

Biodegradation of main carbon sources in vinasse stillage by a mixed culture of bacteria: influence of temperature and pH of the medium

Agnieszka Ryznar-Luty, Edmund Cibis and Krzysztof Lutosławski

ABSTRACT

The aim of the study was to examine how temperature and the pH influence the progress and efficiency of an aerobic biodegradation process, where major organic pollutants are removed from beet molasses vinasse by a mixed culture of *Bacillus* bacteria. It was conducted in an aerated bioreactor with a stirring system in four experimental series, each composed of five processes run at temperatures of 27, 36, 45, 54 and 63 °C. In the first and second series, medium pH was not controlled, the initial pH amounted to 6.5 and 8.0, respectively. In the third and fourth series, medium pH was controlled at 6.5 and 8.0, respectively. Under optimal conditions, the pollution load of the vinasse stillage expressed as soluble chemical oxygen demand was removed with an 88.73% efficiency. The bacterial culture assimilated all organic pollutants simultaneously, but the rate of assimilation was different. An exception was the process of betaine assimilation, which intensified only when readily available carbon sources were depleted in the medium. Synthesis and assimilation of organic acids were observed in all experiments. Advantages of the proposed method include: possibility of its use at high temperatures, and no necessity for medium pH adjustment during the process.

Key words | aerobic biodegradation, *Bacillus*, glycerol, organic acids, reducing substances, vinasse

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INTRODUCTION

Aerobic processes of bio-treatment under thermo- and mesophilic conditions represent an effective method of utilisation of highly contaminated wastewater from the food industry, including also stillage (Cibis *et al.* 2006, 2011; Krzywonos *et al.* 2009, 2017; Ryznar-Luty *et al.* 2015). In these processes the optimum temperature is between 30 °C and 58 °C (LaPara *et al.* 2000), and they involve predominantly bacteria of the genus *Bacillus* because of their significant metabolic potential. In most instances these are mixed cultures characterised by higher stability and activity as compared with monocultures, which is probably due to the synergic interactions among the microorganisms (LaPara *et al.* 2002; Jang *et al.* 2013; Kosseva & Kent 2013; Kim *et al.* 2014; Awasthi *et al.* 2018).

Processes of aerobic thermo- and mesophilic degradation are more suitable for small and medium treatment plants processing highly loaded wastewater from the food

industry. They are characterised by a high rate of organic compounds degradation. Under thermophilic conditions, they additionally result in the inactivation of pathogens and formation of small volumes of biomass. In turn, anaerobic processes are more suitable for large plants treating highly-contaminated wastewater, as they ensure a valuable end-product, i.e. biogas. Unfortunately, this method is not cost-effective for medium and small plants due to high investment costs and time-consumption of the treatment processes (Jang *et al.* 2013).

Comparative studies show that under thermophilic conditions the rate of reduction in the pollution load is higher (Kosseva *et al.* 2001; Collivignarelli *et al.* 2014) and the extent of pollutant removal is lower than under mesophilic conditions (LaPara *et al.* 2000). Studies on the influence of temperature on the efficiency of aerobic biodegradation of industrial effluents have been reported by many researchers. Although during those studies little

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attention was given to the kinetics of assimilation of particular organic pollutants, it was observed that thermophilic cultures had a limited potential for simultaneous metabolism of different substrates as compared with mesophilic cultures (LaPara *et al.* 2000; Kosseva *et al.* 2001; Cibis *et al.* 2002; Krzywonos *et al.* 2009).

In the literature fewer reports are found that pertain to the influence of pH control on the progress and efficiency of high-strength wastewater biodegradation than to the influence of temperature on the same process parameters. According to Henze *et al.* (2002), biodegradation without pH control is justified when the wastewater being treated shows a high buffering capacity, and when the pH is maintained within the range 6.0–9.0, which does not disturb the activity of the bacteria. Research on optimising the initial pH value of fruit stillage (Beltran *et al.* 2001) and starch stillage (Krzywonos *et al.* 2002) treated by aerobic biodegradation has revealed that the highest extent of chemical oxygen demand (COD) reduction is achieved when the initial pH ranges from 7 to 7.8, and that biodegradation of starch-based stillage with controlled pH should be conducted at a constant pH value ranging between 6.5 and 7.0 (Cibis 2004).

The literature contains only a few references to the problem of how the temperature or pH of the aerobic biodegradation process affects the assimilation of organic pollutants that are present in the distillery stillage being treated. Krzywonos *et al.* (2009) gave a detailed description of how temperature influences the sequence and rate of removing major organic pollutants from potato slops during biodegradation processes conducted over the temperature range of 20 to 63 °C at the pH of the medium amounting to 7.0. Cibis (2004) scrutinised the relationship between the pattern of organic matter assimilation in starch-based stillage and the pH (6.5 to 9.0) of the medium being biodegraded at 45 °C. The issue of how the control of the pH of the medium influences the assimilation of the organic vinasse stillage components at 38 °C and initial pH of 8.0 was the focus of the study reported by Lutosławski *et al.* (2011), and also of the investigations carried out by Ryznar-Luty *et al.* (2008) at the temperature of 58 °C and initial pH of 8.35. To the authors' knowledge, no attempts have been reported in the literature to examine the effect of temperature, initial pH of the medium, and pH control on the kinetics of organic matter assimilation during aerobic biodegradation of vinasse stillage. The aim of this work here was to investigate how these three factors affect the assimilation of main organic pollutants during aerobic biodegradation of

vinasse stillage by a mixed culture of bacteria of the genus *Bacillus*.

MATERIALS AND METHODS

Microorganisms

The study was conducted with a mixed culture of bacteria which included seven strains of the *Bacillus* genus. Two belonged to the species *B. circulans*, whereas the other five belonged to the species *B. laterosporus*, *B. filicolonicus*, *B. stearothermophilus*, *B. acidocaldarius* and *B. licheniformis*. The origin of the culture, its composition and method of its storage were provided in our previous works (Cibis *et al.* 2011; Ryznar-Luty *et al.* 2015).

Vinasse characterisation

Vinasse subjected to the biodegradation process originated from CHEKO plant, Włocławek, Poland. Its characteristics determined by authors of this manuscript were as follows: pH 4.97 ± 0.01 , density 9.40 ± 0.10 degrees Balling, soluble COD (SCOD) 57.39 ± 0.35 g O₂/L, SCOD_{sum} 104.64 ± 2.48 g O₂/L (SCOD_{sum} = SCOD determined with the dichromatic method + theoretical COD of betaine which is not detected with the dichromatic method), biochemical oxygen demand (BOD₅) 36.40 ± 2.14 g O₂/L, and total organic carbon (TOC) 30.75 ± 1.23 g/L. The concentration of suspended solids was 4.641 ± 0.44 g/L, betaine was 22.53 ± 1.17 g/L, pyroglutamic acid was 5.568 ± 0.178 g/L, lactic acid was 2.913 ± 0.148 g/L, acetic acid was 1.509 ± 0.082 g/L, glycolic acid was 0.871 ± 0.032 g/L, valeric acid was 0.242 ± 0.011 g/L, butyric acid was 0.151 ± 0.007 g/L, formic acid was 0.128 ± 0.006 g/L, citric acid was 0.025 ± 0.0022 g/L, reducing substances before hydrolysis was 3.350 ± 0.071 g/L, reducing substances after hydrolysis was 7.710 ± 0.221 g/L, glycerol was 3.333 ± 0.117 g/L, coloured substances was 19.73 ± 1.43 g/L, total nitrogen was 4.004 ± 0.165 g/L, ammonia nitrogen was 0.187 ± 0.005 g/L, total phosphorus was 0.056 ± 0.0021 g/L, orthophosphate as phosphorus was 0.011 ± 0.0002 g/L, potassium was 7.160 ± 0.201 g/L, and magnesium was 0.011 ± 0.0004 g/L. Values following the sign '±' denote standard deviation, $n = 3$.

To eliminate potential microbiological contaminations, induced during transport and preparation for storage, vinasse was cooked for 15 min. Culture medium was not sterilised. Considering insufficient phosphorus concentration in the vinasse, it was supplemented with

$\text{NH}_4\text{H}_2\text{PO}_4$ in a dose of 0.9 g/L after cooling. Afterwards, its pH was adjusted to values assumed in the experimental design using 33% NaOH.

Biodegradation processes

Four series (spanning 168 h each) of periodical biodegradation processes were conducted in a 5 L stirred tank reactor type Biostat[®]B bioreactor (B. Braun Biotech International). Aeration reached 1 vvm (volume of air/(volume of medium·minute)), and stirring rate reached 900 rpm. Each series was composed of five processes run at temperatures of 27, 36, 45, 54 and 63 °C. In the first and second series, medium pH was not controlled and the initial pH amounted to 6.5 and 8.0, respectively. In the third and fourth series, medium pH was controlled at 6.5 and 8.0, respectively.

Stirring rate, process temperature and pH, concentration of dissolved oxygen (pO_2), aeration rate, and volume of utilised neutralising solution were continuously monitored. The aeration rate was set manually, whereas the stirring rate, temperature and pH were controlled automatically. The pH value was controlled only when needed, accordingly to study design. Then, its stable value was maintained using 2M H_2SO_4 and 2M NaOH. Liquid level and foam level in the bioreactor were also controlled automatically. Vinasse stillage losses due to evaporation were replaced with distilled water.

Analytical methods

Suspensions were separated from the collected samples by centrifugation at 13,000 rpm for 40 min in a Sigma[®] 4K15 centrifuge. Suspended solids were determined with the gravimetric method, by drying the sample at a temperature of 50 °C for 1 day and then at 105 °C until constant weight was reached. Supernatants were used for analyses. Concentrations of COD, BOD_5 , TOC, total phosphorus and orthophosphate as phosphorus were determined using Hach-Lange spectrophotometric cuvette tests (*Handbuch der photometrischen Betriebsanalytik 2002*). Concentrations of reducing substances were assayed with the Lane–Eynon method. Concentration of total nitrogen was determined with the Kjeldahl method, that of ammonium nitrogen with the distillation method, and that of potassium and magnesium with flame photometry. Concentrations of glycerol, coloured substances and betaine were assayed spectrophotometrically (*Cibis et al. 2011*), whereas organic acids – with the high-performance liquid chromatography method (Varian Pro Star, USA; column type Aminex

HPX-87 H, column size 7.8 mm i.d. 300 mm, eluent 0.004 M H_2SO_4 , flow rate 1.2 mL/min).

RESULTS

Regardless of whether the process was conducted with or without pH control of the medium, the mixed culture of *Bacillus* bacteria had the ability to provide high-efficiency aerobic biodegradation of beet molasses vinasse over a wide range of temperature. The highest extent of SCOD_{sum} removal (88.73%) was achieved at 36 °C in the process with controlled pH, which amounted to 6.5 (*Table 1*).

Among the organic substances being components of the vinasse, glycerol was removed by the bacteria at the fastest rate in the majority of experiments (*Figure 1*, which refers to processes with no pH control; $\text{pH}_0 = 8.0$). In all processes with uncontrolled pH, except two of them, more than 90% of the glycerol removed in the entire process was biodegraded before 48 h. The exception lies in the processes conducted at high temperatures ($T = 54$ °C, $\text{pH}_0 = 6.5$ and $T = 63$ °C, $\text{pH}_0 = 8.0$), where 90% of glycerol removed was not biodegraded until 72 h. When the processes were carried out with controlled pH amounting to 6.5, more than 90% of glycerol was assimilated within 24 h at the temperatures of 54 °C and 63 °C; at controlled pH of 8.0, within the same time span, equally high quantities of glycerol were utilised by the bacteria at 27, 36, 54 and 63 °C. As for the other experiments, such results were achieved in the time span of 24 h to 48 h (data not shown). Only in one of the 20 experiments, the extent of glycerol biodegradation fail to exceed 90% ($T = 54$ °C; $\text{pH} = 6.5$, controlled) (*Table 1*). Control of the pH did not significantly increase the efficiency of glycerol removal from the vinasse stillage ($p\text{-value} = 0.7728$ for two-way t -test and $p = 0.3864$ for one-way t -test). In six cases the reduction in the glycerol content of the medium was even found to decline (by several per cent) as compared to relevant experiments with no pH control (*Table 1*).

A constant pH accounted for an increase in the removal of reducing substances determined both before and after hydrolysis. For those determined upon hydrolysis, the rise in removal efficiency was as high as 23.49% at $T = 54$ °C and $\text{pH} = 8.0$ (*Table 1*). Only in the experiment performed at 36 °C and $\text{pH} = 8.0$ was the extent of reducing substances removal lower than in the experiment carried out at the same temperature but with no pH control (at $\text{pH}_0 = 8.0$) (*Table 1*). In nearly all experiments, reducing substances

Table 1 | Removal of SCOD_{sum} and major vinasse components under various biodegradation conditions [%] (data obtained during processes conducted with controlled pH (Cibis et al. 2011), extent of reduction in SCOD_{sum} and betaine during processes conducted with uncontrolled pH (Ryznar-Luty et al. 2015))

PARAMETER	pH	pH ₀ = 6.5					pH ₀ = 8.0				
		27 °C	36 °C	45 °C	54 °C	63 °C	27 °C	36 °C	45 °C	54 °C	63 °C
SCOD _{sum}	NC	36.42 ± 2.78 ^a	83.86 ± 0.53	83.61 ± 0.41	40.72 ± 2.47	33.72 ± 3.87	77.74 ± 0.82	85.41 ± 0.51	39.47 ± 2.70	35.44 ± 2.67	35.02 ± 3.78
	C	84.47 ± 0.60	88.73 ± 0.68	87.02 ± 0.43	41.32 ± 2.53	38.07 ± 2.60	84.71 ± 0.62	86.44 ± 0.88	86.86 ± 0.54	83.68 ± 0.58	40.00 ± 2.78
Betaine	NC	0 ± 6.66	100 ± 0	100 ± 0	0 ± 6.90	0 ± 9.88	86.37 ± 0.95	100 ± 0	0 ± 6.79	0 ± 7.23	0 ± 9.77
	C	93.36 ± 0.69	100 ± 0	100 ± 0	0 ± 6.12	0 ± 5.74	93.64 ± 0.74	100 ± 0	100 ± 0	100 ± 0	0 ± 5.98
Sum of organic acids	NC	90.70 ± 0.87	92.34 ± 0.95	94.33 ± 0.74	78.76 ± 0.48	80.03 ± 0.31	90.94 ± 0.65	93.49 ± 0.94	96.63 ± 0.76	61.88 ± 0.39	49.71 ± 0.89
	C	88.35 ± 0.31	89.09 ± 1.18	88.90 ± 0.55	90.71 ± 0.44	90.55 ± 0.37	89.11 ± 0.58	94.72 ± 0.33	91.93 ± 0.92	93.03 ± 0.42	88.41 ± 1.08
Pyroglutamic acid	NC	99.62 ± 0.10	99.67 ± 0.08	99.78 ± 0.12	98.42 ± 0.13	95.86 ± 0.09	99.71 ± 0.13	99.79 ± 0.11	100 ± 0	71.39 ± 0.09	100 ± 0
	C	99.74 ± 0.13	100 ± 0	99.80 ± 0.09	100 ± 0	100 ± 0	99.71 ± 0.15	99.78 ± 0.13	99.80 ± 0.11	100 ± 0	100 ± 0
Lactic acid	NC	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	97.39 ± 0.65
	C	100 ± 0	100 ± 0	100 ± 0	98.75 ± 0.87	99.12 ± 0.23	100 ± 0	100 ± 0	100 ± 0	81.76 ± 1.35	98.22 ± 0.36
Acetic acid	NC	87.83 ± 0.87	100 ± 0	96.48 ± 0.75	96.98 ± 0.49	100 ± 0	91.67 ± 0.76	100 ± 0	91.21 ± 0.82	95.95 ± 0.90	ic
	C	95.05 ± 0.63	100 ± 0	100 ± 0	99.23 ± 0.21	97.04 ± 0.63	96.70 ± 0.94	100 ± 0	100 ± 0	97.21 ± 0.75	86.87 ± 1.25
Glycolic acid	NC	25.13 ± 1.23	43.89 ± 1.50	70.74 ± 1.16	100 ± 0	78.02 ± 1.09	15.74 ± 1.34	40.20 ± 1.28	85.30 ± 1.61	100 ± 0	78.28 ± 1.04
	C	ic	ic	ic	ic	ic	ic	51.20 ± 1.19	12.51 ± 1.68	100 ± 0	ic
Valeric acid	NC	100 ± 0	100 ± 0	58.4 ± 1.32	25.82 ± 0.95	96.09 ± 1.28	100 ± 0	100 ± 0	88.62 ± 1.09	53.69 ± 0.86	100 ± 0
	C	85.58 ± 1.55	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	70.52 ± 1.86	100 ± 0	67.98 ± 1.31
Butyric acid	NC	ic	ic	ic	ic	ic	ic	ic	45.32 ± 0.91	ic	ic
	C	45.12 ± 1.97	80.91 ± 0.95	ic	60.83 ± 1.80	ic	23.58 ± 2.11	27.87 ± 1.54	43.50 ± 1.76	43.21 ± 1.83	ic
Formic acid	NC	100 ± 0	100 ± 0	100 ± 0	ic	ic	100 ± 0	100 ± 0	100 ± 0	ic	100 ± 0
	C	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
Citric acid	NC	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
	C	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
Reducing substances after hydrolysis	NC	67.29 ± 1.05	80.69 ± 0.89	64.61 ± 0.72	56.09 ± 1.15	46.94 ± 1.34	75.93 ± 0.86	79.99 ± 0.95	69.27 ± 0.69	48.04 ± 1.31	55.38 ±
	C	80.24 ± 1.03	87.59 ± 0.81	87.63 ± 0.71	71.44 ± 1.24	67.11 ± 0.94	87.73 ± 0.61	75.19 ± 1.31	72.68 ± 0.95	71.53 ± 0.73	70.46 ± 0.76
Reducing substances before hydrolysis	NC	87.07 ± 0.79	87.53 ± 0.82	84.11 ± 0.98	84.03 ± 0.61	84.01 ± 0.94	83.84 ± 0.59	84.01 ± 0.66	84.72 ± 0.71	82.23 ± 0.84	83.53 ± 0.68
	C	93.68 ± 0.85	94.42 ± 0.97	94.24 ± 0.67	88.64 ± 0.75	87.95 ± 0.68	92.89 ± 0.61	90.17 ± 0.98	86.36 ± 0.79	85.35 ± 0.63	87.89 ± 0.74
Glycerol	NC	92.35 ± 0.83	100 ± 0	91.52 ± 0.70	95.57 ± 0.48	90.62 ± 0.97	95.60 ± 0.51	98.52 ± 0.64	91.26 ± 0.42	90.98 ± 0.57	96.92 ± 0.61
	C	92.13 ± 0.60	92.18 ± 1.22	95.31 ± 0.38	88.34 ± 0.90	94.20 ± 0.26	93.34 ± 0.38	96.94 ± 0.47	96.46 ± 0.41	96.33 ± 0.68	93.53 ± 0.33

NC, non-controlled pH; C, controlled pH; ic, increased content.

^aValues following the sign ' ± ' denote standard deviation, *n* = 3.

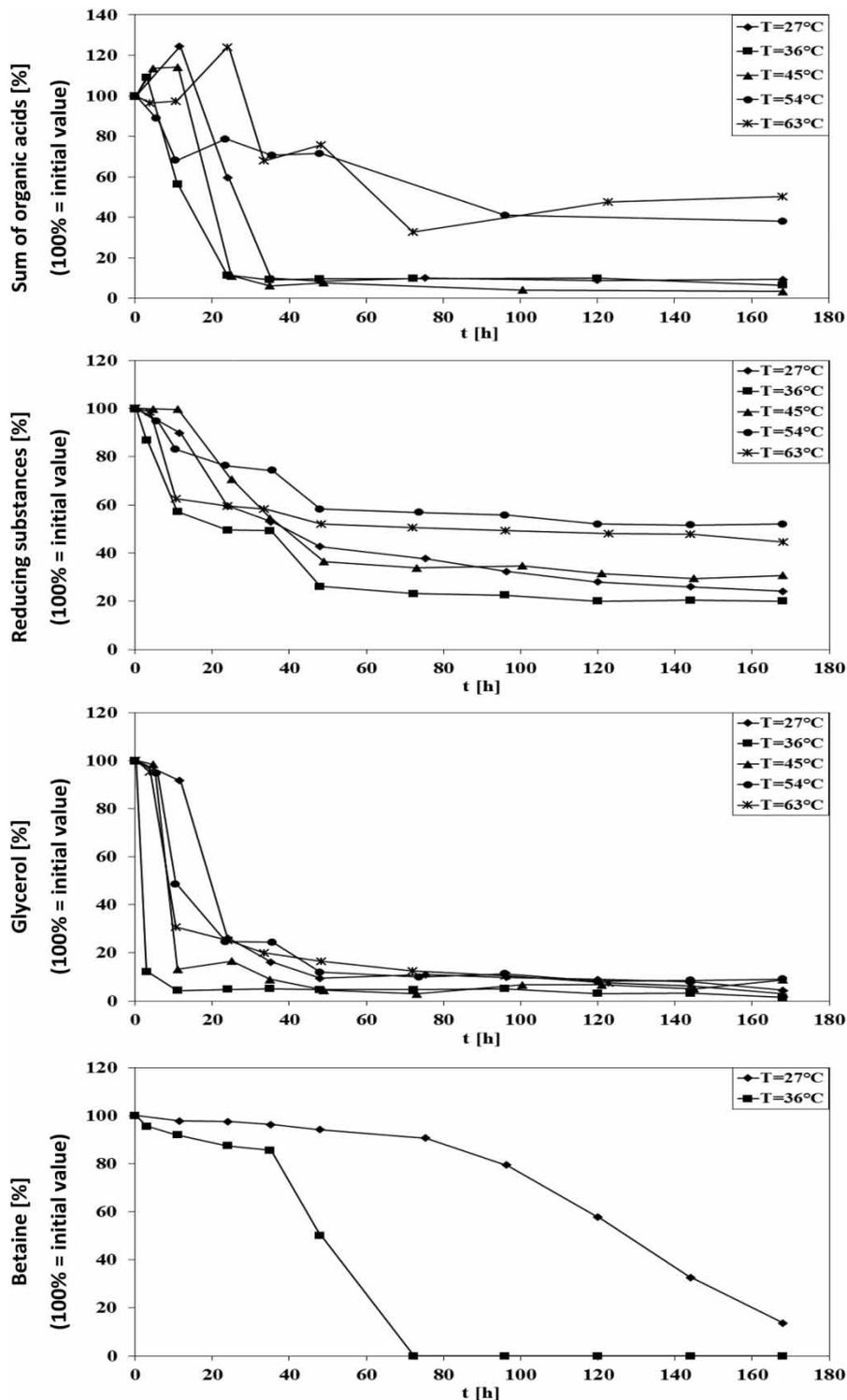


Figure 1 Assimilation kinetics for the sum of organic acids, reducing substances determined upon hydrolysis, glycerol and betaine during vinasse stillage biodegradation in the bioreactor, without pH control, at initial pH of 8.0.

were assimilated at a slower rate than was glycerol (Figure 1, which refers to processes with no pH control; $\text{pH}_0 = 8.0$).

Variations in the content of the sum of organic acids observed in the medium (Figure 1) during biodegradation were associated with the occurrence of the phenomenon

of their assimilation and synthesis. In most instances, at the beginning of the phase of intense microbial growth, lactic acid was synthesised (Figure 2) (at $T = 63\text{ }^{\circ}\text{C}$ and uncontrolled pH at $\text{pH}_0 = 8.0$, lactic acid was formed in the highest amount, which was 3.91 g/L). A few exceptions to that rule, when lactic acid was not produced, include processes with pH control, which were conducted at $27\text{ }^{\circ}\text{C}$ and $\text{pH} = 6.5$; at $45\text{ }^{\circ}\text{C}$ and $63\text{ }^{\circ}\text{C}$ with $\text{pH} = 8.0$, as well as the process without pH control, which was performed at $36\text{ }^{\circ}\text{C}$ with $\text{pH}_0 = 6.5$.

The period of increased oxygen demand was concomitant with the initiation of valeric acid production observed in all experiments. In those without pH control, the largest amounts were synthesised at $54\text{ }^{\circ}\text{C}$ and $63\text{ }^{\circ}\text{C}$ (ranging from 1.88 ($T = 54\text{ }^{\circ}\text{C}$, $\text{pH}_0 = 8.0$) to 3.78 g/L ($T = 54\text{ }^{\circ}\text{C}$, $\text{pH}_0 = 6.5$)), and the lowest (0.05 g/L) were obtained at $36\text{ }^{\circ}\text{C}$ and $\text{pH}_0 = 6.5$. When the pH was controlled at the level of 8.0 , the highest quantities of valeric acid (2 g/L) were produced at $45\text{ }^{\circ}\text{C}$; with pH controlled at the level of 6.5 , the amount of valeric acid formed did not exceed 0.22 g/L (data not shown).

Acetic acid was produced in large amounts in all of the experiments performed. The highest quantities, which ranged from 1.01 to 4.05 g/L , were obtained in three processes at $45\text{ }^{\circ}\text{C}$: two without pH control ($\text{pH}_0 = 6.5$ and $\text{pH}_0 = 8.0$, respectively) and one with pH controlled at 6.5 ; as well as in two processes at the temperature of $63\text{ }^{\circ}\text{C}$: one without pH control ($\text{pH}_0 = 8.0$) and one with pH controlled at 8.0 (Figure 3 referring to processes with no pH control).

Synthesis of lactic, valeric and acetic acids was followed by their assimilation in the medium. After a short period they either were totally utilised by the microorganisms present in the medium or occurred there in very small amounts. There were, however, two exceptions to this pattern. One was in the process performed without pH control at $T = 63\text{ }^{\circ}\text{C}$ and $\text{pH}_0 = 8.0$, where acetic acid content of the medium continued to increase (to the final value of 6.14 g/L) (Figure 3). The other one was in the experiment carried out at $54\text{ }^{\circ}\text{C}$ with controlled pH of 8.0 , where after 120 h lactic acid was synthesised again (Figure 2).

Butyric acid was synthesised in each experiment, but the quantities produced were low (0.3 g/L at the most, with $T = 27\text{ }^{\circ}\text{C}$ and $\text{pH}_0 = 6.5$, uncontrolled). In the processes with no pH control, only once (at $T = 45\text{ }^{\circ}\text{C}$ and $\text{pH}_0 = 8.0$) was this acid partly utilised by the microorganisms in the medium (45.32%). In the other processes the final content of butyric acid was higher than the initial one. Under conditions of controlled pH, after the concentration of the acid in the

medium reached the maximum level, the synthesis of butyric acid was followed by its assimilation in most of the experiments. Only in the two processes conducted at $63\text{ }^{\circ}\text{C}$, as well as in the experiment performed at $45\text{ }^{\circ}\text{C}$ and $\text{pH} = 6.5$, the final butyric acid content of the medium exceeded the initial one (data not shown).

Synthesis of formic acid was observed in the two processes at $54\text{ }^{\circ}\text{C}$ with uncontrolled pH, as well as in the experiment performed at $63\text{ }^{\circ}\text{C}$ and $\text{pH}_0 = 6.5$, also uncontrolled. At the temperature of $54\text{ }^{\circ}\text{C}$, formic acid was synthesised up to the termination of the process; at $63\text{ }^{\circ}\text{C}$ and $\text{pH}_0 = 6.5$, upon termination of the experiment, the final content of formic acid was only slightly lower than the maximal value (Figure 3). In the three processes mentioned, the final content of formic acid varied from 1.51 ($T = 63\text{ }^{\circ}\text{C}$, $\text{pH}_0 = 6.5$) to 2.39 g/L ($T = 54\text{ }^{\circ}\text{C}$, $\text{pH}_0 = 8.0$); in the other processes formic acid was not synthesised, and the bacteria utilised the total amount of this acid that was present in the vinasse stillage (Table 1).

Glycolic acid content of the vinasse stillage being treated by processes with controlled pH was found to decrease solely at $\text{pH} = 8.0$ over the temperature range of 36 to $54\text{ }^{\circ}\text{C}$. Complete removal was observed at $54\text{ }^{\circ}\text{C}$. In the other experiments glycolic acid content of the medium increased (at the most by 0.4 g/L with $T = 36\text{ }^{\circ}\text{C}$ and $\text{pH} = 6.5$). When the pH was not controlled, the extent of glycolic acid utilisation increased with the rise in temperature from 27 to $54\text{ }^{\circ}\text{C}$. At $54\text{ }^{\circ}\text{C}$, glycolic acid was utilised completely, whereas at $63\text{ }^{\circ}\text{C}$, for both $\text{pH}_0 = 6.5$ and $\text{pH}_0 = 8.0$, it was assimilated by approximately 78% (Table 1).

The removal rates of organic acids were strongly influenced by the rate at which pyroglutamic acid was assimilated. In the vinasse stillage reported on here pyroglutamic acid occurred in the highest amounts, accounting for approximately 49% of the overall content of organic acids. The extent of its removal exceeded 95.8% in nearly all the biodegradation processes, and in seven of them pyroglutamic acid was utilised with 100% efficiency (Table 1). A low extent of pyroglutamic acid assimilation (71.39%) was noted only during the experiment without pH control conducted at $T = 54\text{ }^{\circ}\text{C}$ and $\text{pH}_0 = 8.0$. It was observed that at uncontrolled pH ($\text{pH}_0 = 6.5$ and $\text{pH}_0 = 8.0$), and when its value was kept at the constant level of 8.0 , at the temperatures of 54 and $63\text{ }^{\circ}\text{C}$ pyroglutamic acid was assimilated with the slowest rate. But at the same temperatures and pH of 6.5 (controlled), assimilation of pyroglutamic acid proceeded at the fastest rate (Figure 4).

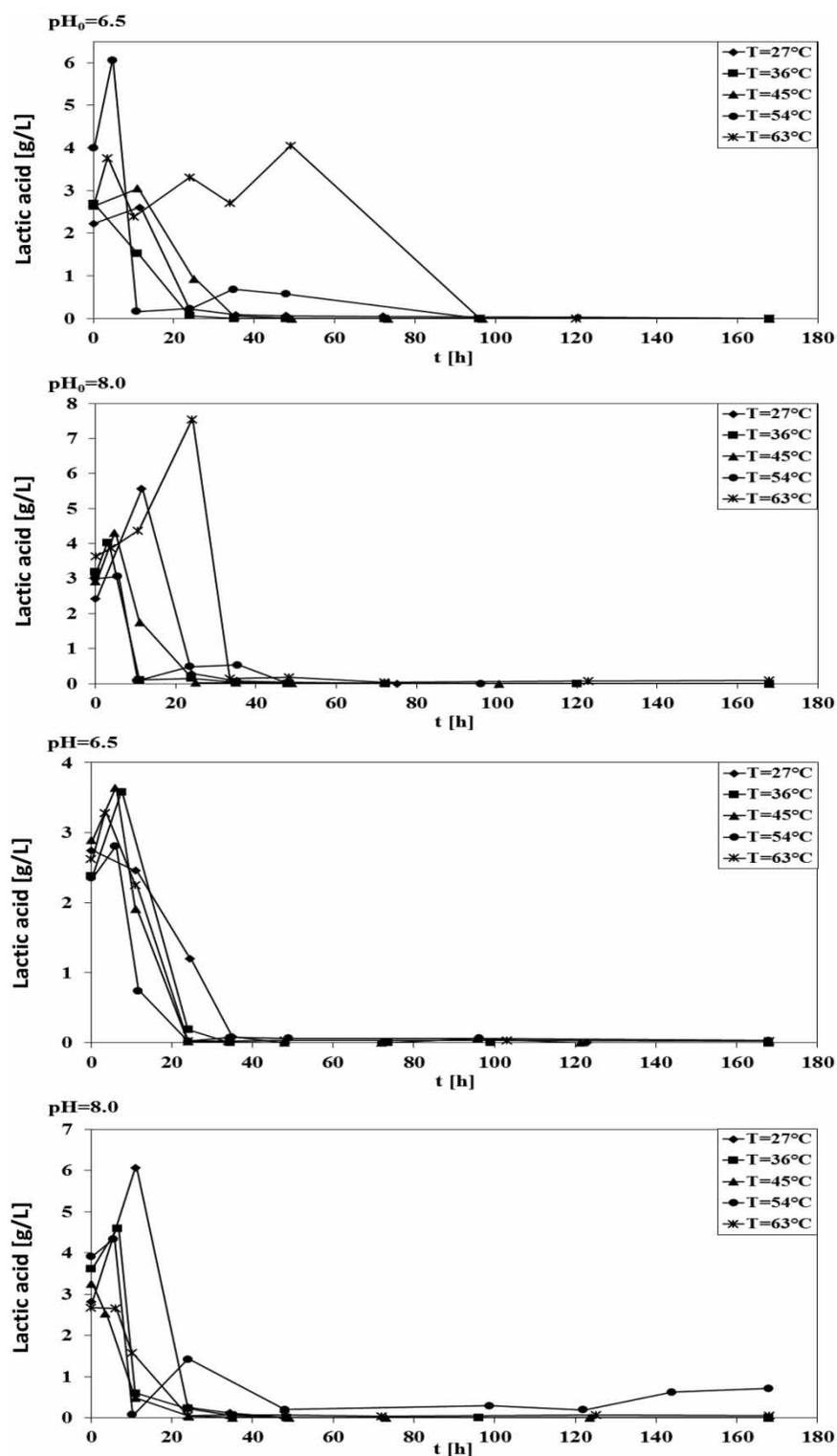


Figure 2 | Temperature-related lactic acid concentrations in the medium during vinasse stillage biodegradation.

Reduction in the sum of organic acids in the medium was found to be noticeably slower at higher (54 and 63 °C) than lower (27, 36 and 45 °C) temperatures in the processes

without pH control, both at $\text{pH}_0 = 6.5$ and $\text{pH}_0 = 8.0$, as well as in the processes with pH control, at $\text{pH} = 8.0$ (Figure 1, which refers to processes with no pH control; $\text{pH}_0 = 8.0$).

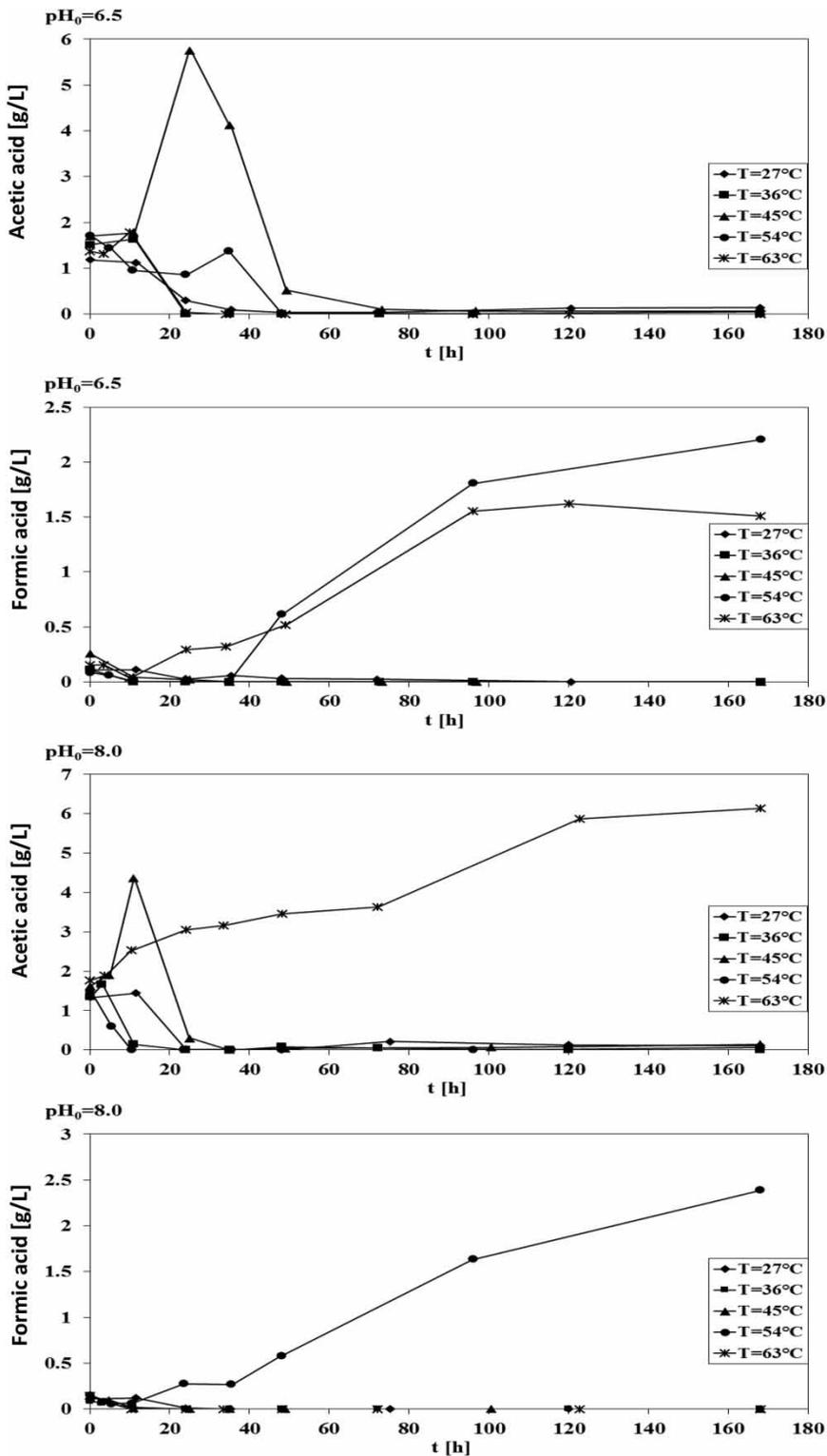


Figure 3 | Temperature-related concentrations of acetic acid and formic acid in the medium during vinasse stillage biodegradation without pH control.

The phenomenon of acetic acid or formic acid synthesis, and also the low extent of pyroglutamic acid assimilation (71.39% at $T = 54^\circ\text{C}$ and $\text{pH}_0 = 8.0$) are the contributing

factors in the decline of the sum of organic acids (far less pronounced than at lower temperatures) in the experiments conducted with no pH control at 54 and 63°C (Table 1,

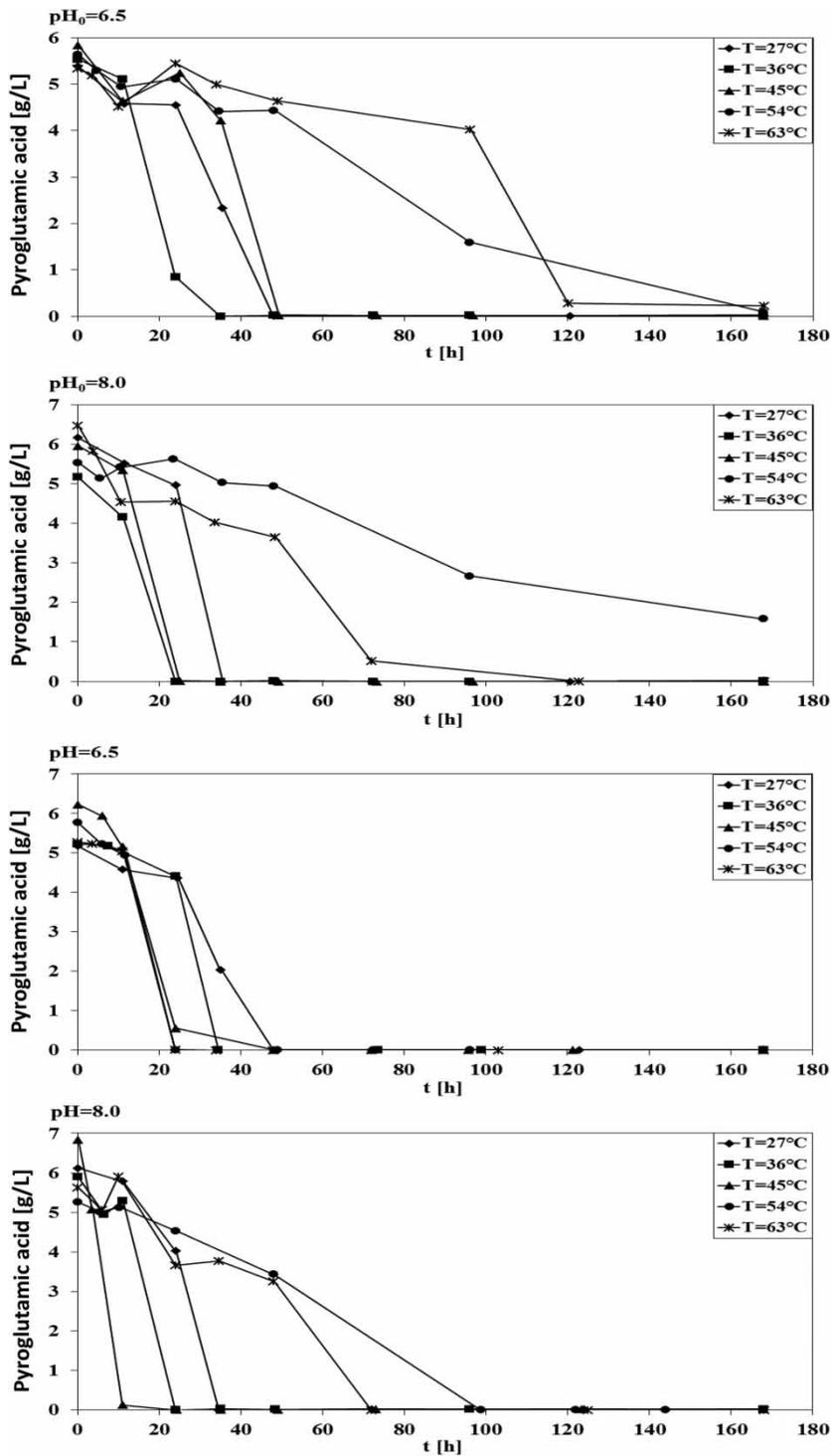


Figure 4 | Temperature-related pyroglutamic acid concentrations in the medium during vinasse stillage biodegradation.

Figures 3 and 4). In the experiments with controlled pH of 6.5, the rate of reduction in the concentration of the sum of organic acids increased with the rise in the temperature of the biodegradation process (data not shown).

DISCUSSION

Sequential assimilation of particular organic pollutants was observed by Cibis *et al.* (2002) and Krzywonos *et al.* (2009),

during degradation of potato slops under thermophilic conditions, and thermo- and mesophilic conditions, respectively, with the same mixed bacterial culture as the one used in this work here. In their experimental studies, organic acids were utilised first, followed by glycerol and reducing substances. But when potato slops with a low initial pollution load were biodegraded at 45 °C, glycerol, reducing substances and organic acids were removed from the medium simultaneously (Cibis *et al.* 2006). Seemingly, the difference in the mode of main pollutants' assimilation by the microorganisms can be attributed to the variable structure of the mixed bacterial culture used (Cibis *et al.* 2006), but it may as well be related to the concentrations of the pollutants, because the inhibition phenomenon may not have occurred when the concentrations of those pollutants were low. Sequential assimilation of pollutants at increased temperature was also reported by LaPara *et al.* (2000) and Kosseva *et al.* (2001). LaPara *et al.* (2000) observed during biodegradation of model wastewater that at 25 °C gelatine and α -lactose were removed simultaneously, whereas at 55 °C they followed a sequential removal pattern. Kosseva *et al.* (2001), in their study on whey biodegradation, noticed that at 45 °C proteins were utilised simultaneously with other organic compounds, while at the temperatures of 55, 60 and 65 °C the utilisation of proteins was followed by that of the other organic substances.

During treatment of the vinasse stillage its organic components were assimilated by the bacteria at the same time, but the rates of their assimilation were different. Glycerol was removed from the medium at the fastest rate, followed by reducing substances and organic acids. Betaine showed a specific pattern of assimilation. In the majority of instances the process intensified only after the medium was lacking in easily available carbon sources (Figure 1, which refers to processes with no pH control; $\text{pH}_0 = 8.0$). Furthermore, betaine was assimilated over the temperature range of 27 to 45 °C with one exception. This was in one of the four experiments carried out at 54 °C with pH controlled at 8.0 (Table 1). More details are found in the papers by Cibis *et al.* (2011) and Ryznar-Luty *et al.* (2015). The temperature-related differences in the metabolic activity of thermo- and mesophilic bacterial cultures are attributed by LaPara *et al.* (2000) to the variability of their structure. According to Vogelaar *et al.* (2002), those differences are attributable to the inability of the microorganisms to simultaneously produce enzymes indispensable for the assimilation of particular organic substances at elevated temperature.

Volatile organic acids that are synthesised during high-temperature biodegradation originate at the stage of oxygen deficit, when the level of dissolved oxygen in the medium is in decline. At the subsequent stage of the process, when the dissolved oxygen level is high, they are removed by the bacteria in the majority of instances (Cibis 2004; Ugwuanyi *et al.* 2005; Cibis *et al.* 2006; Ryznar-Luty *et al.* 2008; Krzywonos *et al.* 2009). A biochemical model describing the metabolism of volatile organic acids in autothermal thermophilic aerobic digestion for sewage sludge treatment was proposed by Liu *et al.* (2012). Most of the studies reported in the literature indicate that acetic acid is synthesised in the largest quantities (Ugwuanyi *et al.* 2005; Liu *et al.* 2012; Jin *et al.* 2016). However, Cibis (2004) noted that, during biodegradation of rye-, maize- or waste-feedstock-based stillage from rural distilleries, butyric acid was synthesised in the largest amounts. According to Ugwuanyi *et al.* (2005), the composition of the medium is a decisive factor in the type of acid being synthesised.

During their study on the biodegradation of potato slops, Krzywonos *et al.* (2009) made the following observations: at the temperature of 20 °C formic acid was synthesised in the largest amounts; the largest quantities of isobutyric acid and malic acid were produced at 30 °C and 45 °C, respectively, whereas the production of acetic acid was the highest at 35, 40, 50, 55, 60 and 63 °C. These findings give evidence to suggest that the parameters of the biodegradation process can also be rated as significant contributory factors, deciding which of the organic acids is synthesised in larger amounts than are others. The focus of this work here was to demonstrate the influence of three parameters, temperature, pH value and pH control, on the type of acids synthesised in the medium during aerobic biodegradation of distillery wastewater.

The largest quantities of acetic acid were synthesised during three experiments at the temperature of 45 °C, where two of them were conducted without pH control, and one with pH controlled at the level of 6.5, as well as during two experiments at the temperature of 63 °C, where one of them had a pH controlled at 8.0, and the other one an uncontrolled pH with $\text{pH}_0 = 8.0$. The highest amounts of butyric acid were produced at two temperatures: 36 °C with uncontrolled pH at $\text{pH}_0 = 6.5$, and 27 °C with pH controlled at the level of 6.5. Synthesis of valeric acid was the highest in two experiments: at 54 °C with uncontrolled $\text{pH}_0 = 6.5$, and at 45 °C with pH controlled at 8.0. The highest production of formic acid was noted at the temperature of 54 °C and uncontrolled $\text{pH}_0 = 8.0$. In the other experiments, maximal increment was observed in the content of

non-volatile lactic acid. In most of the experiments, the acids formed were removed from the medium after the period of increased oxygen demand. In some experiments, however, an increase in the quantities of certain acids was noted in the medium upon termination of the biodegradation process (Table 1). What seems worth noting here is the synthesis of formic acid at both 54 °C and 63 °C in the experiment with uncontrolled pH at $pH_0 = 6.5$, and at 54 °C in the experiment with uncontrolled pH at $pH_0 = 8.0$, as well as the synthesis of acetic acid at 63 °C in the experiment with uncontrolled pH at $pH_0 = 8.0$ (Figure 3). It is essential to add that under such conditions both the acids were synthesised despite the high oxygen content of the medium. Kurian *et al.* (2006), who examined the thermophilic aerobic treatability of high strength oily pet food wastewater, revealed that higher-molecular-weight volatile organic acids were biodegraded while acetic and propionic acids remained in the medium.

Scientific literature points to a few possibilities of vinasse management. In Western Europe countries, it is used directly or in the condensed form mainly as feedstuff for animals. In Brazil (cane vinasse), France, Spain, Italy and in Eastern Europe countries, it is sprinkled on arable fields as a fertiliser. However, the low nutritional value and high potassium content of vinasse make management of its entire volume produced by distilleries impossible. Attempts have been conducted under laboratory conditions to develop a method that would enable using vinasse as a substrate in biomass production or biosynthesis of organic compounds (Ryznar-Luty *et al.* 2009). Another approach is to treat vinasse as wastewater and subject it to the degradation process. Anaerobic methods of vinasse treatment are used on the industrial scale in India, where ethanol is mainly produced from sugarcane and cane molasses. However, anaerobic degradation of vinasse under industrial conditions has failed to ensure high removal percentage of contaminants (Rais & Sheoran 2015). The reduction of contaminant loads from vinasse, demonstrated in the present study, makes it possible to hypothesise that the effluent from biodegradation mixed with other less-loaded wastewater could be discharged to the sewerage system and subjected to successive stages of the treatment process. Another solution could be to perceive the proposed method as the first stage of the treatment process of this highly contaminated wastewater. Undisputed advantages of this method include the possibility of its use at high temperatures, no need for cooling the vinasse (which is hot directly after being produced), and no need for medium pH adjustment throughout the process.

CONCLUSIONS

Both the temperature and the pH of the medium and also control of the second parameter influence the progress of aerobic biodegradation of organic pollutants of vinasse. The temperature primarily influences the betaine removal from biodegraded medium. The compound was utilised mainly in the processes conducted in the lower temperature. Controlling the pH of the medium brought about an improvement in the removal of reducing substances, but had no significant effect on the extent of glycerol removal. As for the sum of organic acids, the control of this parameter at a constant level made it possible to significantly improve the extent of their biodegradation during processes conducted at elevated temperature. Conditions of biodegradation processes also affect the kind and the amount of organic acids synthesised by the bacteria.

During aerobic biodegradation of beet molasses vinasse with a mixed culture of bacteria of the genus *Bacillus*, the main organic pollutants were removed at the same time, but with diverse rate of removal. An exception to this rule was the assimilation of betaine, which intensified only after the medium was lacking in easily available carbon sources.

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