toxic effects of ethanol and other above-mentioned inhibitors, were studied.

Belonging to the group of facultative anaerobes formed close optima of temperatures, pH, concentrations of O_2 and D-xylose in the medium for the growth of both strains. Thus, the highest growth rate of *C. shehatae* Y-1632 culture was observed at temperatures of 24–30 °C, pH = 4.5–5.5, D-xylose concentrations of at least 0.15 g/l and no more than 110.3 g/l under conditions of complete saturation of the fermentation medium with oxygen. For the *P. tannophilus* Y-1533 strain, only minor shifts in the optimal temperature ranges (24–32 °C), pH (3.5–5.0), and D-xylose concentrations in the medium (3.01–109.6 g/l) were noted. However, the value of the Mono substrate saturation constant (k_x = 4.44 g/l) indicated a lower affinity of *P. tannophilus* Y-1533 enzymes for D-xylose and, consequently, a lower depth of consumption of this sugar during microaerobic fermentation. The similar characteristic of *C. shehatae* Y-1632 was 0.27 g/l. At the same time, the specific growth rate of the latter strain μ, equal to 0.15 h^{-1}, was significantly lower than that of P.tannophilus Y-1533 (0.25 h^{-1}).

The most striking differences were revealed when analyzing the resistance of xylose assimilating yeasts to inhibitors. At similar maximum permissible ethanol concentrations of 45.5 g/l (*C. shehatae* Y–1632) and 46 g/l (*P. tannophilus* Y-1533), the dependence of the growth rate of the strains on alcohol concentration was not the same. The value of the constant α for *C. shehatae* (1.53) indicated an inversely proportional relationship, while a similar value for *P. tannophilus* (3.02) indicated a hyperbolic relationship. Thus, the higher sensitivity of *C. shehatae* Y-1632 to the toxic effects of alcohol was confirmed. In addition, the maximum permissible concentrations of inhibitors for the growth of *C. shehatae* Y-1632 (g/l): 0.02 (F), 0.13 (OP), 0.44 (LOA), 0.76 (SLC) were significantly lower than those of *P. tannophilus* Y-1533: 0.1 (F), 0.27 (OF), 0.50 (LOA) and 1.74 (SLC). Consequently, it is advisable to carry out bioconversion of industrial waste from plant biomass (such as sulfite liquor, byproducts of the pulp and paper industry) using *P. tannophilus* yeast adapted to growth on hexose-poor plant substrates with metabolic inhibitors (Boidin and Adzet, 1957). Therefore, at the final stage of the work, the parameters of alcoholic fermentation of a sample of sulfite liquor with a periodic culture of *P. tannophilus* Y-1533 were determined (Table 1).

The recalculation of alcohol production per 1200 ml of mash obtained during rectification indicated an economic yield of 12.52 g of ethanol or 15.5 ml of ethyl alcohol with a strength of 96%.

According to theoretical calculations, up to 42.6 liters of ethanol can be obtained from 100 kg of sugars of sulfite liquor from hardwood containing 55.6% D-xylose, 24.7% D-glucose, 8.7% D-mannose, 7.6% D-galactose and 3.7% L-arabinose (Korolkov, 1990), increasing the total alcohol production by 70% compared to the traditional technology of hydrolysis production (Sharkov, 1973). Processing of sulfite liquor from coniferous wood containing 46.3% D-mannose, 24.6% D-glucose, 20.5% D-xylose, 7.9% L-arabinose, 1.6% D-rhamnose (Korolkov, 1990) will provide 55.5 liters of ethanol, which will improve the total alcohol production by 28% (Sharkov, 1973).

Table 1. Alcoholic fermentation of sulfite liquor by strain *P. tannophilus* Y-1533. The statistical error at each experimental point did not exceed 5.0%.

Fermentation parameters			
Time, h	Degree of sugar utilization, %	Ethanol production	
		Volumetric velocity, g/l×h	Concentration, g/l
76	76	0.58	14.7

Conclusion

1. A comprehensive biotechnological, biochemical and physiological analysis of the features of D-xylose catabolism in various types of xylose-assimilating yeast allowed us to formulate requirements for bioethanol producers from pulp and paper industry waste. The possibility of practical implementation of this process is determined by the degree of resistance of xylose assimilating strains to toxic impurities of sulfite alkalis and lignocellulose hydrolysates (furfural, oxymethylfurfural, volatile organic acids and substances of the lignofuran complex). The level of bioethanol production from D-xylose, the main pentatomic sugar of PPM waste, correlates well with the activity value, as well as the coenzyme specificity of the enzyme's xylose reductase and xylitol dehydrogenase.

2. The experimental results prove the prospects of using xylose assimilating yeast strain *P. tannophilus* Y-1533 to optimize techno-ecosystems in the regions where PPI is present. However, the development of a technological concept for biorefining sulfite alkalis and lignocellulose hydrolysates requires preliminary selection of alcohol-producing strains based on *P. tannophilus* collection cultures, which have never been used as industrial biocatalysts before.

3. The successful implementation of the circular economic model will ensure the formation of regulatory feedback by closing resource flows at PPI enterprises. This will significantly reduce the toxic effect of organic waste on the lithosphere and hydrosphere of the North-West of the Russian Federation, as well as create an opportunity to obtain cheap renewable bioenergy.

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