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Organic Compounds Generated in Bioethanol Production from Agave Bagasse

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Abstract

In bioethanol production through lignocellulosic residues fermentations are generated by-products such as organic compounds (OCs). The organic compounds (OCs) had been well studied in wine and beer industry, but little is known about their presence in bioethanol industry, even when these affect yeasts physiologic state, and are considered as economically desirable in the chemical industry. In this work was evaluated the production of OCs in bioethanol production processes through separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) of different agave bagasse residue (ABR). Fermentations were carried out by the *Kluyveromyces marxianus* SLP1, *K. marxianus* OFF1 and *Saccharomyces cerevisiae* Ethanol Red yeasts strains. The main OCs detected were ethyl acetate, methanol, 1-propanol, isobutanol, butanol, isoamyl-alcohol, ethyl-lactate, furfuryl-alcohol, phenyl-acetate, and 2-phenyl ethanol. A higher number of OCs was found in the SSF process when were used the *K. marxianus* OFF1 and SLP1 yeasts. This study provides better knowledge of the kind and concentrations of OCs produced by fermentation of the lignocellulosic ABR, which allow propose bioethanol by-products as potential source of economically desirable compounds.

Indexing Terms/Keywords: Agave Bagasse; Bioethanol; By-Products; Fermentation; Organic Compounds

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INTRODUCTION

Yeasts are used to produce chemicals, pharmaceuticals, and other products such as bioethanol [1-4]. Bioethanol production through lignocellulosic wastes is considered a promising process [5]. The agave bagasse residue (ABR) is a lignocellulosic waste with potential to bioethanol production [6, 7, 8, 9]; however low have been described about other organic compounds (OCs) produced in the fermentation process of that lignocellulosic residue. On the one hand, the OCs are toxic for sugar yeast fermentation rendering in consequence decreased ethanol yield [10, 11]; but on the other hand, the OCs have multiple industrial applications such as food additives, pharmaceutical, and cosmetic excipients [4]. In yeast fermentation OCs like esters, aldehydes, ketones, carbonyls, furans, and terpenes are produces [4]. The OCs production is affected by pH, temperature, carbon source, and yeast strain [12, 13, 14]. The *Kluyveromyces marxianus* genus has high potential for industrial production of OCs as volatiles compounds [11], as well as fast growth rate [15], and GRAS status.

The aim of this work was determinate the OCs obtained by the fermentation of hydrolyzes ABR, utilizing variables of process such as separate hydrolysis fermentation (SHF) and simultaneous saccharification and fermentation (SSF) stages, using the native yeast strains of *K. marxianus* SLP1 and OFF1 and comparing with the industrially-utilized yeast *S. cerevisiae* (Ethanol Red). To our knowledge, this is the first work in report the OCs produced in the fermentation of the lignocellulosic ABR. According with our results, the ABR bioethanol process could be considered as a potential way for produces economically desirable OCs.

MATERIAL AND METHODS

Yeast strains

Yeast strains were obtained from the culture collection of the CIATEJ (*Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco, México*) [16] and from the ATCC (American Type Culture Collection, Rockville, MD, USA). The *K. marxianus* yeasts strains (SLP1 and OFF1) were isolated at handcraft mezcal distilleries in the Mexican State of San Luis Potosi and Guerrero, respectively. The Ethanol Red yeast was acquired from the ATCC.

Agave bagasse residue and saccharification process

The agave bagasse residues (ABR) classified as masonry oven, autoclave and diffuser were obtained from the distilleries "Casa de Piedra", "Gonzalez-Gonzalez", and "La Madrileña", respectively. The three distilleries are located in Jalisco, Mexico. The tequila industries have different pine agave treatment as is mentioned by Cedeño-Cruz [17], and Casas [18]. The ABR were submitted to thermo-acid treatment showing higher sugar release under conditions previously determinate (Table 1). Enzymatic hydrolysis was done utilizing the commercial cellulases complexes CTec2, HTec2, and Rapidase (Novo enzymes) at 1.5 g of enzyme/g ABR (dry weight).

Table 1. Conditions for the agave bagasse residue thermo-acid treatment

ABR	Autoclave	Masonry oven	Diffuser
H_2SO_4 (%)	3	1	1
Temperature (°C)	110	130	110
Time (min)	40	30	10

ABR, Agave Bagasse Residue.

Fermentation processes

The enzymatic hydrolysates ABR were fermented through separate hydrolysis and fermentation (SHF) or simultaneous saccharification and fermentation (SSF), using the *K. marxianus* yeasts SLP1 and OFF1, or the *S. cerevisiae* Ethanol Red. 1 X 10^7 cells/ml suspensions was added with the enzymes mix or after 48 h of enzymatic hydrolysates of ABR, to SSF or SHF, respectively. The fermentations were done at 40°C and 100 rpm. Samples were taken at 24 h and 48 h after yeast inoculation. The OCs were detected by gas chromatography, as mentioned below.

Analysis of organic compounds

The OCs determination was carried out as described previously [19]. Briefly, after 48 h of fermentation OCs were quantified using a Hewlett–Packard 6890 gas chromatograph (Palo Alto, CA, USA) with flame ionization detector (FID) equipped with an HP-Innowax PEG column (60 m, 0.320 mm). The initial column temperature was 45°C, then ramped at 10°C/min to 160°C, followed by a 20°C/min ramp to 220°C, maintained during 4 min. Injector and detector temperatures were maintained at 250°C. The injection system consisted in a head-space (Hewlett–Packard 7694E). The preparation program and injection simple started with vial temperature at 80°C, loop temperature at 110°C, and transfer line temperature of 115°C. The cycle time of head space and gas chromatograph was of 40 min, with vial equilibrium time of 5 min, pressurization time 0.2 min, filling loop time 0.2 min, loop equilibrium time 0.5 min, injection time and agitation time of 1 min. The OCs measured in this study were ethyl-acetate, methanol, ethyl-butyrate, 1-propanol, isobutanol, isoamyl-acetate, butanol, isoamyl-alcohol, ethyl-hexanoate, ethyl-lactate, ethyl-octanoate, ethyl-decanoate, furfuryl-alcohol, phenyl-acetate, and 2-phenylethanol. As external standard were used compounds purchased from Sigma–Aldrich.

Yeasts cell growth

Cell number was determined by cell counting in Neubauer chamber by a sample taken at 24 h and 48 h after yeast fermentation. The counting was done according to Strober [20].

RESULTS

Total organic compounds produced

The *K. marxianus* yeast OFF1 showed more number of higher total organic compounds (TOCs) concentrations than the other yeasts. The TOCs concentrations were from 11 to 374 mg/L. SLP1 yeast obtained the highest concentration generated (Table 2). Although the higher TOCs concentrations were through SHF (Table 2), a higher number of OCs was generated in the SSF process. The compounds ethyl acetate, 1-propanol, isobutanol, butanol and isoamyl-alcohol were detected in the SSF of autoclave ABR, while in the SHF of the same ABR were no detected. In general was detected a lesser number of OCs as well as TOCs concentrations, in the fermentation of masonry oven ABR, compared with the detected in fermentations of autoclave and diffuser ABR (Table 2). The phenyl-acetate and furfuryl-alcohol were not detected in the fermentations of diffuser ABR while in the rest of the samples has variable concentrations around 15 and 50 mg/L, respectively. The ethyl acetate concentration was the most influenced by the ABR kind; when was used the autoclave ABR this compound was detected only in the SSF process; with the masonry oven ABR the presence of the ethyl-acetate was variable, while with the diffuser ABR this compound was present in all the samples. Others OCs presents in all the conditions were the methanol and 2-phenylethanol.

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Table 2. Total organic compounds (TOCs) in separate hydrolysis and fermentation (SHF) or simultaneous saccharification and fermentation (SSF) of the agave bagasse residue (ABR).

	TOCs (mg/L)																	
Yeast	SLP1						OFF1						Ethanol Red					
Enzyme	C		H		RAP		C		H		RAP		C		H		RAP	
Process	SHF	SSF	SHF	SSF	SHF	SSF	SHF	SSF	SHF	SSF	SHF	SSF	SHF	SSF	SHF	SSF	SHF	SSF
a)	214	71	150	113	122	175	134	66	135	95	106	184	150	43	136	33	106	203
b)	137	110	158	150	176	34	260	56	61	218	139	196	216	125	168	218	136	11
c)	122	99	158	125	188	374	43	75	112	205	94	237	150	107	216	38	176	36

ABR hydrolysates from autoclave (a), masonry oven (b) and diffuser (c). The fermentations were carried out in shake flasks at 40°C and 100 rpm. Enzyme: C (CTec2), H (HTec2) and RAP (Rapidase). Samples were taken at 48 h of fermentations processes.

Higher concentrations of the OCs detected

Were detected ten OCs with concentrations lesser than 300 mg/L (Table 3). The OC with the higher concentration was ethyl acetate (249 mg/L), followed by the 2-phenylethanol (75.31 mg/L). The *K. marxianus* yeasts SLP1 and OFF1 produced a higher number of OCs and concentrations of seven of the ten compounds detected. The methanol, butanol and ethyl-lactate were the OCs produced in higher concentration by the *S. cerevisiae* Ethanol Red yeast. While K. marxianus yeasts generated the 70% of the higher OCs concentrations, 60% were through SSF.

The compounds found in higher concentrations in SHF were methanol, ethyl-lactate, furfuryl-alcohol and 2-phenylethanol. The enzymatic complex showed a significance influence respect the higher concentrations; due 60% of the higher OCs concentrations were generated from hydrolysates by the Rapidase enzyme (Table 3).

Table 3. Higher concentrations of the organic compounds (OCs) detected

OCs	Concentration (mg/L)	Yeast	Process	ABR	Enzyme
Ethyl-acetate	249.38	S	SSF	D	RAP
Methanol	45.97	ER	SHF	D	RAP
1-propanol	12.56	O	SSF	A	RAP
Isobutanol	24.52	O	SSF	A	RAP

ISSN: 2348-6201
Volume: 07 Issue: 01
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Butanol	3.69	ER	SSF	A	RAP
Isoamyl-alcohol	34.81	O	SSF	A	RAP
Ethyl-lactate	65.17	ER	SHF	M	C
Furfuryl-alcohol	57.05	O	SHF	A	C
Phenyl acetate	17.61	S	SSF	M	C
2-phenyl ethanol	75.31	S	SHF	M	C

Yeasts: SLP1 (S), OFF1 (O) and Ethanol Red (ER). Processes: Separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF). Agave bagasse residue (ABR): A (autoclave); M (masonry oven); D (diffuser). Enzymatic complex: CTec2 (C) and Rapidase (RAP).

Yeasts cell grown

In eleven of the eighteen different fermentations conditions generated, the SLP1 yeast increased their cells number at 48 h with respect to 24 h of fermentation; in the seven rest conditions preserve their cells number at 48 h. In contrast, in six conditions the *K. marxianus* OFF1 yeast showed a reduction in the cells number from 24 h to 48 h of fermentation. The industrial *S. cerevisiae* yeast (Ethanol Red) showed a reduction in their cells number in three samples, preserves their cells number in ten conditions, and increased the cells number in five conditions of the eighteen generated, comparing at 48 h with respect 24 h of fermentation (Table 4)

Table. 4 Yeast cells number (1 X 10^6 cells/ml).

Yeast	SLP1																	
Enzyme	C						H						RAP					
ABR	A		M		D		A		M		D		A		M		D	
Time (h)	24	48	24	48	24	48	24	48	24	48	24	48	24	48	24	48	24	48
SHF	266±15	336±49	18±7	24±11	153±23	171±58	203±55	392±67	22±9	47±23	57±23	125±56	57±27	117±65	21±11	29±16	48±26	305±40
SSF	103±26	150±21	89±18	94±43	139±26	202±26	94±24	137±21	53±14	59±6	53±20	322±56	89±19	121±36	36±16	41±7	49±11	133±19
Yeast	OFF1																	
SHF	62±17	79±26	18±8	59±11	98±25	176±35	69±28	29±11	72±17	54±16	77±15	93±21	89±24	89±53	41±25	32±11	62±18	162±44

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SSF	75±32	45±25	141±45	57±31	94±20	81±30	126±35	50±17	63±24	100±33	70±14	157±36	167±38	265±74	81±41	81±24	70±26	189±56
Yeast	Ethanol Red																	
SHF	462±80	227±80	33±10	15±5	383±77	54±19	54±39	99±50	41±11	52±25	71±29	180±45	62±24	236±54	22±7	42±11	152±40	348±67
SSF	244±51	286±79	33±5	51±18	160±61	91±46	57±21	69±24	41±7	52±17	92±41	97±54	142±57	268±75	42±12	62±11	66±30	149±56

The cells were counted after 24 h and 48 h of separate hydrolysis and fermentation (SHF) or simultaneous saccharification and fermentation (SSF). Agave bagasse residue (ABR): A (autoclave); M (masonry oven); D (diffuser). Enzymatic complex: CTec2 (C), HTec2 (H) and Rapidase (RAP).

DISCUSSION

The utilization of lignocellulosic wastes as substrate in bioprocesses has increased in recent years due their renewable capacity, low-priced, and abundance [21, 22]. In Mexico the lignocellulosic agave bagasse is produced in tequila industry as waste with a rate of 360 thousand of dry tons [17, 23]. The agave bagasse residue (ABR) had been reported as a promising lignocellulosic biomass for production of fermentable sugars [7], and bioethanol [6, 7, 8, 9, 24]; however, there is not information about bioethanol by-products from ABR.

Organic compounds (OCs) are produced in the secondary yeast metabolism and can be considered as bioethanol contaminants [25], which could be yeast toxic [10, 11, 26]; moreover, these compounds has potential industrial use with high commercial interest in the food, cosmetics, detergent and pharmaceutical industries [4]. *K. marxianus* yeasts are some of the best organic volatiles compounds (OVCs) yeasts producer [11], and theirs use to produced these compounds through cassava bagasse fermentation is a feasibility process [27]. As well as cassava bagasse, sugar cane bagasse has been used for OVCs production [28, 29], without considered at the moment the ABR.

In this work we studied the OCs produced as bioethanol by-product from ABR fermentation using two *K. marxianus* yeasts (SLP1 and OFF1) and the industrially utilized yeast *S. cerevisiae* (Ethanol Red). Ethanol production from lignocellulosic biomass like ABR includes four main steps: pretreatment, saccharification, fermentation and distillation [30]. This study was focus in the OCs present in the fermentation step, using the ABR after theirs saccharification.

In our results were detected a lesser number of OCs than the reported López-Alvarez et al. [31] when used the *K. marxianus* yeast UMPe-1 to ferment agave must; this effect is due the agave must has higher sugar concentration than the ABR [6], and a major sugar concentration can result in higher OCs concentration [32].

Cedeño-Cruz [17], and Casas [18] explained that in tequila industries autoclave, masonry oven or diffuser could process the agave pines. We observed that agave pine process could affect the TOCs concentrations, as well as the kind of OCs produced (Table 2). This effect could be results of the kind and concentration of sugars in the ABR after agave pine processed.

The compound ethyl acetate is considered a solvent with many industrial applications. *K. marxianus* yeasts are some of the most potential yeasts for the production of ethyl acetate in an industrial scale [33]. In this work the higher ethyl acetate concentration was 249 mg/L, concentration lesser than the reported as *K. marxianus*

growth inhibitor (17 g/L) [10]; moreover copper limitation can increase ethyl-acetate synthesis in *Kluyveromyces* yeasts [34].

Yeasts are considered the most promising producers of 2-phenylethanol. The 2-phenylethanol is one of the more commercially OVCs due to their rose-like aroma [35], and in our results (Table 3) was the second more abundant compound. Although *Kluyveromyces* strains are considered as good producers of this compound [36], their resistance to this compound is lower than that of *S. cerevisiae* [37]. A concentration of 2 g/L of 2-phenylethanol is toxic to *K. marxianus* yeasts, and ethanol generates a synergistic interaction amplifying its cytotoxicity [38]. The higher 2-phenyl ethanol concentration produced in this work was of 75 mg/L, which is similar than the produced using molasses-based medium by the *K. marxianus* CBS 600 (89 mg/L) reported by Etschmann et al. [39]. Compound levels could increase through addition of exogenous L-phenylalanine [37, 40], or through solid-phase *in situ* product removal [41].

The higher alcohols detected in the ABR fermentations showed concentrations from 3.69 to 34.81 mg/L. The 1-propanol and isobutanol were detected in agave Tequilana fermentation with the same *K. marxianus* yeasts used in our work [42]. While the isobutanol can be used for production of bio-based product packaging [43], the butanol is considered a fuel additive [44]. The butanol concentration (20 mg/L) detected in the "Tequila Blanco" beverage obtained using the *K. marxianus* UMPe-1 [31], was higher than the detected using the ABR (3.69 mg/L). With the exception of butanol, in this work the *K. marxianus* yeasts showed major production of higher alcohols than the *S. cerevisiae* yeast, results that are in agreement with the reported by López-Alvarez et al. [31]. Although amino acids availability influences higher alcohols production, the uptake and assimilation of these substrates determines the final concentration [4]. According with our results the uptake and amino acid assimilation could be better in the *K. marxianus* yeasts (SLP1 and OFF1) than the *S. cerevisiae* yeast (Ethanol Red).

The ethyl-lactate that is used as solvent and "building block" to produce degradable plastic polymers [45] was not found in the fermentations by the SLP1 yeast, while the Ethanol Red yeast produced 2 mg/L, and the OFF1 yeast 60 mg/L. This last concentration is similar than the reported by Arellano et al. [42], during mezcal fermentation using the same yeast strain (OFF1). When López-Alvarez et al. [31] compared the production of this compound between the *K. marxianus* UMPe-1 and the *S. cerevisiae* baker's Pan1, the *S. cerevisiae* yeast produced seven times more than the *K. marxianus* yeast, result that is in disagreement with the results in this work, due the production by the *K. marxianus* OFF1 was higher than the produced by the *S. cerevisiae* yeast Ethanol Red. Due the last, we suggested that the production of ethyl-lactate could depend in great of every yeast strain, even in yeasts of the same genus.

While the *K. marxianus* SLP1 yeast showed more adaptability avoiding reduces their cell number at 48 h of fermentation, the yeast strain with the lesser adaptability was the OFF1, reducing their cell number main in the SHF, process where were detected the higher concentrations of furfuryl-alcohol (Table 4). Although the concentrations of OCs detected were lesser than their yeast toxic values, the OCs mix could exert a synergic toxic effect with other stress conditions as pH and temperature [38, 46]. Yeast robustness and physiological fitness is of high importance to efficient fermentation process [4]. A promising approach in yeasts OCs adaptation could be the study of the membrane fatty acids, due the membrane is one of the first OCs targets [47].

In this work we found that ABR fermentation could be a source of OCs, moreover, immobilized cells of *S. cerevisiae* produced higher amount of OCs than cells in suspension [48], and Rossi et al. [49] got higher overall concentration of VOCs when mixed different carbon and nitrogen sources, therefore, OCs production as bioethanol by-products from ABR could be improved through the uses of similar techniques. In addition, continuously OCs removing from ABR fermentation is an option to reduce their toxic effect and increases bioethanol production.

According with the results of this work, *K. marxianus* yeasts in bioethanol production from ABR generated acetate esters and higher alcohols with higher concentrations through SSF. Although bioethanol production from ABR is as potential source of by-compounds economically desirable, improve their production should be considered.

Conflicts of Interest

The authors declare no conflicts of interest in this work.

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