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Intensification of higher alcohols biosynthesis – an advanced feedstock for biofuel production

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Abstract

At present the problem of alternatives to fossil fuel is so critical that it sets an objective to search optimal renewable feedstock for biofuel. Being an alcohol production by-product fussel oil consists of higher volatile alcohols and can become such feedstock. Fusel oil is theoretically possible to process by the alcohol to jet (AtJ) method converting it into biojet fuel. Thus, it is reasonable to intensify the higher alcohols biosynthesis to increase the efficiency of biofuel production. However, it tends to be problematic to reach higher than average 0.35 % of ethanol yield of higher alcohols within industrial conditions. This paper shows the increase of up to 0.82 % of ethanol under modeled industrial conditions. Also the theoretical maximum of achievable increasing of up to 3.5 % of ethanol has been calculated.

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Keywords: fusel oil; higher alcohols; biofuel; intensification

1. Introduction

The interest to biofuels has increased through the last years. One of the most important questions in biofuel production is the searching of potential renewable feedstock. The feedstock ranges from different wastes, macro- and microalgae to plant oils, potato, beef tallow and tobacco [1–5]. It has been estimated that these types of feedstock tend

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to decrease CO_2 emissions from transport [6]. One of such feedstocks can be higher volatile alcohols (HVA) producing in ethanol fermentation of carbohydrate substrates by yeast. Higher alcohols are separated from ethanol during rectification and traditionally named "fusel oil". Fusel oil essentially contains isobutyl and isoamyl alcohols in ratio 1:4. Fusel oil yields produced during fermentation can range from 0.1 to 1.1 % (average 0.35 %) of ethanol depending on the processing feedstock and fermentation conditions [7–9]. With the annual worldwide fuel ethanol production of 97.2 billion liters (in 2015 [10]) the amount of fusel oil produced can be estimated as 340 million liters per year.

Fusel oils are currently treated as by-product and are not used efficiently. Therefore, methods of utilization by adding fusel oil to fossil fuel [7–9, 11] or its converting into biodiesel [12, 13] are developing. There are also ways of converting alcohols into hydrocarbon fuels [14, 15]. One of these methods is AtJ (Alcohol to Jet) the 3-stage processing including such operations as dehydration, oligomerization and hydrogenation [15].

Higher alcohols formation is considered to occur with amino acids assimilation by Ehrlich pathway in anaerobic process of alcohol fermentation. By this pathway assimilation of valine results in isobutanol formation, leucine – 3-metylbutanol-1, isoleucine – 2-metylbutanol-1 (isoamyl alcohol isomers) [16, 17].

Taking into account the interest to fusel oil the awareness of its biosynthesis regulation can allow to change technological parameters of fermentation to intensify higher alcohols yield with their further use in biofuel production.

2. Materials and Methods

2.1. Yeast strains & Medium

The industrial culture S. cerevisiaeY-2396 was obtained from Russian National Collection of Industrial Microorganisms (VKPM).

A wort of 18 % dry matter obtained by saccharifying of semolina was used as substrate for fermentation. Enzymatic hydrolysis of semolina starch was carried out under traditional industrial regime in two stages. Thermostable α-amylase was used at the first stage (pH 6.5, T = 90 ºC, dosage 0.25 ml/kg of starch) and glucoamylase at the second one (pH 5.0, T = 60 ºC, dosage 0.8 ml/kg of starch).

2.2. Analytical methods

The concentrations of isobutanol and isoamyl alcohol were determined by means of gas-liquid chromatography. The measurements were carried out in sample distillates using HP-4890 chromatograph by Hewlett-Packard packed with FFAP column 50 x 0.32 x 0.25. Helium flow rate was 7.0 ml/min. The oven temperature was set to isothermal regime of 200 ºC. The injector temperature was 220 ºC.

The inoculate concentration used was 5 g/l in all cases. The fermentation temperature was 30 ºC except for the study of fermentation temperature effect.

3. Results and discussion

3.1. Formation of higher alcohols with the use of diverse nitrogen sources

Since HVA are considered to be formed from amino acids the experiment in addition of corresponding amino acids (leucine and valine) to the fermentation media was carried out. Also, the effect of addition of ammonia nitrogen (in the form of ammonium sulphate $(NH_4)_2SO_4$) was studied in the experiment.

Table 1 shows the results of fermentation with diverse concentration of amino acids and ammonia nitrogen.

Table 1. The results of fermentation of substrates with diverse nitrogen sources.

Additive type	Fermentation rate, g/l·h	Nitrogen added, mg/l	Nitrogen used, mg/l	Concentration of higher alcohols, mg/l		HVA— ethanol, %
				Isoamyl	Isobutyl	
Leucine 2 g/l	1.8	213	256	1435.0	117.6	1.90
Leucine 3 g/l	3.0	321	368	2118.0	126.0	2.81
Valine 2 g/l	1.6	240	196	282.0	798.0	1.35
Valine 3 g/l	1.7	360	349	212.5	1625.4	2.29
Ammonium sulphate 400 mg/l	2.0	400	24	97.0	46.0	0.19
No additive	0.15	–	17	78.0	34.0	0.14

According to the fermentation rate (see Table 1), valine and leucine are complete nitrogen sources for yeasts. The assimilation of these amino acids results in accumulation of considerable HVA amounts (more than 2.5 % of ethanol). The uptake of nitrogen for valine and leucine in the absence of limitations is 350–370 mg/l. This value is close to the entire yeast demand in nitrogen during fermentation. The data reported allow us to draw the conclusion that the HVA yield is not likely to exceed 3.5 %. Providing that the nitrogen consumed by biomass during fermentation is assimilated by Ehrlich pathway and potential culture demand of nitrogen is about 350–450 mg/l.

Amino acids are products of protein hydrolysis and present in the form of mixture in industrial substrates. Cereal wort contains considerable protein amount that can provide fermenting culture with nitrogen in plenty after hydrolysis with proteolytic enzymes.

Table 2 shows the results of fermentation with the adding of different concentrations of neutral bacterial protease.

Table 2. The results of fermentation of protease hydrolyzed substrates.

No	Enzyme concentration, g/l	Fermentation rate, g/l·h	Concentration of higher alcohols, mg/l		HVA — ethanol, %
			Isoamyl	Isobutyl	
1	0.05	1.7	170.0	99.6	0.34
2	0.1	2.5	303.6	95.4	0.49
3	0.5	3.5	279.6	123.6	0.50
4	0.1 g/l protease + 400 mg/l ammonium sulphate	2.5	135.6	56.4	0.24
5	0.1 g/l protease + 1.0 g/l leucine	3.5	1009	107	1.39

As shown in Table 2 the increasing concentration of proteolytic enzyme promotes considerable raise of fermentation rate that points to good supply of culture with nitrogen while there is no significant effect on HVA yield. The leucine and enzyme addition increase HVA biosynthesis. Otherwise, the ammonium addition still decreases higher alcohols yield.

Low HVA yield is easily explained with parallel assimilation of ammonium or amino acids that are not HVA formable. Experiments on substrates supplemented with binary leucine and valine mixtures with ammonium and the most common amino acids have clarified the nature of parallel assimilation.

Table 3. The results of fermentation of substrates with diverse nitrogen sources binary mixtures.

Nitrogen nutrition composition	**Fermentation rate, g/l·h**	**HVA yield, %**
Leucine, 3 g/l	3.2	100
Valine, 3 g/l	3.0	100
Leucine, 3 g/l + Glutamic acid, 3 g/l	3.0	92
Valine, 3 g/l + Glutamic acid, 3 g/l	3.0	85
Leucine, 3 g/l + Aspartic acid, 3 g/l	3.2	87
Valine, 3 g/l + Aspartic acid, 3 g/l	3.0	80
Leucine, 3 g/l + Glutamine, 3 g/l	3.5	103
Valine, 3 g/l + Glutamine, 3 g/l	3.2	90
Leucine, 3 g/l + Asparagine 3 g/l	3.5	32
Valine, 3 g/l + Asparagine 3 g/l	3.5	25
Leucine, 3 g/l + Alanine, 3 g/l	2.8	97
Valine, 3 g/l + Alanine, 3 g/l	2.5	90
Leucine, 3 g/l + Tryptophan, 3 g/l	3.0	84
Valine, 3 g/l + Tryptophan, 3 g/l	2.5	90
Leucine, 3 g/l + Phenylalanine, 3 g/l	2.8	80
Valine, 3 g/l + Phenylalanine, 3 g/l	2.5	85
Leucine, 3 g/l + Ammonium sulphate, 3 g/l	2.7	35
Valine, 3 g/l + Ammonium sulphate, 3 g/l	2.2	27

As it is seen from Table 3 asparagine and ammonia nitrogen decrease HVA yield significantly. These results allow to suppose ammonium and amino acids are alternative nitrogen sources for yeasts. However, in terms of target products that are higher alcohols they can be regarded as inhibitors of HVA formation process.

The Inhibition of the process by ammonium and asparagine is well described by the kinetics of uncompetitive suppression. Analysis of the fermentation parameters made by Lineweaver-Burk graphic method allows calculating the constants of the inhibition. The constants are shown in Table 4.

Table 4. The kinetic constants of inhibition.

Substrate – Inhibitor	Maximum of substrate assimilation rate, mg/l·h	Inhibition constant, mg/l
Leucine – Asparagine	120	750
Leucine – Ammonium sulphate	120	730
Valine – Asparagine	85	650

Table 4 shows that the maximums of substrate assimilation rate and constant of inhibition of valine are considerably lower than of leucine. Thus, isobutanol formation is always lower than isoamyl alcohol formation. This aspect characterizes known isobutanol:isoamyl alcohol ratio of 1:4 in fusel oil.

Close values of constants of inhibition for asparagine and ammonium sulphate highlight that inhibition with ammonium forming during deamination of asparagine takes place in all cases.

The conclusions from experiments in inhibition by amino acids shows that the considerable increase in higher alcohols yield is hard to achieve using substrates supplemented with hydrolyzed cereal protein. Asparagine is one of the essential components of cereal and yeast biomass protein. However, the biomass protein contains less asparagine than the cereal one. Being amides asparagine and glutamine dissociate with ammonium formation in acid hydrolysis. Thus, the use of any protein hydrolyzates expels the possibility of the elimination of ammonium or asparagine inhibition of HVA biosynthesis.

It is possible to add mixtures of amino acids resulting from different protein types to increase the fusel oil yield in alcohol production. For example, it can be cereal or biomass protein obtained in aerobic cultivation of yeast. Biomass protein is more preferable since the ratio leucine:asparagineis 1.4 for it against 0.5 for cereal protein.

Table 5 shows the results of wort with diverse concentration of yeast autolysate fermentation.

Table 5. The results of fermentation of substrates with diverse adding of yeast autolysate.

No	Autolysate concentration, % (nitrogen mg/l)	Fermentation rate, g/l·h	Concentration of alcohol, mg/l		HVA — ethanol, %
			Isoamyl	Isobutyl	
1	2.0 (60.0)	1.5	318	110	0.53
2	5.0 (150.0)	2.5	474	184	0.82
4	7.0 (210.0)	4.2	470	192	0.82
5	10.0 (300.0)	3.7	456	186	0.80
6	15.0 (450. 0)	4.5	218	67	0.35
7	20.0 (600.0)	4.5	150	47	0.25
8	Autolysate 10 % + Leucine 1.0 g/l	5.0	976	97	1.34

As it is shown in Table 5 the autolysate use resulted in the increase of the HVA yield up to 0.82 % of ethanol. This increase of the HVA biosynthesis is significant taking into account the average 0.35 %. One can notice that the HVA yield dependence on the autolysate concentration in substrate has extreme type with the extreme point between 5 % and 7 %. The increase of the HVA yield and the fermentation rate is seen until the concentration of autolysate of 7 %. Raising the concentration of autolysate up to 10 % and more decreases the HVA yield with remaining fermentation rate. This is the consequence from asparagine inhibition.

3.2. The effect of pH

The fermentation process rate is known to be determined by fermentation conditions. According to this, a number of experiments to estimate the pH effect on the HVA biosynthesis processes were carried out.

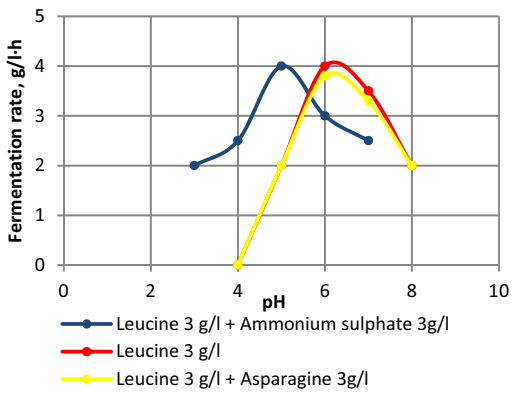

Fig. 1. Fermentation rate against pH plot.

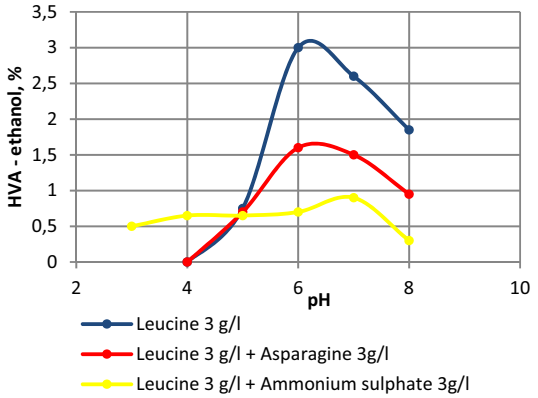

Fig. 2. HVA of ethanol yield against pH plot.

Fig. 1 and Fig. 2 show plots of the fermentation rate and the HVA biosynthesis change against the pH. The optimums of the fermentation rate and the HVA yield lie in area of pH 6.0 with leucine as the only nitrogen source. The addition of ammonium causes the optimum of the fermentation rate shifting to pH 5.0 and the optimum of the HVA yield shifting to pH 7.0. The fermentation stops at pH 4.0 with amino acids as nitrogen source, then the fermentation goes at acceptable rate even at pH 3.0 with addition of ammonium.

3.3. The effect of temperature

The temperature effect on the HVA biosynthesis has typical extreme type which is common to all biosynthesis processes with the extreme point at 20 ºC. This result correlates with fermentation temperature optimum of used S. cerevisiae strain Y-2396 (from VKPM).

Table 6. The results of fermentation at diverse temperatures.

No	Fermentation temperature, ºC	HVA concentration, mg/l	HVA — ethanol, %
1	12	93.6	0.13
2	20	269.7	0.35
3	30	195.5	0.25
4	38	158.9	0.28

4. Conclusions

The amino acids assimilation and the higher alcohols formation are regulated by nitrogen metabolism in yeast cells. Asparagine, ammonium and low pH values of fermenting media act as inhibitors of higher alcohols formation.

The intensification of biosynthesis of higher alcohols within alcohol production can be performed by means of amino acids addition with minimum of asparagine and ammonia nitrogen content. A prospective way to intensify the HVA biosynthesis is the development of S. cerevisiae strain aiming to minimization of asparagine assimilation.

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