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Stillage as a Source of Growth Promoting Biofactors and a Stimulator of Levan and Extracellular Levansucrase Synthesis for *Zymomonas mobilis*

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Summary

In the present work, the fermentation of simultaneous production of ethanol and levan by *Zymomonas mobilis* grown on different growth media has been studied. Yeast extract, rye stillage or sugar beet molasses stillage were used as additives to the basic sucrose media and the chemical composition, including vitamins, of the cultivation liquids have been determined. It has been shown that 0.5 % of yeast extract dry weight additive could be substituted by 10.0 % of native stillage additive. It was established that molasses stillage stimulates the ethanol synthesis, but rye stillage additive is more preferable for levan production. The extracellular levansucrase obtained from the culture liquid resulted in similar fructooligosaccharide-producing activities using all the above-mentioned media additives.

Key words: Zymomonas mobilis, stillage media, vitamins, ethanol, levan, levansucrase

Introduction

It is well known that many industrial fermentation media are supplemented with different sources of vitamins, amino acids, and other biologically active substances. The most popular growth media additives are corn extract, hydrolysate of soy, yeast extract and other natural products. Selection of biologically active additives depends mainly on the specific needs of a particular culture-producer as well as on the price. The wastes or by-products of industrial technologies including stillage from the ethanol production are promising *(1).* With conventional techniques, ethanol fermentation usually proceeds with a low concentration of reactants (that is, 12–20 % of aqueous feedstock solutions). Thus, the generation of stillage may amount to 10 times the production volume of alcohol *(2).* Bacteria *Zymomonas mobilis* is an interesting organism as a producer of ethanol and different by-products: levan, fructooligosaccharide (FOS), sorbitol, gluconic acid *(3,4).* The levan producer *Zymomonas mobilis* 113 »S« has been isolated in our laboratory and its cultivation media must contain several vitamins: pantothenic acid, thiamine, pyridoxine, biotin, and nicotinic acid *(5).* Usually yeast extract is used as an additive to the fermentation media in laboratory scale experiments *(4).* This raw material raises the price of the industrial fermentations and therefore the stillage was investigated as a substitute of the yeast ex-

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tract. From literature it is well known that stillage from grain as well as from molasses contains vitamins *(6)*. However, Doelle *et al. (7)* reported that the ethanol synthesising activity of *Z. mobilis* decreases in the sugar cane molasses media due to the high concentration of mineral salts. Our investigations *(8)* have shown that growth of *Z. mobilis* in sucrose media and the ethanol production activity are inhibited at high osmotic pressure, but levan producing activity is stimulated *(8)*.

The aim of this study was to investigate the chemical composition, including vitamins, of the cultivation liquid during *Z. mobilis* fermentation using sucrose media supplemented with the yeast extract, rye stillage, or molasses stillage, and to evaluate the influence of the growth media on the culture growth, ethanol and levan synthesis, and the FOS forming activity of extracellular levansucrase.

Materials and Methods

Raw materials and culture

The sugar beet molasses stillage (further-molasses stillage) was obtained from the company »RNS-D-Preili« distillery in Livani (Latvia) and rye stillage from the »Jaunpagasts Plus« Ltd. distillery (Talsi region, Latvia). Both stillages were centrifuged at 5000 rpm during 30 min, the liquid fraction was sterilised at 1 atm for 20 min and used as substitutes for the yeast extract (SIGMA, Y-1625 Lot 95HO186) in sucrose media. *Zymomonas mobilis* 113 »S« culture was used as ethanol and levan producer (*5*). The culture was maintained in a liquid media (*9*) containing 50 g/L sucrose, re-seeded after every second week and stored at 4 °C.

Preparation of media

The media for culture inoculation and fermentation were prepared from sucrose and mineral salts as described previously (*9*). The media contained 2.5 g/L KH_2PO_4, 1.6 g/L $(NH_4)_2SO_4$, 1.0 g/L Mg $SO_4 \cdot 7H_2O$. The amount of 0.5 % of yeast extract was added to the control medium. The amount of the molasses and rye stillages, as a substitute for the yeast extract, was determined in the previous experiments, using 5, 10 and 20 % (natural mass) of the volume of the media. The culture growth in the yeast extract media was practically similar to that obtained using 10 % of molasses stillage. The experiments were carried out using 10 % of molasses or rye stillage as the yeast extract substitutes.

Fermentation of Z. mobilis on sucrose media

Fermentation was carried out in 0.5 L flasks (300 mL media) without aeration and mixing. The cultivation of inoculum (the first stage of fermentation) was carried out in media with 100 g/L of sucrose at 30 °C during 24 h, levan synthesis (the second stage of fermentation) was carried out in media with 150 g/L of sucrose at 25 °C during the next 24 h. At the beginning of the second fermentation stage sucrose was added to the cultivation media.

Extracellular levansucrase isolation and determination of FOS-forming activity

The extracellular levansucrase and levan were sedimented with ethanol (75 %) from the cell free culture liquid (*10*). The FOS forming activity of the extracellular levansucrase was assayed by the incubation of levan-enzyme sediment with 60 % sucrose syrup as a substrate. Levansucrase and levan were isolated from 200 mL of the cell-free culture liquid by sedimentation with ethanol (75 %) and 5 g of wet sediment was incubated in 100 mL of sucrose syrup at 45 °C for 24 h.

Analytical methods

The cell mass in the culture liquid was determined after centrifugation for 15 min (at 6000 rpm), washing and subsequent drying at 105 °C. The optical density was measured at 590 nm in a solution diluted 10 times. The concentration of ethanol was measured by gas chromatography (Chrom 4; column Inerton AW-HDS + 5 % PEG, T_1=80 °C, T_2=200 °C).

Levan was precipitated with ethanol (75 % of volume) and determined as fructose after hydrolysis of polysaccharides (*3*). Sugar content was determined after hydrolysis using the Lane-Eynon method (*11*). Soluble solids were determined gravimetrically after dehydration of the sample at 105 °C.

The concentrations of glucose, fructose, and sucrose were determined by HPLC (column Pinacle Amino 5 μm, 250 × 4.6 with mobile phase, acetonitrile:water 75:25, refractive index detector). The yield of FOS was calculated from the estimated fructose, glucose, and sucrose content in the levan-free solution before and after hydrolysis (*12*).

The content of the principal components – carbohydrates, proteins, and lipids, in stillage was analysed spectroscopically (*13*). The concentrations of vitamins in the cultivation media: pantothenic acid, thiamine, pyridoxin, biotin and nicotinic acid were determined with microbiological methods (*14,15*).

Results and Discussion

The chemical composition of media

Grain, molasses as well as stillages after ethanol fermentation are not only valuable carbon and nitrogen sources, but also other significant microorganism growth media components (*16,17*). The infrared absorption spectra of rye stillage and molasses stillage have been compared (Fig. 1). Both IR-spectra could be divided into three spectral fingerprint ranges: 3000–2800 cm^{-1} (fatty acid region), 1660–1400 cm^{-1} (proteins, amides, nitrogen and phosphate region) and 1200–900 cm^{-1} (phosphate and carbohydrate region). In the spectra of rye stillage the most intensive broad absorption band indicates unconverted alleurone endosperm carbohydrates and starch. Nitrogen compounds are presented by a broad band in 1300–1500 cm^{-1} region. Proteins and negligible amounts of fats are also among the main rye stillage compounds. The IR-spectra of molasses stillage differ quantitatively from the one previously discussed. The main components of molasses stillage are non-protein nitrogen, pro-

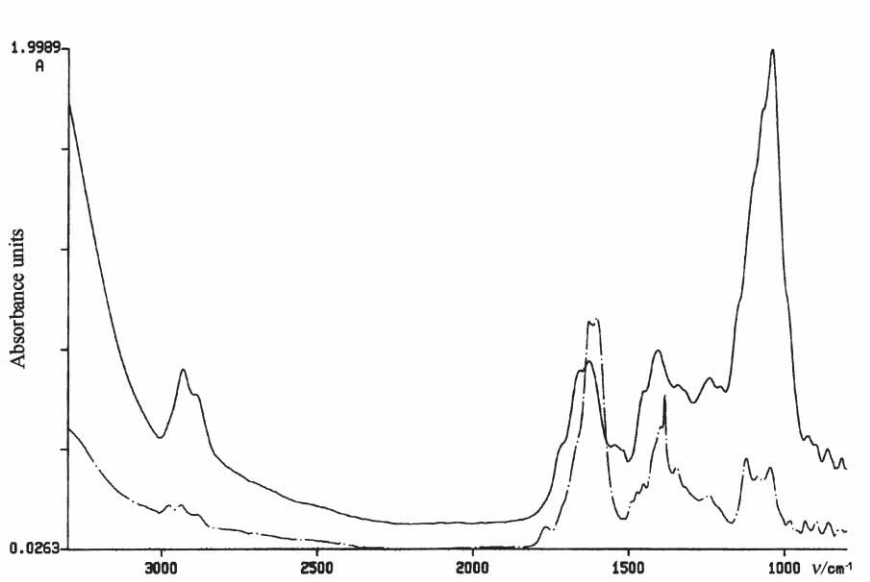

Fig. 1. FT-IR absorption spectra of stillages: — rye stillage; -·-·- molasses stillage

teins and carbohydrates, mainly sucrose. The absorption band in 1450–1300 region with maximum at 1400 cm^{-1} is characteristic for betaine. This is in agreement with the literature data (*6,17*) that nitrogen in molasses originates mainly from betaine, free amino acids and proteins, 74, 8.2 and 3.0 % of total nitrogen, respectively.

The content of vitamins in media

All three kinds of the prepared cultivation media have been analysed to determine the vitamins stimulating the growth of *Z. mobilis*. The results are shown in Table 1. The optimal concentrations of vitamins in the cultivation media for *Z. mobilis* growth are: 1 mg/L of thiamine, pyridoxine, nicotinic acid, biotin and 5 mg/L of panthotenic acid (*5*). Our results have shown that some of the limiting vitamins, necessary for *Z. mobilis* growth, appear in the tested media: in yeast media – panthotenic acid and biotin; in rye stillage media – pyridoxine, panthotenic acid and biotin; in molasses stillage media – thiamine, pyridoxine and biotin.

Results of the first fermentation stage

During the first fermentation stage (24 h) *Z. mobilis* biomass was inoculated and the concentration reached 1.49, 0.85, and 1.2 g/L in yeast (I), rye stillage (II), and molasses stillage (III) media, respectively (Fig. 2). It is necessary to note that the concentration of reducing substances (RS) reached the highest content after 12 h of cultivation and was 1.2–2.68 % in dependence of the used media. The highest RS (2.68 %) gave the molasses stillage media. This fact, probably, indicates that the chemical composition of the molasses stillage has activated the hydrolytic activity of levansucrase. During 24 h of cultivation RS in all growth media samples was practically »0«. Thus, after 24 h of inoculation the media carbohydrates were mainly converted and only the synthesised levan and sucrose remain in media.

Negligible decrease of the ethanol concentration can be seen in the rye stillage media after the first fermentation stage in correspondence with molasses stillage media. This corresponds with the decreased *Z. mobilis* biomass content in rye stillage media. High levan concentrations were obtained in yeast extract and rye stillage media and were 1.61 and 1.59 %, correspondingly. For the first fermentation stage the most important characteristics are biomass concentration and system productivity (Q_x). Calculated system productivity (Q) and product yield (Y) are shown in Table 2. The yeast extract additive resulted in the highest levan productivity of 0.3958 $g \cdot L^{-1} \cdot h^{-1}$.

Results of the second fermentation stage

During the second fermentation stage (24–48 h) mainly the synthesis of ethanol and levan occurs. Chemical composition of the fermentation media at the end of the second fermentation stage is shown in Table 3. The yeast extract additive resulted in the most effective levan synthesis while the molasses stillage stimulated ethanol synthesis. Levan production was reduced in molasses media as compared with the yeast extract and rye stillage media. The system productivity of the second

Table 1. The content of vitamins in the *Z. mobilis* growth media with yeast extract, rye stillage, and molasses stillage additives

Vitamin	The mass concentrations of vitamins/(mg/L)		
	Yeast extract media	Rye stillage media	Molasses stillage media
Thiamine	2.60	0.93	0.40
Pyridoxine	0.90	0.20	0.37
Nicotinic acid	5.40	13.40	20.00
Panthotenic acid	3.30	3.20	19.80
Biotin	0.66	0.18	0.78

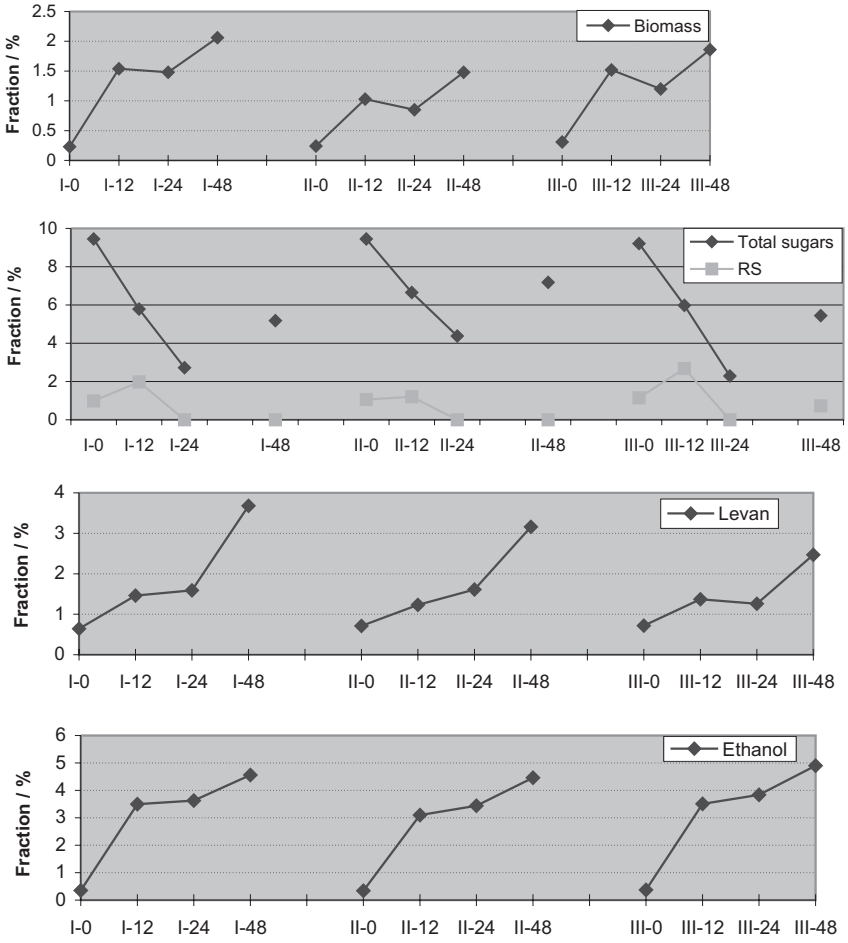

Fig. 2. Biomass and product content changes during fermentation in different cultivation media: I-yeast extract media; II- rye stillage media; III-molasses stillage media. The first fermentation stage lasts 24 h. At the beginning of the second fermentation stage sucrose has been added to the cultivation media.

fermentation stage as well as the product yields are shown in Table 2.

The aim of the second fermentation stage was to achieve high productivity of ethanol and levan synthesis as well as to obtain active levan-levansucrase sediment. In the present study, the highest ethanol production level ($Q_{P/eth}$ = 0.4613 $g \cdot L^{-1} \cdot h^{-1}$) has been produced using molasses stillage media while the highest levan production showed the yeast extract media. It can be seen that ethanol productivity at the second fermentation stage is

Table 2. The system productivity ($g \cdot L^{-1} \cdot h^{-1}$) and yields (g/g) during the first and second fermentation stages

Parameter	Yeast extract media		Rye stillage media		Molasses stillage media	
	1st stage	2nd stage	1st stage	2nd stage	1st stage	2nd stage
Q_x	0.0521	0.0252	0.0254	0.0274	0.0371	0.0287
$Q_{P/eth}$	1.3675	0.4017	1.2917	0.4439	1.4473	0.4613
$Q_{P/lev}$	0.3958	0.9087	0.3750	0.6739	0.2250	0.5261
Y_{prod}	0.5510	0.3272	0.6701	0.3170	0.5377	0.2771

Q_x = productivity of biomass production ($g \cdot L^{-1} \cdot h^{-1}$) $Q_{P/eth}$ = productivity of ethanol synthesis ($g \cdot L^{-1} \cdot h^{-1}$)

$Q_{P/lev}$ = productivity of levan synthesis ($g \cdot L^{-1} \cdot h^{-1}$) Y_{prod} = total product yield (ethanol + levan) (g/g)

Table 3. Composition/% (w/V) of the second stage fermentation media

Media	pH	RS	Total sugars	Ethanol	Levan	Biomass	Soluble solids
Yeast extract	4.30	0	5.28	4.55	3.68	2.06	5.78
Rye stillage	4.25	0	7.18	4.46	3.16	1.48	6.74
Molasses stillage	4.46	0	5.44	4.90	2.47	1.86	7.17

Table 4. Influence of the cultivation media on the FOS syrup composition/% (w/V)

Additive to the cultivation media	RS	Total sugars	Glucose	Fructose	Sucrose	FOS
Yeast extract	9.69	60.31	8.78	2.21	25.00	19.73
Rye stillage	6.80	60.20	6.85	1.36	27.12	19.47
Molasses stillage	21.49	58.8	16.46	3.46	13.20	19.91

lower than at the first stage. Levan productivity is significantly higher at the second fermentation stage. This could be explained by increased amount of extracellular levansucrase at the second fermentation stage. Total product yields (ethanol + levan) were slightly better when yeast extract or rye stillage media were used.

The changes of *Z. mobilis* biomass, total sugars, ethanol and levan content during the first and second fermentation stages using three growth media additives is shown in Fig. 2. The increase of total sugar content after 24 h of cultivation is a result of sucrose additive. The content of total sugars at the end of fermentation is high in all three fermentation media. It could be reduced by increasing the fermentation time or by utilisation of liquid fraction after obtaining ethanol and levan precipitation as water substitute for media preparation in distillery.

The activity of extracellular levansucrase

Our further experiments were carried out to study the correlation between the activity of extracelluar levansucrase and composition of the growth media. Table 4 shows that for all of the applied growth media additives the obtained FOS content in syrup was practically similar.

The ratio fructose:glucose in the FOS was approx. 4:1. It has been shown that the applied growth media additives did not influence significantly the activity of extracellular levansucrase. The results of the present study have shown that by-products from the ethanol production, rye and molasses stillage, are suitable sources of biologically active components (including vitamins of group B) of the industrial *Z. mobilis* cultivation media.

Conclusions

The FOS synthesising activity of levan-levansucrase complex does not depend on the source of biologically active compounds, yeast extract or stillages, used as additives to *Z. mobilis* cultivation media. Both stillages, molasses and rye, could be used as yeast extract substitutes in the media to obtain active levan-levansucrase sediment. The ethanol yield could be increased using molasses stillage.

References

1. T. Senn, H. Pieper, Ethanol – classical methods. In: *Biotechnology, vol.6.*, M. Rehm (Ed.), VCH Verlagsgesellschaft mbH, Weinheim (1996) pp. 59–120.
2. N. Kosaric, Ethanol – potential source of energy and chemical products In: *Biotechnology, vol.6.*, M. Rehm, (Ed), VCH Verlagsgesellschaft mbH, Weinheim (1996) pp. 121–203.
3. L. Viikari, *Appl. Microbiol. Biotechnol*. 19 (1984) 252–255.
4. M. Bekers, J. Shvinka, L. Pankova, M. Laivenieks, I. Mezhbarde, *Appl. Biochem. Biotechno.*, 24/25 (1990) 265–274.
5. M. Bekers, J. Shvinka, J. Raipulis, M. Laivenieks, L Pankova, I. Mezhbarde, *Zymomonas mobilis strain-producer of levan. LV Patent 5709* (1993)
6. M. Denshikov: *The Wastes of Food Industry and their Use*. Pischevaja Promishlenostj, Moscow (1963)
7. M. B. Doelle, P.F. Greenfield, H. W. Doelle, *Proc. Biochem. Int*. 11 (1996) 151–156.
8. A. Vigants, P. Zikmanis, M. Bekers, *Acta Biotechnol.*, 16 (1996) 321–327.
9. M. Bekers, E. Ventina, J. Laukevics, E. Kaminska, D. Upite, A. Vigants, *Acta Biotechnol.* 3 (1997) 265–275.
10. M. Bekers, J. Laukevics, D. Upite, E. Kaminska, A. Vigants, U. Viesturs, *LV Patent application P-00-173.* (2000a).
11. E. Velikaya, V. Suchodol, V. Tomashevich: *General Control Methods in Fermentation Industry.* Pischevaja promishlennostj, Moscow (1964).
12. M. Bekers, A. Vigants, J. Laukevics, M. Toma, A. Rapoports, P. Zikmanis, *Int. J. Food Microbiol*. 55 (2000) 147–150.
13. M. Grube, J. Zagreba, E. Gromozova, M. Fomina, *Vibr. Spectr*. 19 (1999) 301–306.
14. E. N. Odincova: *Microbiological Determination of Vitamins*. USSR Academy of Sciences, Moscow (1959)
15. L. Vulfa: *Methodical Guidelines for Determination of Vitamin B_6*. University of Latvia, Riga (1977)
16. R. I. Gvozdjak, E. F. Grigoriev, M. S. Matyshevskaja, I. I. Maiko, O. A. Litvischuk, V. I. Bessmertnaja, *Microbiologycheskij zurnal,* 48 (1986) 14–17.
17. E. Stoppork, K. Buchholz, Sugar-based raw materials for fermentation applications. In: *Biotechnology, vol.6.*, M. Rehm (Ed.), VCH Verlagsgesellschaft mbH, Weinheim (1996) pp. 5–30.

Džibra kao izvor biofaktora promocije rasta i stimulator sinteze levana i ekstracelularne levansaharoze u *Zymomonas mobilis*

Sažetak

U radu je istraživana istodobna proizvodnja etanola i levana fermentacijom *Z. mobilis* na raznim podlogama. Ekstrakt kvasca, džibra od rižine melase ili melase šećerne repe upotrijebljeni su kao dodaci osnovnoj šećernoj otopini, a određen je kemijski sastav podloge za uzgoj, uključujući vitamine. Pokazalo se da se 0,5 % udjela suhe tvari kvaščeva ekstrakta kao aditiva može zamijeniti s 10,0 % izvorne džibre. Utvrđeno je da melasna džibra stimulira sintezu etanola, a džibra je rižine melase povoljnija za proizvodnju levana. Ekstracelularna levansaharoza dobivena iz podloge za uzgoj, koristeći sve navedene dodatke, pokazala je sličnu aktivnost proizvodnje fruktooligosaharida.