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ORIGINAL PAPER

Bioprospecting of thermo- and osmo-tolerant fungi from mango pulp–peel compost for bioethanol production

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Abstract The persistent edaphic stress on microbial succession due to dynamic changes during composting was explored for selection of multi-stress tolerant microbe(s) desirable for ethanol production. A total of 23 strains were isolated from mango compost using four successive enrichments in YP broth (g l^{-1}): glucose, 100; 150; 250 with ethanol (40) and cycloheximide (0.4) at 40 °C, pH 6.0. Based on multi-gene ribotyping, 14 yeasts (61 %) of *Saccharomycetaceae*, 2 filamentous fungi (8.6 %) and 7 bacteria (30.4 %) were obtained. Phenetic and phylogenetic analysis of the 14 yeasts revealed 64.3 % tolerant to 500 g l^{-1} glucose, growth at 45 °C and resemblance to *Candida* sp. (14.3 %), *Kluyveromyces marxianus* (35.7 %), *Pichia kudriavzevii* (21.4 %) and *Saccharomyces cerevisiae* (28.6 %). Assessment of the 14 yeasts in glucose fermentation medium (pH 4.5 at 40 °C) showed ethanol productivity of ≥92 % by 12 yeasts with theoretical yields of 90–97 %. Fermentation of molasses (150 g l^{-1} glucose equivalent) by *P. kudriavzevii* D1C at 40 °C resulted in 73.70 ± 0.02 g l^{-1} ethanol and productivity of 4.91 ± 0.01 g l^{-1} h^{-1}. Assessment of *P. kudriavzevii* D1C revealed multi-stress tolerance towards 5-hydroxymethyl furfural, ethanol (20 %, v/v), high gravity and H_2O_2 (0.3 M) indicating suitability for ethanol production using high gravity molasses and pre-treated lignocellulosic biomass fermentation.

Keywords Compost · Multi-stress tolerance · *Pichia kudriavzevii* · *Kluyveromyces marxianus* · *Saccharomyces cerevisiae*

Introduction

Global energy crisis and environment security from the excessive use of fossil fuels has driven unprecedented spurts in renewable resources for the production of 'green' bio-based fuels (Balat and Balat 2009). Fermentative production of ethanol using *Saccharomyces cerevisiae* is reported earlier for its proven industrial process robustness and exceptional physiological and x-*omics* characterization for improved lignocellulosics utilization (Nevoigt 2008). However, *Saccharomyces*-based ethanol production is limited due to its lack of multi-stress tolerance to (i) polycyclic aromatic compounds generated during biomass pre-treatment, (ii) high fermentative temperatures (≥40 °C), (iii) low pH and (iv) high sugar concentrations. Consequently, *Saccharomyces*-based fermentation demonstrated inadequate (i) substrate utilization spectrum, (ii) genetic stability, (iii) ethanol productivity and (iv) ethanol tolerance (Chaudhari et al. 2012). On the contrary, tailor-made multi-stress tolerant

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genetically modified ethanologens for high ethanol productivity are impractical at present. Alternatively, exploration of naturally endowed ethanologen(s) from the vast biodiversity of extreme environments with desired attributes for ethanol production are anytime superior and preferred. Based on this, several earlier reports attempted to screen ethanologens from a wide variety of ecological habitats viz. soils (Kiransree et al. 2000a; Limtong et al. 2007), hot spring drainage (Ryohei et al. 2003), sugar mills (Anderson et al. 1986), sugarcane juice (Dhaliwal et al. 2011), traditional brews and wines (Blieck et al. 2007; Pereira et al. 2010), Long-pang (Laopaiboon et al. 2009), bioethanol plants (Pereira et al. 2010), etc. However, very few reports exist on isolation of ethanologens with both thermo- and osmo-tolerant peculiarities (Brooks 2008; Kiransree et al. 2000a, b; Watanabe et al. 2010). Hence, the major impetus for the exploration of ethanologenic yeasts from various habitats stems to search desirable yeasts for ethanol production.

Compost, an artificial extreme environment entails dynamic microbial succession predominantly due to changes in temperature, oxygen concentration, moisture content and nutrient availability in the decomposition of high-solid organic matter. The composition and selection of microbial biodiversity involved in composting is dependent on the (i) choice of organic material and (ii) alternate mesophilic and thermophilic phases (≥60 °C) (Ryckeboer et al. 2003). The mango peel and pulp waste material consists of high levels of residual polyphenolics and carbohydrates (Ajila et al. 2007) imposing a unique milieu for selection of microbes tolerant to phenolics, high C5 and C6 sugars, complex organic content, high temperatures (≥60 °C), low pH and a_w during composting. Although, various compost material have been explored for ecological and functional biodiversity but no reports exist about microbial consortium in mango-fruit waste compost. Hence, the mango (*Mangifera indica* L.) peel and pulp compost was identified as a potential habitat for selection of multi-stress-tolerant fermentative microbes.

In view of these requirements desirable for ethanol production, the present study examined the mango pulp–peel compost to selectively screen and isolate robust, ethanologenic, multitude stress factor (osmo-, thermo-, ethanol and inhibitor) resistant microbes (yeast, bacteria and molds) for fermentative production of ethanol using glucose and molasses as simple and complex substrates.

Materials and methods

Sampling

Compost samples ($n = 5$) were collected from the mango pulp composting unit of a near-by fruit-pulp manufacturing industry (Lat, Lon: 20°56′41″N, 75°33′12″E). Composting heaps (90 days old) were excavated at a depth of 1.5 m from top to collect sample (~100 g) and composite sample was processed immediately for enrichment.

Media, enrichment and isolation

Enrichment of indigenous microflora was performed using modified procedure of Peres et al. (2001). Compost sample (25 g) was suspended in 225 ml YPD10 medium consisting of (g l^{-1}) yeast extract, 10; peptone, 20; dextrose, 100; pH 6.0 and incubated at 40 °C, 160 rpm for 48 h. The culture broth repeatedly grown for two times in YPD10 medium was successively transferred (5 %) in each Erlenmeyer flask (500 ml capacity) containing YP medium supplemented with (i) step 1: dextrose (150 g l^{-1}; YPD15); (ii) step 2: dextrose (150 g l^{-1}) and ethanol (40 g l^{-1}); (iii) step 3: dextrose (150 g l^{-1}), ethanol (40 g l^{-1}) and cycloheximide (0.4 mg ml^{-1}) to enrich bacteria, molds and antibiotic resistant yeast; and (iv) step 4: dextrose (250 g l^{-1}), ethanol (40 g l^{-1}) and cycloheximide (0.4 mg ml^{-1}) and incubated at 40 °C for 36–48 h on rotary shaker (150 rpm). Triplicates of different dilutions (1:10, 1:100 and 1:1,000) from each medium from each step was spread on to corresponding agar medium and incubated at 40 °C for 48 h. Each isolated colony differing in morphological characteristics was subcultured and maintained at 5 °C on same medium.

Identification and characterization of the microbes

Yeast isolates were characterized morphologically and biochemically for utilization of carbon and nitrogen sources and each test was interpreted as per Kurtzman et al. (2011). The genotypic characterization of yeast and mold isolates was performed as per Kurtzman and Robnett (1998) after sequencing internal transcriber spacers (ITSs) (ITS1-5.8S-ITS2) and the D1/D2 domain of 26S (LSU) ribosomal DNA (rDNA). Additionally, the 18S rDNA was also

sequenced in order to compare them with the previously reported ethanologenic microbes. Similarly, bacterial isolates were characterized based on 16S rDNA gene sequence.

Genomic DNA extraction

Genomic DNA extraction of the yeast isolates was performed using modified Bust n' Grab protocol (Harju et al. 2004). The growth medium was replaced by YPD15 for each yeast isolate and additional freeze-thaw cycles (3*x*, −80 to 80 °C at 15 min intervals) followed by treatment of 60 µl lyticase (2.5 mg ml^{-1}) and incubation at 37 °C for 2 h. DNA extraction of the mold isolates was performed with initial vortex treatment with sterile glass beads and replacing lyticase with 40 µl of chitinase. The bacterial genomic DNA isolation was performed by single colony lysis as per Ausubel et al. (2003). The DNA was quantified and assayed for purity spectrophotometrically (Nanodrop ND1000 UV/VIS spectrophotometer) and visualized by 0.7 % agarose gel electrophoresis. All the chemicals and reagents were procured from Sigma Aldrich, USA.

PCR amplification and DNA cycle sequencing

All the three potentially informative sequences of rDNA (18S, ITS1-5.8S-ITS2 and D1/D2 domain of LSU) were sequenced for phylogenetic analysis. The primers and the PCR conditions used for amplification of the target rDNA gene used in the study are summarized in Tables S1 and S2, respectively. The PCR conditions and primers used for sequencing the D1/D2 domain of LSU were as per Kurtzman and Robnett (1998). The partial 18S rDNA was amplified using *Taq* DNA polymerase (Qiagen) and the primers NS1 and NS8 in a reaction mixture (25 µl) containing 2 µl template DNA, 1.25 µl of each primer (100 pmol), 2.5 µl dNTP (0.25 mM) and 2.5 µl *Taq* buffer ($MgCl_2$, pH 8.0). The ITS region was PCR amplified in 25 µl reaction mixture containing the same components as for 18S rDNA except the primer sets ITS1 and ITS4 as per White et al. (1990). Bacterial 16S rDNA (~ 960 bp) was PCR amplified from total chromosomal DNA using 530F (5′-GTGCCAGCMGCCGCGG-3′) and 1490R (5′-GGTTACCTTGTTACGACTT-3′) primers in a reaction mixture (25 µl). All the PCR amplifications were carried out using Applied Biosystems 9700 Thermal Cycler. Sequencing reaction of each PCR product was performed as per manufacturer's instructions using a fluorescence-based BigDye™ v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and respective internal primers. Reaction products were purified using BigDye® X-Terminator™ Purification Kit (Applied Biosystems, USA). Single strand sequencing was performed on fully automated Applied Biosystems ABI 3730 DNA Analyzer.

Sequence, phylogenetic analysis and nucleotide sequence accession numbers

All sequences were base called and analyzed for quality (Sequence Scanner v1.0, Applied Biosystems, CA). Each nucleotide sequence was searched and compared with available sequences in NCBI GenBank database using BLAST to determine phylogenetic affiliation and sequence similarities. Phylogenetic analysis of the molecular dataset was carried out using sequences of the related taxa acquired from NCBI GenBank database using MEGA v5.04 Software (Tamura et al. 2011). Each sequence was deposited to NCBI GenBank (accession numbers JF715166–201 and HM357878–86, $n = 45$).

Growth and CO_2 release by yeast strains

Biomass (15 g dry wt l^{-1}) of each isolate was inoculated separately into 125 ml capacity bottles filled with 80 ml YPD15 and fermentation medium (150 g l^{-1} dextrose), sealed, purged with nitrogen (99.9 %) and incubated at 40 °C for 48 h. Total gas evolved was measured in an inverted measuring cylinder connected to the bottles, while CO_2 (%) production was estimated by headspace gas chromatography (GC, Nucon 5765 Gas Chromatograph, New Delhi) using standard CO_2 (Alchemie, Mumbai). Absolute CO_2 (ml) was calculated as a product of total gas evolved (ml) at 24 and 48 h and CO_2 (%) estimated from headspace GC divided by 100.

Growth and fermentation conditions

The batch fermentative performance of each isolate was examined initially in 100 ml fermentation medium consisting of (g l^{-1}): dextrose, 150, ammonium sulphate, 2 and yeast extract, 1.0 at pH 4.5 and 40 °C under static condition with an initial cell concentration

of 14 g dry wt l^{-1}. For this purpose, each pre-grown culture was cultivated separately to 10 ml and subsequently to 100 ml YPD15 medium in 500 ml Erlenmeyer flask under aerobic condition (150 rpm) at 40 °C for 24 h and finally, cell mass was grown in 200 ml YPD15 medium for another 18 h at 40 °C, 150 rpm. The pellet was harvested (5,000 × *g*, 10 min), washed thrice with sterile distilled water and used as inoculum for batch fermentation.

Similarly, batch fermentation was conducted in 100 ml fermentation medium containing pre-treated molasses (MFM15) at pH 4.5 and 40 °C under static conditions. Appropriate dilutions using Pearson-square method were prepared for the fermentation medium to achieve 150 g l^{-1} total reducing sugar (TRS) content and supplemented with ammonium sulfate (2 g l^{-1}) and final pH of 4.5. All the isolates were pre-acclimatized to the molasses fermentation medium by repeated inoculations (Banerjee et al. 1981).

Aliquots of each sample were withdrawn at an interval of 5 h for determination of ethanol concentration, residual sugar (Miller 1959) and cell mass (gravimetric). Ethanol from fermentation broth was recovered by distillation as per AOAC (Horwitz 1975).

Batch fermentation kinetics

The data for kinetic analysis of batch culture were analyzed in terms of growth yield ($Y_{x/s}$) and ethanol yield ($Y_{p/s}$). Ethanol productivity (P) was expressed as gram ethanol produced per gram of TRS utilized per hour calculated from the final ethanol concentration (Eq. 1). Ethanol yield ($Y_{p/s}$) was calculated as grams of ethanol produced (E_{max}) to per gram of sugar concentration (TRS_o) (Eq. 2). Ethanol yield (% of theoretical) was calculated from Gay-Lussac's equation for ethanol yield for the theoretical maximum fermentative conversion of glucose to ethanol by yeast (0.511, Eq. 3). Growth yield ($Y_{x/s}$) pertaining the growth associated with fermentation was calculated as the ratio of increase in biomass during the course of fermentation to the corresponding substrate concentration in the fermentation medium (Eq. 4). Thus,

$$\text{Ethanol productivity } \left(\text{g}\,\text{l}^{-1}\,\text{h}^{-1}\right),\ P = E_{max}/t_f \quad (1)$$

$$\text{Ethanol yield } \left(\text{g}\,\text{g}^{-1}\right),\ Y_{p/s} = E_{max}/TRS_o \quad (2)$$

$$\text{Ethanol yield } (\%\ \text{of theoretical}),\ ETY\ (\%) = \left(Y_{p/s} \div 0.511\right) \times 100 \quad (3)$$

$$\text{Growth yield } \left(\text{g}\,\text{g}^{-1}\right),\ Y_{x/s} = (X - X_o) \div (TRS_o - TRS) \quad (4)$$

where t_f is the time to achieve complete fermentation (h), X and X_o are the initial and final amounts of biomass (dry wt, g l^{-1}) and TRS_o and TRS are the initial and corresponding TRS content of the fermentation broth.

Stress tolerance

Ethanol and oxidative stress tolerance towards H_2O_2 were assessed using YPD15 medium using modified procedures described by Lewis et al. (1997) separately by inoculating the yeast cells (3×10^7 ml^{-1}) in YPD15 broth containing 20 % (v/v) ethanol and H_2O_2 (0.3 M) each for 1 h at 45 °C in triplicates. Ethanol stress was alleviated by 10-fold dilution in YPD15 medium while cell pellets were washed (thrice) for relieving H_2O_2 stress. Tolerance towards acetic and formic acid, furfural, guaiacol and vanillin was estimated separately by incorporating different concentration of each inhibitor in YPD15 broth. Cell count of treated and untreated (control) samples was estimated by plating on YPD15 agar medium at 40 °C.

Analytical methods

Residual sugar content in each sample was determined using 3,5-dinitrosalicylate reagent (Miller 1959). Physico-chemical characterization of mango peel–pulp compost material was performed in conformity to APHA (Eaton et al. 2005).

The CO_2 released during the fermentation in medium (YPD15 and fermentation medium) was estimated using GC by injecting headspace gas sample (0.5 ml) collected at 24 and 48 h intervals into Porapak Q (80–100 mesh) column, eluted with helium as a carrier gas and monitored with thermal conductivity detector. Temperature at injector was ambient, column at 60 °C and detector at 150 °C. Data was measured by WinChrome06Ex Advanced Chromatography Software v2.0.

Each fermentation wort sample was centrifuged to separate the biomass and distillate was mixed with one

volume of isopropanol. Calibration curves of absolute ethanol (slope = 0.19) were used to determine the ethanol content of distillate sample. For GC analysis, each distillate sample was injected into Porapak Q column, nitrogen (99.99 %) at 30 ml min^{-1} flow rate (1.96 bars) and monitored using FID detector. Temperature at injector was 175 °C, column oven at 200 °C and detector at 250 °C. GC responses of each distillate were measured by the same software. The retention time for ethanol and isopropanol was estimated to be 1.7 and 2.8 min, respectively under similar conditions. GC analysis of each distilled sample was performed in duplicate.

HPLC analysis of 5-hydroxymethyl furfural (5-HMF) in MFM15 medium (0 and 50 h fermentation) was performed by injecting 20 µl of each sample in a Young Lin Acme 9000 HPLC isocratic system equipped with a UV visible detector using mobile phase of methanol:water (44:55, v/v) and NeoSphere C-18 (5µ, 250 × 4.6 mm, stainless steel) stationary reverse phase column at a flow rate of 0.8 ml min^{-1}. Reduction in peak area of 50 h sample as compared to control and reference 5-HMF (Hi-media, Mumbai) was analysed.

Statistical analysis

All experiments were performed in triplicates unless otherwise mentioned. Means and standard deviations were calculated using Microsoft MS-Excel Spreadsheet Software.

Results and discussion

Characteristics of mango-fruit pulp compost

The composition of microbiota during composting is determined by many factors. Heat evolved during composting may per se inhibit microbes through enzyme inactivation or oxygen unavailability. The yeasts are generally less tolerant to high temperature during composting (Ryckeboer et al. 2003). At 60 °C, yeasts are either killed or transiently persist as ascospores and recur when temperature drops down to 54 °C (Choi and Park 1998; Peters et al. 2000; Ryckeboer et al. 2003). The proliferation of yeast during thermophilic composting was attributed to composition of food waste and low pH (Choi and Park 1998). The direct isolation of microbes from composting may possibly favor (i) natural selection of ethanologens for desirable attributes, (ii) high temperature (61–67 °C) metabolism of carbohydrates at varying rates and (iii) screening for polyphenols degradation activity. The chemical analysis of mango pulp–peel compost sample was performed for various parameters and summarized in Table 1. Earlier report about analysis of mango peel showed the presence of 21–28 % total carbohydrates and 54–109 mg g^{-1} polyphenols in different varieties of mango fruit (Ajila et al. 2007). The presence of carbohydrate (glucose and fructose) and low pH in mango peel–pulp compost may likely offer protection to yeast cells against thermal inactivation. Thus, compost of mango-fruit pulp and peel may be suitable for selection of better thermo-tolerant ethanologens due to the presence of (i) high temperature, (ii) carbohydrates and (iii) acidic pH during composting process. Hence, compost material of mango pulp and peel was predicted as a fertile habitat for screening ethanologens of interest.

Selective enrichment and isolation of ethanologens

Several approaches like metagenomic libraries and high-throughput solid-phase screening were explored for selection of desired microbes from different ecological niches (Banat et al. 1992; Fracchia et al. 2006; Gardner et al. 2012). Furthermore, earlier investigations employed either high concentration of glucose alone or in combination with high temperature

Table 1 Physico-chemical characterization of the mango peel–pulp compost

Parameter	Observation
pH	4.99 ± 0.39
Temperature (°C)	64.0 ± 4.0
Moisture content (%)	42.22 ± 10.81
Total organic carbon (%)	22.41 ± 5.19
Total nitrogen (%)	0.79 ± 0.13
C:N ratio	28.96 ± 7.26
Total solids (%)	57.78 ± 10.81
Phosphorus (%)	0.49 ± 0.14
Potassium (%)	0.48 ± 0.19
Sodium (%)	0.01 ± 0.00
Volatile solids (%)	48.10 ± 7.69
Ash (%)	13.33 ± 4.66

of incubation for isolation of ethanologens (Banat et al. 1992; Dhaliwal et al. 2011; Kiransree et al. 2000a). Accordingly, serial dilution with four successive transfers initially in YPD10 medium (225 ml) was used for isolation of ethanologenic microbes from mango peel compost (25 g) at 40 °C, 150 rpm and pH 6.0 for 48 h. Then the culture broth (5 %) was grown under similar conditions successively in (i) YPD15, (ii) YPD15 and cycloheximide (0.4 mg ml^{-1}), (iii) YPD15 fortified with cycloheximide (0.4 mg ml^{-1}) and ethanol (40 g l^{-1}) and (iv) finally, YPD25 supplemented with cycloheximide (0.4 mg ml^{-1}) and ethanol (40 g l^{-1}). The culture broth (48 h) was subsequently purified on same medium at 40 °C. After 48 h incubation, a total of 78 yeasts, two molds and seven bacterial isolates were obtained. Of the 78 yeast strains showing typical microscopic yeast-like morphological forms were selected, grouped and then each strain was examined for utilization of sugar (150 g l^{-1}) in YPD15 medium at 40 °C under aerobic condition at 150 rpm and monitored for 5 days. Strains showing meager glucose consumption within 5 days were abandoned. Among the selected 14 yeasts, 2 strains (YD1 and YD2) showed slow growth and delayed consumption of sugar (lag of 72 h), but the 12 yeast isolates utilized sugar in 60 h with increase in growth. The two mold strains too showed a similar pattern of rapid glucose consumption, while the seven bacterial isolates showed meager growth and almost negligible consumption of sugar (data not shown). Hence, yeast isolates (12) were screened and examined for further study.

Biochemical and physiological characteristics

Assessment of biochemical characteristics of ethanologenic yeast isolates further distinguished intra-species metabolic capabilities (Table S3). Yeast strains (14) of four genus were distinctly separated based on (i) growth and fermentation of carbohydrates, (ii) growth at ≤50 °C, (iii) tolerance to ≤50 % (w/v) glucose concentrations and (iv) formation of the pseudohyphae on corn meal agar by Dalmau plate technique and ascospore formation.

From Table S3, the strains Y4, Y16 and D1C were characterized as belonging to the genus *Pichia* based on (i) occasional pellicle formation, (ii) assimilation of ethanol and glycerol, (iii) growth at temperatures above 37 °C (observed up to 50 °C), (iv) multi-lateral budding, (v) absence of nitrate assimilation and (vi) urease production. These traits were found similar to the characteristics described by Kurtzman et al. (2011). However, the strains showed meager utilization of D-xylose, a desirable trait for ethanol fermentation from pre-treated lignocellulosic biomass (Chaudhari et al. 2012).

Similarly, the strains of Y1, Y2, Y9, Y12 and Y15 displayed distinctive intra-species differences in sugar utilization, pseudohyphae formation and growth in high gravity (50 %) D-glucose. The strains utilized hexose and pentose mono-, di-, tri- and polysaccharides. The yeast isolates showed (i) presence of multilateral budding, (ii) pseudomycelium formation, (iii) absence of nitrate assimilation, (iv) urease production and (v) cycloheximide resistance (0.1 %). Vigorous fermentation of D-xylose and cellobiose and tolerance to high gravity (50 %) glucose concentration were observed indicating that the yeast isolates belong to *Kluyveromyces marxianus*.

The strains B1M, C2M, D3A and D3C showed phenotypic characteristics similar to *Saccharomyces* sp. as described by Kurtzman et al. (2011). It includes (i) absence of raffinose fermentation and (ii) assimilation of maltose. However, tolerance to high temperature (50 °C) and D-glucose concentration (50 %) was observed for the strains.

The yeast isolates YD1 and YD2 exhibited distinct peculiarities like (i) maltose fermentation, (ii) growth on melezitose, (iii) formation of pseudomycelium and (iv) ring pellicle formation indicating its affiliation to *Candida* sp.

Phylogenetic analysis

Phenetic characteristics of isolates alone are unable to differentiate inter-species divergence among the strains. Hence, alternative approach like sequencing of rDNA is employed for molecular characterization of yeasts exhibiting slower rates of mutation and regarded as evolutionary chronometers (Kurtzman 1992). The sequences and similarity indices of the isolates are summarized in Table 2. The percent similarity differed among the yeast strains with respect to rDNA but clustered to their nearest neighboring taxa. Phylogenetic analysis of 14 yeasts revealed similar clustering pattern in three rDNA regions with variable degree of % similarity. Based on sequencing of 18S, ITS and D1/D2 domain of 26S rDNA gene,

Table 2 GenBank accession numbers for the nucleotide sequences of rDNA of yeast isolates and respective percent similarity patterns observed from the nucleotide database

Phylogenetic affiliation	Strain	SSU (18S)			ITS1-5.8S-ITS2			LSU 26S (D1/D2 domain)		
		GenBank accession numbers	Phylogenetic neighbor (accession numbers)	% Similarity[a]	GenBank accession numbers	Phylogenetic neighbor (accession numbers)	% Similarity[a]	GenBank accession numbers	Phylogenetic neighbor (accession numbers)	% Similarity[a]
K. marxianus	Y1	JF715166	X89523	99 (99)	–	–	–	JF715177	CR382124	99 (99)
	Y2	JF715167	X89523	97 (99)	JF715191	JQ425346	99 (100)	JF715178	CR382124	99 (99)
	Y9	JF715168	X89523	99 (100)	JF715192	JQ425346	99 (99)	JF715179	CR382124	99 (100)
	Y12	JF715169	X89523	99 (99)	JF715193	JQ425346	99 (98)	JF715180	CR382124	99 (96)
	Y15	JF715170	X89523	99 (100)	JF715194	JQ425346	99 (99)	JF715181	CR382124	99 (97)
P. kudriavzevii	Y4	JF715171	EF550360	99 (100)	JF715195	AB369918	99 (99)	JF715182	DQ377649	100 (99)
	Y16	JF715172	EF550360	100 (99)	JF715196	AB369918	98 (96)	JF715183	DQ377649	99 (98)
	D1C	JF715173	EF550360	99 (100)	JF715197	AB369918	97 (72)	JF715184	DQ377649	99 (98)
S. cerevisiae	B1M	JF715174	EU011664	99 (99)	JF715198	AY130313	99 (99)	JF715185	BK006945	99 (99)
	C2M	–	–	–	JF715199	HF545670	98 (99)	JF715186	BK006945	99 (100)
	D3A	JF715175	EU011664	99 (99)	JF715200	JX094776	99 (98)	JF715187	BK006945	100 (87)
	D3C	JF715176	EU011664	99 (99)	JF715201	AB533539	99 (96)	JF715188	BK006945	100 (99)
Candida sp.	YD1	–	–	–	JF715189	JX463262	100 (100)	–	–	–
	YD2	–	–	–	JF715190	HF545671	96 (93)	–	–	–

[a] Figures in the parenthesis represent the nucleotide query coverage (%)

yeast isolates (61 %) delineated to the family *Saccharomycetaceae* and identified as members of *Candida* sp. (14.3 %), *K. marxianus* (35.7 %), *Pichia kudriavzevii* (21.4 %) and *S. cerevisiae* (28.6 %). Among the genus (i) *Saccharomyces*, strain B1M was found to be most distinct from the other strains but clustered with the genus *Saccharomyces* and (ii) *P. kudriavzevii* (syn. *Issatchenkia orientalis*; Kurtzman et al. 2008, 2011), strain D1C was most divergent but clustered with the genus *Pichia* clade. On the contrary, characteristic feature of each isolate was clearly inferred from the sequencing of ITS region with 99 and 97 % similarity for strain B1M and D1C with the genus *Saccharomyces* and *P. kudriavzevii*, respectively. The resemblance of the strains Y4, Y16 and D1C to previously reported ethanologenic *I. orientalis* MF121 (Hisamatsu et al. 2006) was 98 % (Y4) and 99 % (Y16 and D1C), 99 % with *I. orientalis* YS22 (Rao et al. 2008), 98 % (strain Y16) and 99 % (Y4 and D1C) with *P. kudriavzevii* Y1-N-10 (Dhaliwal et al. 2011) with the respective 18S, ITS and 26S rDNA sequences and hence, may be different from the previously reported strains.

The mold isolates (02) showed growth rapidly at 40 °C (within 48 h), however, meagerly fermenting 150 g l^{-1} as well as 250 g l^{-1} glucose containing YPD medium. Analysis of 18S rDNA gene sequencing revealed phylogenetic affiliation to *Aspergillus* and *Rhizopus* sp. sharing 98 % similarity with *Aspergillus fumigatus* (AB008401) and the zygomycete, *Rhizopus microsporus* var. *chinensis* CCTCC M201021 (EU410 422).

Ethanol production by yeast isolates

Preliminary screening

Multi-stress resistance towards elevated temperature, alcohol concentrations, enzyme inhibitors, low external pH, high sugar concentrations, weak organic acids (lactic and acetic) and furfurals are considered as desirable traits in ethanologenic microbe(s) (Chaudhari et al. 2012). Yeasts are generally better candidates for higher ethanol productivity in the presence of multitude stress factors. Initially, all isolates viz. yeasts (14), bacteria (07) and molds (02) were analyzed for sugar utilization in YPD15 medium under aerobic conditions (150 rpm) at 40 °C. Except bacterial isolates, the efficiency of sugar utilization was found to be above 90 % in 5 days. Of the 14 yeast isolates, 02 strains of Candida sp. showed (i) growth after 3 days and (ii) slow glucose consumption (below 40 %). While, mold isolates (02) exhibited better utilization of glucose at 40 °C but produced negligible ethanol (data not shown).

The CO_2 production for the selected 12 yeast isolates was investigated in 125 ml capacity sealed bottles containing YPD15 and fermentation medium with 150 g l^{-1} glucose using 15.0 g dry wt l^{-1} biomass at 40 °C under static condition for 24 and 48 h. The results are shown in Fig. 1. The absolute CO_2 evolution was found more than 90 and 135 ml with *K. marxianus* Y15 and *P. kudriavzevii* Y4, Y16 and D1C and *S. cerevisiae* B1M in YPD15 medium after 24 and 48 h. On the contrary, pattern of absolute CO_2 evolution was more than 114 ml observed with *P. kudriavzevii* Y4, Y16, D1C and strains of *S. cerevisiae* in fermentation medium (glucose, 150 g l^{-1}) after 24 h. Interestingly, CO_2 evolution was so vigorous after 48 h that the bottles violently de-sealed and poised the GC measurement.

Fermentation of D-glucose at 40 °C by yeast isolates

The preliminary screening of 12 yeast isolates showed ethanol production from fermentation medium (pH

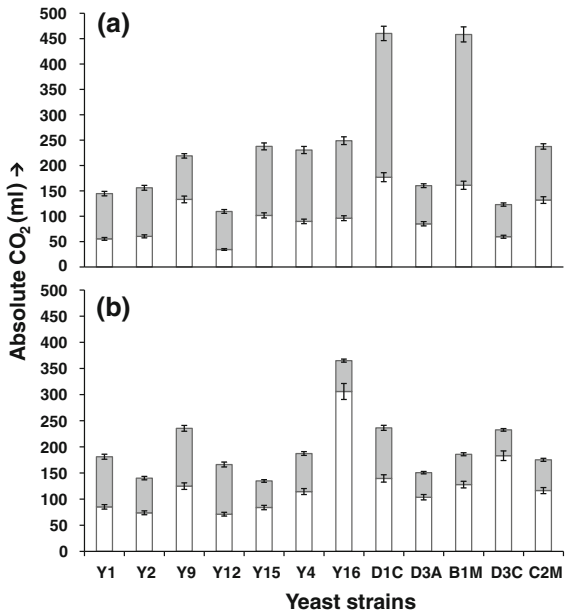

Fig. 1 CO_2 production profile of each yeast strain on **a** YPD15 and **b** glucose fermentation medium in 24 h (*white*) and 48 h (*grey*) at 40 °C

4.5) containing glucose (150 g l^{-1}). Accordingly, each isolate was pre-grown in YPD15 medium to 14.0 g dry wt l^{-1} for fermentation at 40 °C. The results obtained are set out in Fig. 2 and Table 3. From the results, it was clear that yeast isolates (12) showed ethanol production with fermentation efficiencies >92 % and ethanol productivity in the range of 0.82–7.43 g l^{-1} h^{-1}.

Among the *K. marxianus* strains, isolate Y9 achieved (i) the highest ethanol productivity (*P*) of 2.50 g l^{-1} h^{-1}, (ii) ethanol concentration (E_{max}) of 74.6 g l^{-1}, ethanol yield ($Y_{p/s}$) of 50.8 g g^{-1} and (iii) fermentation efficiency of 99.80 % (of theoretical) in 30 h. The *P* of Y12, Y1, Y15 and Y2 strains was found in the range 0.82 < 0.85 < 0.63 < 0.96 g l^{-1} h^{-1}, respectively. While, the growth yield ($Y_{x/s}$) of the strain Y2 was minimum (0.002 g g^{-1}) compared to Y15 strain (0.079 g g^{-1}). Ethanol production of most strains of *K. marxianus* is reported to be <60 g l^{-1} while fermenting glucose (150 g l^{-1}) at 40 °C (Banat

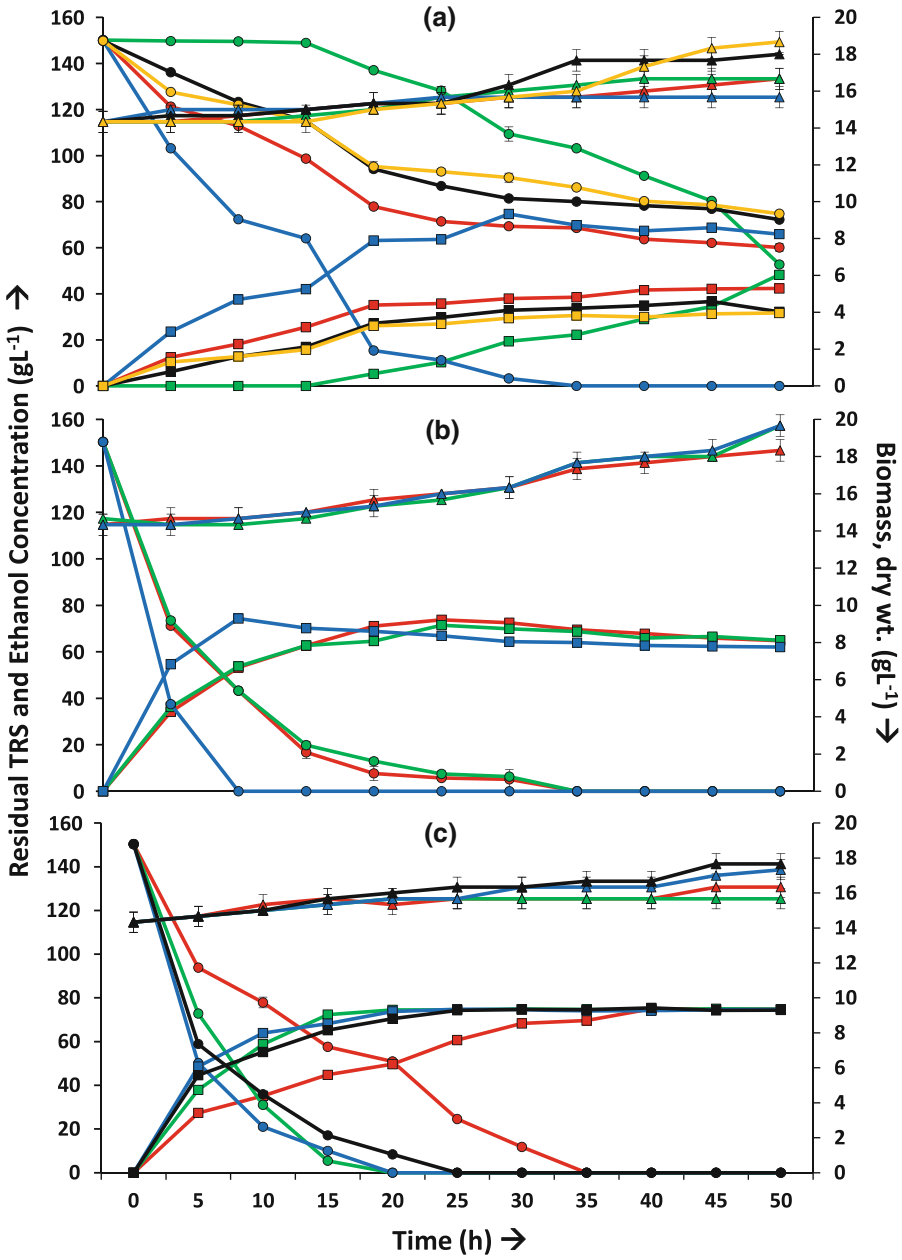

Fig. 2 Fermentative performance of yeast isolates **a** *K. marxianus*, **b** *P. kudriavzevii* and **c** *S. cerevisiae* on fermentation medium containing 150 g l^{-1} glucose at 40 °C showing residual TRS (*circles*), ethanol produced (*squares*) and biomass, dry weight (g l^{-1}, *triangles*). *Figure* **a** showing strain Y1 (*red*), strain Y2 (*green*), strain Y9 (*blue*), strain Y12 (*black*) and strain Y15 (*orange*) of *K. marxianus*. *Figure* **b** showing strain Y4 (*red*), strain Y16 (*green*) and strain D1C (*blue*) of *P. kudriavzevii*. *Figure* **c** showing strain B1M (*red*), strain C2M (*green*), strain D3A (*blue*) and strain D3C (*black*) of *S. cerevisiae*

Table 3 Comparison of kinetic parameters in batch cultures at 40 °C and 150 g l^{-1} glucose or TRS containing fermentation medium (pH 4.5)

Yeast isolate	Strain	Medium	t_f (h)	E_{max} (g l^{-1})	$Y_{p/s}$ (g g^{-1})	*ETY* (%)	P (g l^{-1} h^{-1})	$Y_{x/s}$ (g g^{-1})
P. kudriavzevii	Y4	YPD15	25	73.76 ± 0.08	0.509 ± 0.00	99.99 ± 1.13	2.95 ± 0.03	0.012 ± 0.00
		MFM15	25	73.01 ± 0.08	0.488 ± 0.00	95.87 ± 0.59	2.92 ± 0.03	0.013 ± 0.00
	Y16	YPD15	25	71.49 ± 0.19	0.500 ± 0.01	98.10 ± 3.15	2.86 ± 0.07	0.009 ± 0.00
		MFM15	25	72.48 ± 0.04	0.485 ± 0.00	95.26 ± 0.28	2.90 ± 0.01	0.016 ± 0.00
	D1C	YPD15	10	74.31 ± 0.08	0.494 ± 0.00	96.91 ± 1.04	7.43 ± 0.08	0.002 ± 0.00
		MFM15	15	73.70 ± 0.02	0.492 ± 0.00	96.61 ± 0.24	4.91 ± 0.01	0.004 ± 0.00
K. marxianus	Y1	YPD15	50	42.34 ± 0.16	0.470 ± 0.02	92.18 ± 4.81	0.85 ± 0.03	0.026 ± 0.00
		MFM15	30	57.96 ± 0.01	0.481 ± 0.00	94.44 ± 0.44	1.16 ± 0.00	0.021 ± 0.01
	Y2	YPD15	50	48.24 ± 0.22	0.494 ± 0.03	97.05 ± 6.34	0.96 ± 0.04	0.024 ± 0.00
		MFM15	50	56.30 ± 0.02	0.486 ± 0.00	95.38 ± 1.15	1.13 ± 0.00	0.035 ± 0.00
	Y9	YPD15	30	74.63 ± 0.06	0.508 ± 0.00	99.79 ± 1.20	2.49 ± 0.02	0.009 ± 0.00
		MFM15	50	52.38 ± 0.06	0.456 ± 0.00	89.52 ± 0.74	1.05 ± 0.01	0.044 ± 0.00
	Y12	YPD15	45	36.70 ± 0.16	0.502 ± 0.03	98.61 ± 5.94	0.82 ± 0.03	0.046 ± 0.03
		MFM15	15	72.16 ± 0.04	0.480 ± 0.00	94.27 ± 0.59	4.81 ± 0.03	0.007 ± 0.00
	Y15	YPD15	50	31.64 ± 0.04	0.421 ± 0.00	82.57 ± 1.74	0.63 ± 0.00	0.058 ± 0.02
		MFM15	20	65.43 ± 0.00	0.481 ± 0.00	94.37 ± 0.30	3.27 ± 0.00	0.010 ± 0.00
S. cerevisiae	C2M	YPD15	30	74.85 ± 0.10	0.497 ± 0.00	97.95 ± 1.33	2.50 ± 0.03	0.009 ± 0.00
		MFM15	35	74.96 ± 0.03	0.499 ± 0.00	97.92 ± 0.45	2.14 ± 0.00	0.013 ± 0.00
	D3A	YPD15	25	74.83 ± 0.08	0.497 ± 0.00	97.59 ± 1.13	2.99 ± 0.03	0.009 ± 0.00
		MFM15	40	74.95 ± 0.02	0.499 ± 0.00	97.91 ± 0.31	1.87 ± 0.00	0.018 ± 0.00
	B1M	YPD15	40	74.98 ± 0.11	0.498 ± 0.00	97.78 ± 0.97	1.87 ± 0.01	0.009 ± 0.00
		MFM15	40	74.88 ± 0.07	0.498 ± 0.00	97.83 ± 1.01	1.87 ± 0.01	0.016 ± 0.00
	D3C	YPD15	30	74.72 ± 0.07	0.496 ± 0.00	97.44 ± 0.98	2.49 ± 0.02	0.013 ± 0.00
		MFM15	30	74.89 ± 0.02	0.498 ± 0.00	97.83 ± 0.35	2.50 ± 0.00	0.018 ± 0.00

et al. 1998; Limtong et al. 2007). Recently, Pang et al. (2010) observed 69 g l^{-1} ethanol using mutant strain GX-UN120 of *K. marxianus.*

In contrast to the strains of *K. marxianus*, the strains of *S. cerevisiae* (D3C, D3A, B1M and C2M) showed a considerably higher % theoretical ethanol yield (97–98 %) with $E_{max} \geq$74.72 g l^{-1}. The maximum E_{max} of 74.98 g l^{-1} was shown by the strain B1M. However, P of strain B1M was much lower (1.87 g l^{-1} h^{-1}) than that of strain D3A (2.99 g l^{-1} h^{-1}). The $Y_{p/s}$ was almost same (0.496–0.498 g g^{-1}) which can be attributed to the negligible growth yield ($\leq$0.008 g g^{-1}) of all the strains.

The strains of *P. kudriavzevii* namely, Y4, Y16 and D1C showed better P than the strains of *K. marxianus* and of *S. cerevisiae* (except strain D3A) at 40 °C. The P was significantly higher with strain D1C (7.43 g l^{-1} h^{-1}) compared to strains Y4 (2.95 g l^{-1} h^{-1}), Y16 (2.86 g l^{-1} h^{-1}), the strains of *K. marxianus* and *S. cerevisiae*. The E_{max} of 74.31 g l^{-1} was attained within 10 h by strain D1C with ethanol yield of 0.494 g g^{-1} close to the strains Y4 (0.509 g g^{-1}) and Y16 (0.5 g g^{-1}). While, the E_{max} of 73.76 and 71.49 g l^{-1} by strain Y4 and Y16, respectively were attained in 25 h. The $Y_{p/s}$ of 0.509 g g^{-1} with 99.99 % theoretical yield was noticed with strain Y4, but produced 73.76 g l^{-1} ethanol lower than strain D1C and the strains of *S. cerevisiae*. The strain D1C was therefore, found to be best performing with respect to the shortest fermentation time (10 h) and E_{max} close to theoretical ethanol yield than the strains of *K. marxianus* and *S. cerevisiae* with D-glucose as a substrate at 40 °C. These results are in accordance with Dhaliwal et al. (2011) and Oberoi et al. (2012) where galactose adapted *P. kudriavzevii* Y1-N-10 cells produced 71.95 g l^{-1} ethanol with P of 4.0 g l^{-1} h^{-1} within

18 h at 40 °C in fermentation wort containing 166 g l^{-1} cane sugar and ethanol production decreased to 58.53 g l^{-1} at 45 °C. Previous studies with *P. kudriavzevii* have indicated emerging multi-stress tolerant non-*Saccharomyces* yeast for ethanol production. Transformant TTK316 of *I. orientalis* MF121-*bgl*1 produced ethanol in repeated batch fermentation of glucose (100 g l^{-1}) at 40 °C, pH 2.5 within 12 h (Kitagawa et al. 2010). In contrast, Hisamatsu et al. (2006) screened *I. orientalis* MF121 to produce 90 g l^{-1} ethanol in fermentation medium containing 200 g l^{-1} glucose and 50 g l^{-1} sodium sulphate at 30 °C, pH 6.0 and the strain further showed lower P ($\sim$95 g l^{-1}) at 30 °C after 5 days. But Gallardo et al. (2011) noticed ethanol production up to 45 g l^{-1} by *I. orientalis* 195B on YPD10 containing 100 g l^{-1} glucose at 42 °C in 30 h.

Fermentation of molasses at 40 °C by yeast isolates

Several fermentation industries use molasses, a residue of sugar juices after sucrose crystallization as a cost-effective substrate. Currently, more than 395 distilleries operate on sugarcane molasses as the principal feedstock for ethanol production in India. Besides, sugar (>46 %), molasses contain various furfurals to inhibit ethanol production by yeasts. The ethanologenic strains were therefore, adapted in molasses medium by repeated sub-culturing prior to the fermentation in order to overcome the inhibitors (Banerjee et al. 1981).

Figure 3 and Table 3 summarizes the performance of yeast isolates on MFM15 medium. Except for the *K. marxianus* strain Y9, all the yeasts showed ethanol productivity ≥94 % (of theoretical). The ethanol productivity for each isolate was ranged from 1.05 < 1.13 < 1.16 < 3.27 < 4.81 g l^{-1} h^{-1} for Y9 < Y2 < Y1 < Y15 < Y12 of *K. marxianus* strains, respectively. While, various *S. cerevisiae* strains showed ethanol productivity of 1.87 g l^{-1} h^{-1} for strain D3A and B1M and 2.14 < 2.5 g l^{-1} h^{-1} for C2M and D3C respectively. But in case of strain Y12, ethanol productivity was 4.81 g l^{-1} h^{-1} on MFM15 medium better than 0.82 g l^{-1} h^{-1} on glucose which possibly may be due to the presence of activators (metal ions, vitamins etc.) and better adaptation of the strain to resist the fermentation inhibitors present in MFM15 medium. On the contrary, *S. cerevisiae* C2M attained ethanol yields ≥97.83 % (of theoretical) with ethanol yield and E_{max} of 97.92 % and 74.96 g l^{-1}, respectively by strain C2M. The performance corroborates with the ethanol productivities on glucose as a substrate with respect to E_{max}, and ethanol yield. However, ethanol productivity of the strains C2M and D3A decreased from 2.5 to 2.14 and 2.99 to 1.87 g l^{-1} h^{-1}, respectively.

The ethanol productivity of strain D1C of *P. kudriavzevii* was 4.91 g l^{-1} h^{-1} (15 h) compared to 7.43 g l^{-1} h^{-1} (10 h) on glucose as substrate. However, marginal decrease in E_{max} from 74.31 to 73.7 g l^{-1} on MFM15 medium was noticed. Relatively consistent ethanol productivities of $\sim$2.9 g l^{-1} h^{-1} and E_{max} (≥72.48 g l^{-1}) were observed for strain Y4 and Y16.

The observed variation of fermentation efficiency within the strains and between the simple and complex substrates viz. glucose and molasses may be attributed to the stress factors encountered by yeasts and their adaptability to the presence of fermentation inhibitors/enzyme activators in MFM15 medium. However, at the same time, thermo-, osmotolerance and ethanol inhibition stress to all the isolates were compelling to high ethanol productivities on molasses as a substrate.

Stress tolerance

An array of yeast growth and fermentation inhibitors like weak organic acids (acetic, formic etc.), furan derivatives (furfural and hydroxymethyl furfural) and phenolic compounds (vanillin, guaiacol etc.) are reported during pre-treatment of carbohydrate feedstock (van Maris et al. 2006). The concentration of the inhibitors varies in the processed feedstock depending upon the composition and pre-treatment process adopted. In the present study, the ethanologenic *P. kudriavzevii* D1C was analyzed for ethanol, oxidative and 5-HMF tolerance. The strain showed (i) survival (%) of 67.57 ± 0.8 and 72.97 ± 1.5 in ethanol (20 %, v/v) and H_2O_2 (0.3 M) respectively and (ii) degradation of 5-HMF as evidenced from substantial decrease in peak area (retention time of 4.47 min) in fermented MFM15 medium (50 h) compared to untreated (0 h) and reference 5-HMF (retention time of 4.46 min) with emergence of additional peak in the fermented broth (retention time of 2.53) (Fig. 4). These results are in accordance with % survivors after stress in *S. cerevisiae* A16 (Lewis et al. 1997) and better than mutant strains of *S. cerevisiae* (Çakar et al. 2005). The growth of strain D1C was totally arrested at (i) 8 g l^{-1} acetic acid, (ii)

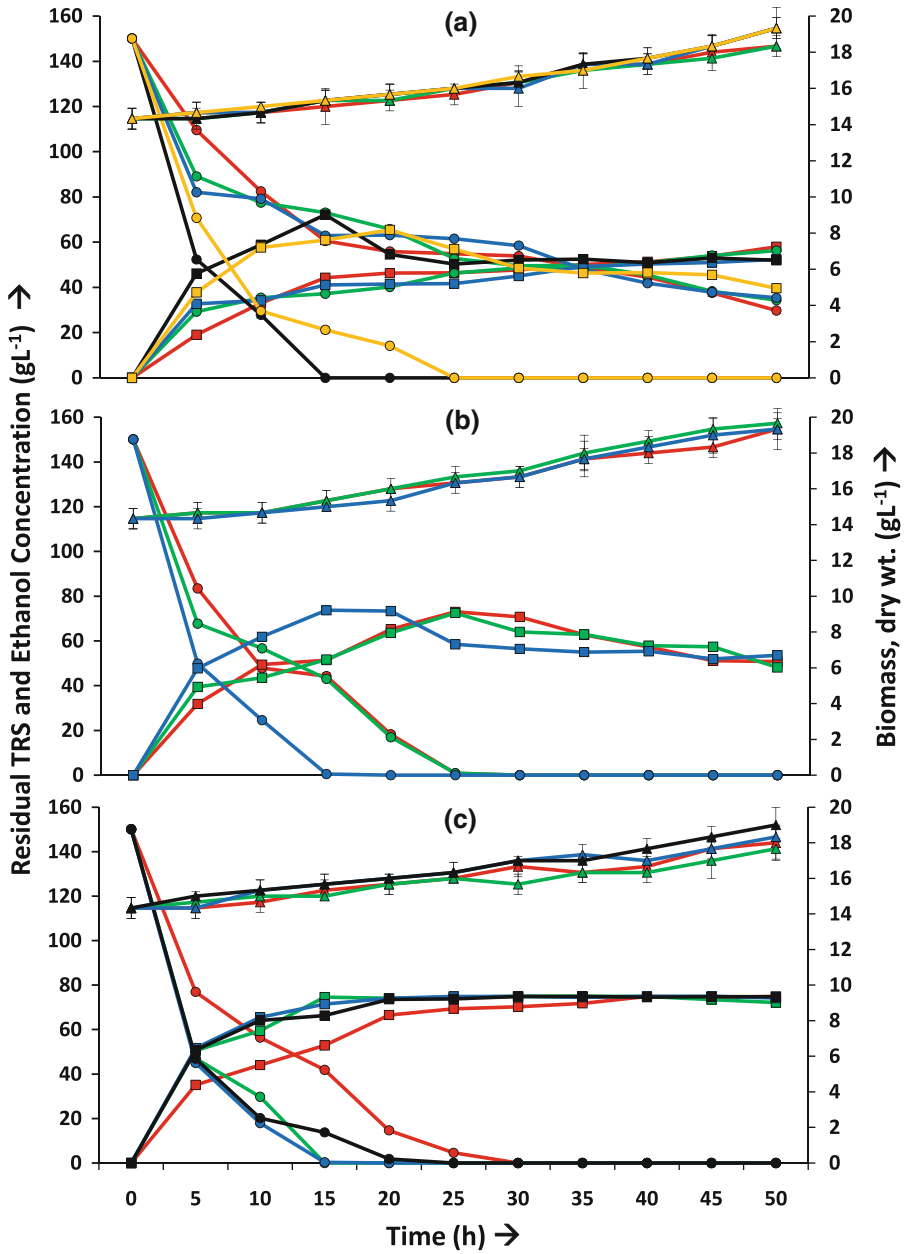

Fig. 3 Fermentative performance of yeast isolates on MFM15 medium containing 150 g l^{-1} TRS at 40 °C showing (g l^{-1}) residual TRS (*circles*), ethanol produced (*squares*) and biomass, dry weight (*triangles*). *Figure* **a** showing strain Y1 (*red*), strain Y2 (*green*), strain Y9 (*blue*), strain Y12 (*black*) and strain Y15 (*orange*) of *K. marxianus*. *Figure* **b** showing strain Y4 (*red*), strain Y16 (*green*) and strain D1C (*blue*) of *P. kudriavzevii*. *Figure* **c** showing strain B1M (*red*), strain C2M (*green*), strain D3A (*blue*) and strain D3C (*black*) of *S. cerevisiae*

2 g l^{-1} formic acid, (iii) 2.5 g l^{-1} furfural, (iv) 12 mg l^{-1} guaiacol and (v) 12 mM concentration of vanillin. The strain D1C was also tolerant to low pH (2.0), a characteristic similar to the *I. orientalis* strain MF121 (Hisamatsu et al. 2006). These findings revealed ethanol, oxidative and inhibitor tolerance of *P. kudriavzevii* D1C thereby demonstrating as most suitable candidate for ethanol production from molasses as well as pre-treated carbohydrate feedstock.

Conclusions

This probably is the first study in which mango-fruit peel–pulp compost was scientifically examined for its composition and composting microflora. The study also demonstrated mango peel–pulp compost as a suitable source for isolation of highly fermentative microbes tolerant to multiple stress factors using a simple selective enrichment strategy. The

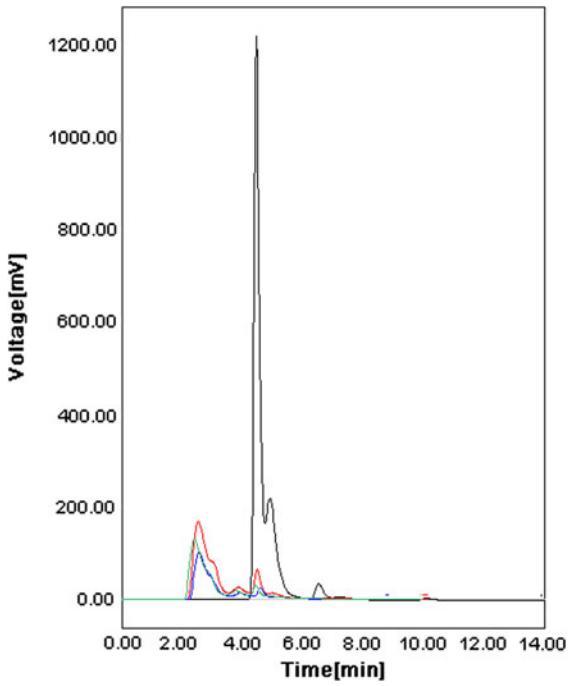

Fig. 4 HPLC profile of 5-HMF in MFM15 medium treated with *P. kudriavzevii* D1C (*red*), *K. marxianus* Y12 (*blue*) and *S. cerevisiae* (*green*) after 50 and 0 h (*black*)

preliminary performance of yeast isolates in batch fermentation of 150 g l^{-1} glucose and molasses at high temperature (40 °C) and low pH (4.5) showed better characteristics desirable for ethanol production. The isolates exhibited rapid fermentation maintaining high ethanol productivities under multitude of stress conditions. In summary, newer potential ethanologenic yeast strains were obtained from mango peel–pulp compost, but its ethanol production potential by fermenter scale-up and lignocellulosics is yet to be demonstrated.

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