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ORIGINAL ARTICLE

High temperature alcoholic fermentation of orange peel by the newly isolated thermotolerant *Pichia kudriavzevii* KVMP10

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Significance and Impact of the Study: Citrus peel waste is one of the most underutilized and geographically diverse residues in the planet. In attempt to develop a citrus peel based biorefinery we report here the isolation of a yeast which exhibited favourable technological characteristics for the production of ethanol through utilization of the specific food waste. *Pichia kudriavzevii* KVMP10 was highly thermotolerant and utilized both hexoses and pentoses for ethanol production, which was achieved at elevated rates, highlighting its great potential for application in ethanol production processes from citrus peel.

Keywords
D-limonene, ethanol, orange peel, *Pichia kudriavzevii*, thermotolerant yeast.

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Abstract

This work explores the potential for the development of orange peel based ethanol bioprocesses through isolation of the thermotolerant *Pichia kudriavzevii* KVMP10. A model solution of hydrolysed Valencia orange peel was employed to determine the ethanologenic potential of the yeast, which was maximized at 42°C producing 54 g l^{-1} of ethanol. The effect of orange peel oil on bioethanol formation was investigated at 30 and 42°C confirming that the minimum inhibitory peel oil content was 0·01% (v/v). *Pichia kudriavzevii* KVMP10 demonstrated significant technological advantages for the production of sustainable bioenergy, such as utilization of both hexoses (glucose, sucrose, fructose and galactose) and pentoses (xylose) at high temperatures, exemplifying its great potential for application in orange peel based biorefineries for ethanol production.

Introduction

The manufacture of citrus juice is a major industrial sector with $31{\cdot}2 \times 10^6$ tonnes of fruits processed on an annual basis worldwide (Lin *et al.* 2013). The juice extraction results in residual peel waste that corresponds to 50% of the fruit, while the high levels of sugars contained usually result in mould growth necessitating its prompt treatment due to the high risk for uncontrolled production of greenhouse gases. Orange peel (OP) consists of soluble sugars, fibre, protein, ashes and fat constituting a rich feedstock for the production of added-value chemicals and biofuels. Various protocols have been developed for peel valorization into single components or through development of integrated biorefineries aiming to generate a series of products. D-limonene, pectin, biogas, bioethanol, single cell protein and succinic acid have been targeted as individual products from OPs (Li *et al.* 2010; Martin *et al.* 2010; Santi *et al.* 2014). However, microwave treatment has been used to produce D-limonene, pectin and mesoporous cellulose (Balu *et al.* 2012), while D-limonene, pectin, biogas and bioethanol have been produced through an integrated biorefinery approach (Pourbafrani *et al.* 2010).

OP is an attractive raw material for the production of bioethanol. However, membrane toxicity is usually imposed from D-limonene on the cell, which should be removed prior to fermentation (Grohmann *et al.* 1994). The negative effect of OP oil has been demonstrated on the ethanologenic potential of three major industrial

strains (*Saccharomyces cerevisiae*, *Kluyveromyces marxianus* and *Zymomonas mobilis*) (Wilkins *et al.* 2007a; Wilkins 2009). Thus, in order to eliminate peel oil inhibition popping treatment has been applied to reduce the D-limonene levels of the feedstock preceding the ethanol production step (Choi *et al.* 2013). This work aimed to isolate an ethanologenic strain capable of resisting the inhibition imposed by the peel oil content of the waste. Ethanol production was explored at different levels of OP oil and temperatures, while the strain was tested for its capacity to generate the biofuel from various sugars.

Results and discussion

Screening and isolation of ethanol producing micro-organisms

Following six sequential cultivations of samples from soil, contaminated OP and activated sludge in liquid media, substantial microbial growth occurred. About 0·1 ml of grown cultures was inoculated into solid media supplemented with OPs resulting in the purification of 21 single colonies. The purified strains were grown into the hydrolysed Valencia OP waste model solution (derived from Wilkins *et al.* 2007a) and six strains produced ethanol. A single strain, derived from the environmental sample collected from soil beneath an apple tree, was the strongest producer and it could generate approx. 30 g l^{-1} of ethanol.

Based on the phylogenetic and physiological characteristics, the strain was designated as *Pichia kudriavzevii* KVMP10 and the internal transcribed spacer (ITS) sequence was deposited in GenBank (accession number KP690977). Thus, a phylogenetic tree based on ITS was constructed (Fig. 1) with the use of the multiple alignment software CLUSTALW (MEGA 5.05) (Thompson *et al.*. 1994) aiming to correlate *P. kudriavzevii* KVMP10, with other *P. kudriavzevii*, *K. marxianus* and *Saccharomyces sp*. that shared ITS gene sequence similarities. *Kluyveromyces marxianus* NBRC 1777 and *Saccharomyces sp* KCH were used as outgroup. As shown on the phylogenetic tree the isolated strain exhibited close similarity to *P. kudriavzevii* d89a (accession number KP674621) and *P. kudriavzevii* B-WHX-12-12 (accession number KC756946.1). Similar to KVMP10 strain, *P. kudriavzevii* B-WHX-12-12 was isolated from apple orchards in China and demonstrated osmotolerant characteristics (unpublished work, NCBI). Furthermore, *P. kudriavzevii* TY11 (KC905770.1), which only slightly varied from the isolated KVMP10 (Fig 1), has also demonstrated significant thermotolerance and ethanol productivity (unpublished work, NCBI). *Pichia kudriavzevii* was previously named as *Issatchenkia orientalis* and it has been characterized as robust and multi-stress-tolerant yeast, resisting low pH values, elevated temperatures and salt contents (Toivari *et al.* 2013). The strain was cultivated under pH values of 4, 4·8, 6 and 7 to identify the optimal conditions for bioethanol production, while citrate buffer consisting of citric acid and sodium citrate were used to adjust the pH. The experiment performed at pH value of 6 produced 20 g l^{-1} of ethanol, while the culture conducted at pH 7 yielded insignificant concentration of ethanol following 48 h of cultivation. However, the tests performed at pH values of 4 and 4·8 demonstrated the highest ethanol concentration (between 24 and 25 g l^{-1}) and thus, the value of 4·8 was used in the experiments.

The effect of D-limonene concentration on bioethanol production

The inhibitory effect of D-limonene on *P. kudriavzevii* KVMP10 was first tested in solid media of the hydrolysed OP waste model solution supplemented with OP oil at concentrations that ranged between 0 and 1% (v/v) under aerobic conditions. Although increased concentrations of D-limonene resulted in longer lag phases and substantial reduction in biomass formation, the micro-organism was capable of growing on all OP oil concentrations tested and the colonies formed exhibited a cream colour (Fig. S1). Furthermore, the colonies were spreading

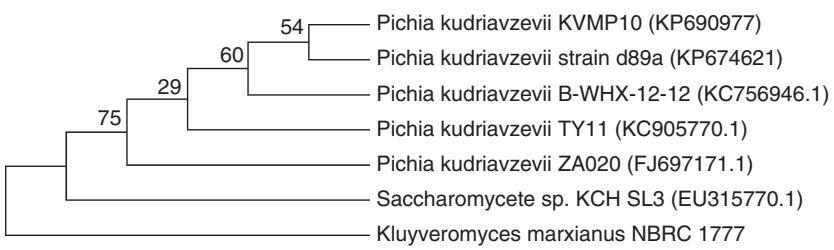

Figure 1 Phylogenetic tree of *Pichia kudriavzevii* KVMP10 obtained by neighbor-joining analysis of internal transcribed spacer sequences, showing the position among different *P. kudriavzevii*, *Kluyveromyces marxianus* and *Saccharomycete sp*. strains. Bootstrap values (expressed as percentages of 1000 replications) are shown at the branch points.

rapidly particularly at lower peel oil contents, while the biomass formed aggregates that raised from the surface of the plate for peel oil concentrations higher than 0·2% demonstrating a potential inhibitory effect. The duration of the lag phase varied between 0 and 48 h, while maximum biomass formation was observed following 72–168 h of cultivation (Table S1).

OP oil was also added to cultures conducted at 30°C (Fig. 2a). Increasing contents of the inhibitor resulted in prolonged lag phases and slight reduction in ethanol formation. Thus, although without the inhibitor the maximum product concentration occurred within 24 h of cultivation, the addition of 0·01, 0·05 and 0·10% (v/v) of OP oil resulted in maximum ethanol generation after 48, 54 and 73 h respectively. Moreover, OP oil slightly reduced ethanol production from 25 g l^{-1} in OP oil free media to 21–22 g l^{-1} with the use of the inhibitor. Various studies have shown the inhibitory effect of D-limonene highlighting the need for its removal prior to the bioprocess (Table 1). The lag phases of *K. marxianus* and *S. cerevisiae* gradually increased for rising contents of OP oil between 0 and 0·2% (v/v), which also inhibited the formation of ethanol (Wilkins *et al.* 2007a). Prolonged lag phases were not observed in simultaneous saccharification and fermentation of citrus peel supplemented with 0·08–0·043% (v/v) of D-limonene using *S. cerevisiae* (Wilkins *et al.* 2007b), while *Z. mobilis* and *Mucor indicus* have shown significant resistance to D-limonene (Wilkins 2009; Lennartsson *et al.* 2012).

Bioethanol production at different temperatures

Temperature is considered as a major parameter of ethanol bioprocesses reducing the costs through more efficient product recovery and cooling of the bioreactor, higher growth and saccharification rates and reduction in microbial contamination (Banat *et al.* 1998). Thus, batch cultures of *P. kudriavzevii* KVMP10 were conducted at different temperatures aiming to determine the optimal conditions for ethanol production. Figure 2b presents the concentration of ethanol in experiments where the temperature ranged between 30 and 42°C. The bioethanol concentration achieved was 54 g l^{-1} at 42°C and it reached 39 and 40 g l^{-1} at 37 and 40°C respectively. An experiment conducted at 45°C resulted in 60 h of lag phase and substantial reduction in ethanol concentration to a maximum of 10 g l^{-1} indicating an inhibitory effect at higher temperatures.

A comparison of the data obtained here with past studies exemplifies that the isolated strain is a highly efficient bioethanol producer (Table 2). *Pichia kudriavzevii* KVMP10 could produce substantially higher ethanol concentrations to the 37, 41 and 43·5 g l^{-1} formed by the industrial strains *K. marxianus*, *S. cerevisiae* and *Z. mobilis* respectively, using the same model solution of OP waste (Wilkins *et al.* 2007a; Wilkins 2009). Moreover, the highest ethanol production was obtained at 42°C, which is considerably higher to the 37°C used in the above studies. However, although *P. kudriavzevii* KVMP10 demonstrated elevated ethanol productivity (2·25 g l^{-1} h^{-1}), *S. cerevisiae* could generate ethanol at a productivity of 3·85 g l^{-1} h^{-1} in fermentations of mandarin peel waste (Choi *et al.* 2013). Although the capacity of *K. marxianus*

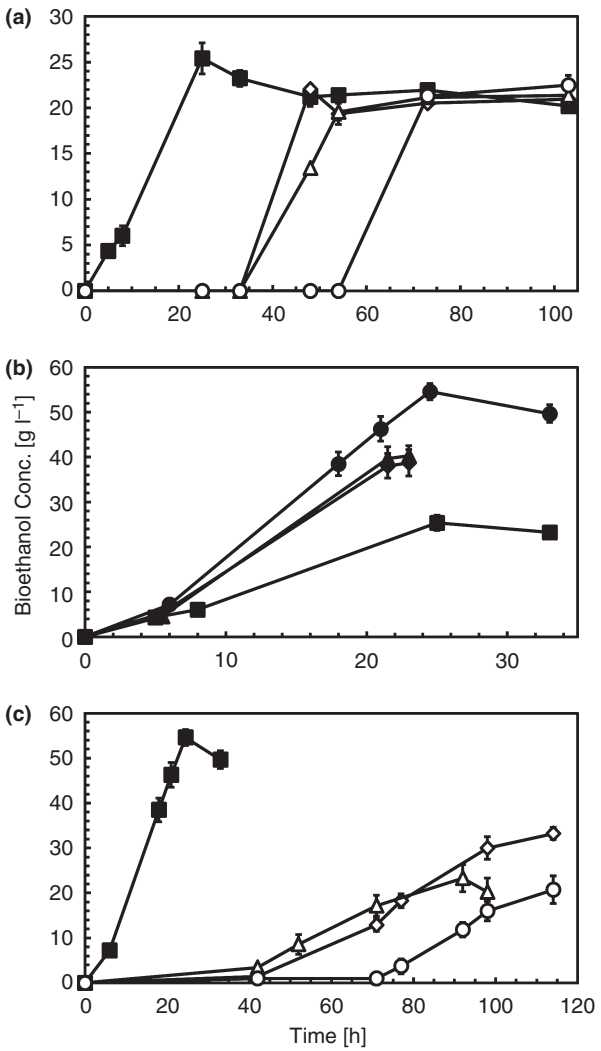

Figure 2 Bioethanol concentration in *Pichia kudriavzevii* KVMP10 fermentations conducted at different conditions. (a) Fermentations at 30°C for different orange peel (OP) oil contents. —■—: 0·00% (v/v) OP oil; —◇—: 0·01% (v/v) OP oil; —△—: 0·05% (v/v) OP oil; —○—: 0·10% (v/v) OP oil. (b) Fermentations at different temperatures. Bioethanol produced at: —■—: 30°C; —◆—: 37°C; —▲—: 40°C; —●—: 42°C. (c) Fermentations at 42°C for different OP oil contents. —■—: 0·00% (v/v) OP oil; —◇—: 0·01% (v/v) OP oil; —△—: 0·025% (v/v) OP oil; —○—: 0·05% (v/v) OP oil.

Table 1 The inhibitory effect of D-limonene in ethanol bioprocesses

Raw material	Micro-organism	Process conditions	Range of initial D-limonene conc. (v/v)	Effect on lag phase	Effect on ethanol conc.	Effect on product yield [$g_{ethanol}$ g_{sugars}^{-1}]	Reference
Orange peel hydrolysate	*K. marxianus*	SF, Aer, 37°C	0·00–0·20%	Gradual increase in lag phase from 0 to 72 h	Gradual decrease from 37 to 13 g l^{-1}	Varying yields between 0·34 and 0·58	Wilkins *et al.* (2007a)
Orange peel hydrolysate	*S. cerevisiae*	SF, Aer, 37°C	0·00–0·20%	Gradual increase in lag phase from 0 to 72 h	Gradual decrease from 41 to 23 g l^{-1}	No effect (0·43–0·45)	Wilkins *et al.* (2007a)
Citrus peel waste	*S. cerevisiae*	SSF, 37°C	0·08–0·43%	No effect (lag phase duration > 24 h)	Gradual decrease from 39 to 7 g l^{-1}	No effect (0·43)	Wilkins *et al.* (2007b)
Orange peel hydrolysate	*Z. mobilis*	SF, 30°C	0·00–0·20%	Gradual increase in lag phase from 0 to 96 h	Substantial reduction for 0·20% (v/v) from 40–43 to 15 g l^{-1}	nd	Wilkins (2009)
Orange peel hydrolysate	*Z. mobilis*	SF, 37°C	0·00–0·20%	Gradual increase in lag phase from 0 to 72 h	Reduction for 0·20% (v/v) from a level of 41–43·5 to 36·5 g l^{-1}	nd	Wilkins (2009)
Orange peel hydrolysate	*Rhizopus sp.*	SF, Aer, 32°C	0–2%	nd	nd	Varying yields between 0·28 and 0·37	Lennartsson *et al.* (2012)
Orange peel hydrolysate	*M. indicus*	SF, Aer, 32°C	0–2%	No effect (26 h lag phase duration)	nd	No effect (0·39–0·43)	Lennartsson *et al.* (2012)
Orange peel hydrolysate	*Rhizopus sp.*	SF, An, 32°C	0–2%	nd	nd	Substantial reduction for 1–2% (v/v) from 0·31–0·39 to 0·20–0·25	Lennartsson *et al.* (2012)
Orange peel hydrolysate	*M. indicus*	SF, An, 32°C	0–2%	No effect (34 h lag phase duration)	nd	Varying yields between 0·36 and 0·43	Lennartsson *et al.* (2012)
Orange peel hydrolysate	*M. indicus*	SF, Aer, 32°C	1% and 2%	Decrease from 55 to 28 h	No effect (19–20 g l^{-1})	Increase from 0·38 to 0·53	Lennartsson *et al.* (2012)
Orange peel hydrolysate	*P. kudriavzevii* KVMP10	SF, 30°C	0·00–0·10%	Gradual increase in lag phase from 0 to 54 h	Varying concentrations between 25 and 21 g l^{-1}	nd	This study
Orange peel hydrolysate	*P. kudriavzevii* KVMP10	SF, 42°C	0·00–0·05%	Gradual increase in lag phase from 0 to 72 h	Gradual decrease from 54 to 21 g l^{-1}	nd	This study

SF, submerged fermentation; SSF, simultaneous saccharification and fermentation; nd, no data; Aer, aerobic conditions; An, anaerobic conditions.

Table 2 Production of ethanol from citrus waste in microbial fermentations

Raw material	Pretreatment method	Process conditions	Micro-organism	Total initial sugar content	Ethanol concentration	Ethanol productivity [g l^{-1} h^{-1}]	Yield [$g_{ethanol}$ g_{sugars}^{-1}]	Reference
Orange peel hydrolysate	nd	SF	*S. cerevisiae*	nd	40–45 (g l^{-1})	0·82–0·90	nd	Grohmann *et al.* (1994)
Orange peel hydrolysate	nd	SF	*E. coli*	111 (g l^{-1})	35–38 (g l^{-1})	0·42–0·80	nd	Grohmann *et al.* (1996)
Orange peel hydrolysate	nd	SF, 37°C	*K. marxianus*	90·6 (g l^{-1})	37 (g l^{-1})	0·51	0·44	Wilkins *et al.* (2007a)
Orange peel hydrolysate	nd	SF, 37°C	*S. cerevisiae*	90·6 (g l^{-1})	41 (g l^{-1})	0·56	0·45	Wilkins *et al.* (2007a)
Citrus peel waste	Steam explosion	SSF, 37°C, 0·08% e.o.	*S. cerevisiae*	0·31 (g g^{-1} dry raw material)	39·03 (g l^{-1})	1·62	0·43	Wilkins *et al.* (2007b)
Orange peel hydrolysate	nd	SF, 37°C, 0·05% e.o.	*Z. mobilis*	90·6 (g l^{-1})	43·5 (g l^{-1})	0·60	0·48	Wilkins (2009)
Mandarin waste and banana peels	Steam depressurization	SSF, 30°C	*S. cerevisiae* and *P. tannophilus*	0·17 (g g^{-1} dry raw material)	26·84 (g l^{-1})	0·55	0·42	Sharma *et al.* (2007)
Orange peel hydrolysate	Two stage acid hydrolysis	SF, 34°C	*S. cerevisiae*	27·54 (g l^{-1})	30·33 (g l^{-1})	3·37	0·46	Oberoi *et al.* (2010)
Mandarin waste	Hydrothermal sterilization	SSF, 37°C	*S. cerevisiae*	74 (g l^{-1})	42 (g l^{-1})	3·50	0·48	Oberoi *et al.* (2011)
Citrus waste	Dilute-acid hydrolysis and pectin recovery	SF, 30°C	*S. cerevisiae*	32·97 (g l^{-1})	39·64 (l tn wet raw $material^{-1}$), 14·17 (g l^{-1})	nd	0·43	Pourbafrani *et al.* (2010)
Mandarin waste	Enzyme hydrolysis	SSF, 40°C	*P. kudriavzevii*	64 (g l^{-1})	33·87 (g l^{-1})	2·82	0·67	Sandhu *et al.* (2012)
Mandarin waste	Steam explosion	SSF, 37°C	*S. cerevisiae*	nd	60 (l tn raw $material^{-1}$)	nd	nd	Boluda-Aguilar *et al.* (2010)
Lemon peel waste	Steam explosion	SSF, 37°C	*S. cerevisiae*	nd	67·83 (l tn raw $material^{-1}$)	nd	nd	Boluda-Aguilar and Lopez-Gomez (2013)
Orange peel waste	Enzyme hydrolysis	Aer, 32°C	*M. indicus*	39 (g l^{-1})	15 (g l^{-1})	0·62	0·39	Lennartsson *et al.* (2012)
Orange peel waste hydrolysate	nd	SF, Aer, 32°C	*Rhizopus sp.*	50 (g l^{-1})	nd	nd	0·37	Lennartsson *et al.* (2012)
Orange peel waste hydrolysate	nd	SF, Aer, 32°C	*M. indicus*	50 (g l^{-1})	nd	nd	0·41	Lennartsson *et al.* (2012)
Orange peel waste hydrolysate	nd	SF, An, 32°C	*Rhizopus sp.*	50 (g l^{-1})	nd	nd	0·39	Lennartsson *et al.* (2012)
Orange peel waste hydrolysate	nd	SF, An, 32°C	*M. indicus*	50 (g l^{-1})	nd	nd	0·43	Lennartsson *et al.* (2012)
Mandarin peel waste	Popping and enzyme hydrolysis	SF, 30°C	*S. cerevisiae*	0·63 (g g^{-1} raw material)	46·2 (g l^{-1})	3·85	0·91	Choi *et al.* (2013)
Orange peel hydrolysate	nd	SF, 30°C	*P. kudriavzevii* KVMP10	101 (g l^{-1})	25 (g l^{-1})	1·08	nd	This study
Orange peel hydrolysate	nd	SF, 42°C	*P. kudriavzevii* KVMP10	101 (g l^{-1})	54 (g l^{-1})	2·25	nd	This study

SF, submerged fermentation; SSF, simultaneous saccharification and fermentation; nd, no data; Aer, aerobic conditions; An, anaerobic conditions; e.o., essential oils.

to produce ethanol at elevated temperatures has received substantial interest (Banat *et al.* 1998; Koutinas *et al.* 2014), several studies have recently tackled the ethanologenic potential of the highly thermotolerant yeast *P. kudriavzevii*, presented here as Supporting Information.

The effect of D-limonene on *P. kudriavzevii* KVMP10 cultures in optimal conditions

In order to understand the capacity of *P. kudriavzevii* KVMP10 for application in OP valorization approaches, the inhibitory effect of D-limonene should be explored under conditions that maximize the production of ethanol. Thus, increasing OP oil contents were added in *P. kudriavzevii* KVMP10 cultures performed with a pH of 4·8 at 42°C (Fig. 2c). The use of 0·01% (v/v) peel oil reduced the maximum ethanol concentration formed from 54 g l^{-1} obtained in the absence of the inhibitor to 33 g l^{-1}. Moreover, the application of higher OP oil concentrations resulted in further reduction in the product concentration, which reached 23 and 21 g l^{-1} for OP oil contents of 0·025 and 0·05% (v/v) respectively. The bioethanol concentration obtained with the use of OP oil at 42°C was similar to that obtained at 30°C (Fig. 2a), demonstrating that the product yield was substantially reduced due to the inhibitor. *Saccharomyces cerevisiae* is inhibited by D-limonene concentrations higher than 0·12% (v/v), while other strains have demonstrated microbial inhibition for D-limonene contents of 0·05–0·25% (v/v) (Wilkins *et al.* 2007b; Lennartsson *et al.* 2012).

Ethanol production by individual sugars

The capacity of *P. kudriavzevii* KVMP10 to produce ethanol from individual sugars was tested in cultures supplemented with 10 g l^{-1} of each carbon source, which were maintained at 42°C with a pH value of 4·8 (Fig. 3). Glucose, sucrose and fructose generated the highest ethanol content that reached 4·5, 4·9 and 5·0 g l^{-1} respectively, while when galactose was fed 3·5 g l^{-1} were formed. Moreover, a culture fed with 10 g l^{-1} of D-xylose generated 1·9 g l^{-1} of the product. One of the most important parameters for the development of cost effective bioethanol processes is the use of yeasts converting both hexoses and pentoses to the product at high rates, yields and final concentrations. Although *P. kudriavzevii* was reported as not capable of assimilating D-xylose (Oberoi *et al.* 2012; Sandhu *et al.* 2012; Dandi *et al.* 2013), similar to this study, *P. kudriavzevii* VVT-C-75010 was a xylose-fermenting strain (Toivari *et al.* 2013), while the catabolic genes of D-xylose have been identified in *P. kudriavzevii* M12 (Chan *et al.* 2012).

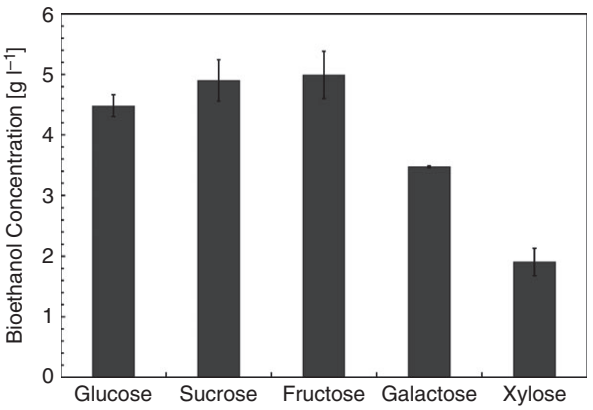

Figure 3 Bioethanol formed in single substrate experiments. Each bar corresponds to the concentration of bioethanol produced using an initial concentration of 10 g l^{-1} of the sugar indicated.

Critical aspects for the use of *Pichia kudriavzevii* KVMP10 in ethanol bioprocesses

The energy demand of distillation could be up to 40% of the energy required for bioethanol manufacture (Nagy and Boldyryev 2013). Moreover, when the bioprocess is conducted at high temperatures, evaporation of significant quantities of ethanol is enabled, which could be further liquefied using a heat exchanger. The cost effective production of fuel-grade ethanol in alcohol distilleries requires low energy demand emphasizing the importance of employing thermotolerant yeasts, such as *P. kudriavzevii* KVMP10. The yeast can ferment sugars at higher temperatures compared to other traditional strains (Yuangsaard *et al.* 2013) and it consumes glucose, sucrose, fructose, galactose and xylose suggesting its potential for application in the development of bioethanol processes based on sugar hydrolysates derived from renewable biomass. Moreover, *P. kudriavzevii* KVMP10 achieved a high product yield, generating higher ethanol concentrations compared to *S. cerevisiae*, *K. marxianus* and *Z. mobilis* using the same model solution of OP waste (Table 2).

Pichia kudriavzevii cultures performed with 150 g l^{-1} of glucose have resulted in ethanol contents higher than 9% (v/v) (Dandi *et al.* 2013) confirming that the yeast may achieve substantially high alcohol concentrations. Thus, although other common strains were more resistant to D-limonene (Table 1), the capacity of *P. kudriavzevii* KVMP10 to ferment a variety of carbohydrates (including xylose) demonstrates that it holds great potential for the development of OP based biorefinery concepts for the production of fuel-grade or potable alcohol. The use of the hydrolysed OP waste model solution in *P. kudriavzevii* KVMP10 fermentations produced 6·8% (v/v) of etha-

nol, which is a typical alcoholic degree achieved industrially, exemplifying its potential for application in potable and fuel-grade alcohol production from OP. Therefore, future studies should focus on bulk trials of KVMP10 in order to confirm at larger scale the enhanced ethanol production achieved herein for low volume shake flask experiments. Furthermore, the data generated in the present work could be employed as the basis for the development of a design of experiments methodology aiming to optimize the production of ethanol from KVMP10.

The valorization of food waste using biotechnological approaches could serve as a sustainable practice for generation of various commodities valuable to society. It has been shown that *P. kudriavzevii* KVMP10 was not highly resistant to the presence of D-limonene. However, a range of favourable characteristics, such as strong ethanologenic capacity, high temperature fermentation and utilization of both hexose and pentose sugars demonstrate that *P. kudriavzevii* KVMP10 is a versatile yeast holding great potential for application in ethanol bioprocesses.

Materials and methods

Medium preparation for strain isolation

The samples required for the isolation of bioethanol producers were collected from the top 10 cm of soil located beneath apple trees (the GPS coordinates of the specific location were N34°56′, E32°57′ and 1242 m of altitude in Kyperounta, Cyprus), fungal contaminated OP and activated sludge. The medium used for strain isolation consisted of (g l^{-1}): $CaCl_2$ 1·0, $MgSO_4{\cdot}7H_2O$ 1·0, $(NH_4)_2SO_4$ 0·5, NaCl 0·5, KH_2PO_4 1·0 and 150 μl of trace elements (composition in 0·01 mol l^{-1} H_2SO_4) (g l^{-1}): $ZnSO_4{\cdot}7H_2O$ 10·0, $CuSO_4{\cdot}5H_2O$ 1·0, $MnSO_4{\cdot}4H_2O$ 1·0, $CoSO_4{\cdot}7H_2O$ 1·0, $Cr_2(SO_4)_3{\cdot}15H_2O$ 0·5, H_3BO_3 0·6; $Na_2MoO_4{\cdot}2H_2O$ 0·5, $NiSO_4{\cdot}6H_2O$ 1·0, $Na_2SeO_4{\cdot}10H_2O$ 1·0, $Na_2WO_42H_2O$ 0·1 and $NaVO_3$ 0·1.

Strain isolation and identification

The above medium was supplemented with 75 g l^{-1} of OPs as the sole carbon source and was pretreated with two different approaches. In the first pretreatment peels were grinded into particles with diameter of 2 mm using a commercial blender (Dolcevita CG1, IMETEC, Greece) and dried at 60°C for 120 h. The second pretreatment aimed at the removal of D-limonene through the addition of water to ground peel at a ratio of 6 : 1 and boiling for 1 h, while prior to their application in fermentation the peels were dried at 60°C for 120 h. Prior to samples' addition, the pH was adjusted to 5 and samples were cultivated at 30°C for 4 weeks in submerged cultures of isolation medium supplemented with OP pretreated with both approaches. In order to isolate the desired strain 0·1 ml of grown cultures were inoculated into solid media containing 15 g l^{-1} of agar and 4 g l^{-1} of OPs treated with the second approach (at 30°C). Subsequently, a single colony of the isolates was transferred into liquid media model of hydrolysed Valencia OP waste, which was prepared in 50 mmol l^{-1} citrate buffer at pH 4·8 and consisted of (g l^{-1}): yeast extract 20·0, peptone 10·0, galactose 8·6, fructose 33·2, glucose 57·4 and sucrose 1·4 (Wilkins *et al.* 2007a). The carbohydrate content of the medium was equal to that obtained through hydrolysis of Valencia OP with cellulase, pectinase and β-glucosidase, which was fermentable by *S. cerevisiae* and *K. marxianus* (Wilkins *et al.* 2005).

DNA extraction was performed as described in Harju *et al.* (2004) and the DNA product was used in the PCR reaction. About 1 μl of template DNA was added in 30 μl of PCR reaction solution, containing 3 μl of Buffer D (5×), 1·8 μl $MgCl_2$ 25 mmol l^{-1}, 0·6 μl dNTPs 10 mmol l^{-1}, 1 μl Primer mix 25 μmol l^{-1}, 0·12 μl KAPA Taq 5 U μl^{-1} and 25·28 μl H_2O. The universal ITS1/ITS4 primers (ITS1 primer targeting the end of 18S rDNA: 5′TCC GTA GGT GAA CCT GCG G3′; ITS4 primer targeting the onset of 28S rDNA: 5′TCC TCC GCT TAT TGA TAT GC3′) were used to amplify the ITS region. The PCR reaction was performed in 34 cycles at 95°C for 30 s, 58°C for 30 s, and 72°C for 60 s. The PCR products were sequenced by Macrogen (the Netherlands).

Fermentation conditions in ethanol production tests

Pichia kudriavzevii KVMP10 was pregrown in the hydrolysed Valencia OP waste model solution at 30°C, while 5% (v/v) of inoculum was used in each culture. The batch experiments were conducted in 250 ml sterile flasks with a working volume of 60 ml in an incubator operated at a temperature according to the specifications of each experiment and reciprocal shaking of 100 rev min^{-1}. All chemicals were obtained from Sigma-Aldrich Company Ltd (Dorset, UK) and were of ANALAR grade. OP oil was procured from a local orange juice factory (Kean Soft Drinks Ltd., Limassol, Cyprus).

Analyses

Gas Chromatography was employed for the determination of ethanol concentration. A Shimadzu GC-2014 (Shimadzu, Milton Keynes, UK) using a flame ionization detector and a 30 m long Zebron ZB-5 capillary column (Phenomenex, Macclesfield, UK) with 0·25 mm internal diameter was employed. The mobile phase applied was nitrogen, while the stationary phase of the

column was 5%-phenyl and 95% dimethylpolysiloxane. Samples were centrifuged for 5 min at 11337 ***g*** and the supernatant was filtered through 0·2 μm filters. Ethanol was extracted into decane by vortexing 1 ml of the filtered sample with 2 ml of the solvent for 1 min. About 1 μl of the extract was injected and the temperature of the column was kept constant at 70°C for 2·5 min followed by an increase of 30°C min^{-1} up to 160°C, while it was maintained at 160°C for an additional 0·5 min. Ethanol concentration was calculated interpolating from a previously established calibration curve and the coefficient of variation for three samples was 4·3% at a concentration level of 1 g l^{-1}.

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Conflict of Interest

No conflict of interest declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Duration of lag phase and time required for maximum biomass formation of *Pichia kudriavzevii* KVMP10 grown in solid media supplemented with increasing concentrations of orange peel oil.

Figure S1 Cultivation of *Pichia kudriavzevii* KVMP10 in solid media supplemented with various orange peel oil concentrations. Shown are pictures taken at the time points specified for cultures containing a peel oil content (v/v) of: (a) 0·0%; (b) 0·1%; (c) 0·2%; (d) 0·3%; (e) 0·4%; (f) 0·5%; (g) 0·6%; (h) 0·7%; (i) 0·8%; (j) 0·9%; (k) 1·0%.