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ORIGINAL ARTICLE

Physiological growth and galactose utilization by dairy yeast *Kluyveromyces marxianus* in mixed sugars and whey during fermentation

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Abstract The dairy yeast *Kluyveromyces marxianus* represents a promising industrial strain useful for the production of bioethanol from cheese whey. Physiology of the five *K. marxianus* strains on galactose was examined during batch cultivation under controlled aerobic conditions on minimal media and one of the strains designated *K. marxianus* strain 6C17 which presented the highest specific galactose consumption rate. A maximum specific growth rate of 0.34 and 0.37 h^{-1}, respectively, was achieved using batch cultivation in a minimal medium and a complex medium amended with galactose (50 g/L) at 37 °C. The sugar was metabolized for the production of ethanol as the chief metabolite with a maximum ethanol yield of 0.39 g/g of galactose. Different growth behaviors were observed when galactose was used with other sugar such as glucose, lactose and fructose. The growth rates on hydrolyzed cheese whey were also measured, and a maximum specific growth rate of 0.39 and 0.32 h^{-1} was observed with glucose and galactose, respectively, with the maximum flux diverted toward ethanol production. This approach of studying the physiology of thermotolerant *K. marxianus* on hydrolyzed whey during fermentation would be helpful in achieving higher yields of ethanol.

Keywords Dairy · Fermentation · Yeast physiology · Galactose

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Introduction

Increase in oil prices, limited availability and environmental concern of petroleum-based fuels led to an increase in the demand for production of renewable resources such as ethanol which has been contemplated as an alternative to them (Mussatto et al. 2010). A number of approaches have been put forward for increasing the productivity of ethanol such as increasing the substrate concentration, optimization rate limiting enzyme, cofactor availability and increasing the activities of all enzymes of the concerned pathway. Although these parameters play a modest role, the increased productivity will further be dependent on the physiology of the yeast strain. So, understanding the physiology of the desired fermentative yeast strain along with advanced fermentor strategies will aid in higher ethanol production.

Kluyveromyces marxianus belongs to the hemiascomycetous group of dairy yeast, better known as sister species of *K. lactis* (Llorente et al. 2000; Lane and Morrissey 2010; Saini et al. 2017a), and has GRAS status. From an industrial point of view, its features are important for its greater thermotolerance, ability to assimilate lactose, higher growth rate and broad spectrum substrate utilization for the production of ethanol (Wu et al. 2016). Although *Saccharomyces cerevisiae* has been the leading representative in fermentation studies related to ethanol production, it does not carry the trait to ferment the lactose like *K. marxianus* which has an inherited property to utilize the lactose and produce ethanol thereby turning out to be an alternative to *S. cerevisiae.* The prime difference between the *S. cerevisiae* and Kluyveromyces species is regarding glucose repression for galactose utilization (Gancedo 1998). The catabolite repression has been observed to be less pronounced in *K. lactis*, compared to *S.cerevisiae*. The

related sister species, *K. marxianus* is expected to possess similar behavior as both the species are adapted to niche containing lactose and galactose (Rubio-Texeira 2005; Guimaraes et al. 2010). Therefore, increasing the knowledge of physiology on galactose will lead to improvement in the fermentation bioprocess.

A rise in the yield of valuable products is essential for industrial processes, and this involves a high rate of utilization of substrate into product. The conversion of a substrate containing galactose as a source of carbon into ethanol requires a balance of flux through the carbon metabolism and can only be achieved if the physiology of the organism is well understood. Hence, prerequisite information is required regarding the sugar metabolism. The major research on *K. marxianus* mainly focuses on application-based biomass-related processes from lactose, whereas the utilization of galactose and mixed carbon source has received much less attention in *K. marxianus*. Therefore, the present work is aimed to study the physiology of *K. marxianus* on galactose as well as galactose mixed with other sugars as a sole carbon source.

Moreover, the main attributes of *K. marxianus* include its greater metabolic diversity because of its presence in various habitats. Cheese whey has been regarded as the prime natural environment where *K. marxianus* has been found growing and also used as a substrate for the production of valuable compounds during fermentation. The chief products are ethanol, organic acid (citric acid, acetic), enzymes (β-galactosidase), amino acids and vitamins (Furlan et al. 2000; Lane and Morrissey 2010; Kokilligadda et al. 2016). The behavior and galactose utilization pattern of the fermentative strain of *K. marxianus* in whey have not been studied so far and therefore is also a part of this present study.

Materials and methods

Strains and their maintenance

Five strains of *K. marxianus* were used in this study. Two *K. marxianus* (MTCC 1388, MTCC 4059) strains were procured from Microbial Type Culture Collection (IMTECH-MTCC) Chandigarh, India, and another three thermotolerant lab isolates, namely *K. marxianus* 6C8, *K. marxianus* 6C17 (= MTCC 25011) and *K. marxianus* 6C23, were isolated from the dairy cream. Partial 18S rRNA gene sequences of the later three strains have been deposited in GenBank under accession KF815064, KF815067 and KF815070, respectively. All the strains were initially cultivated on YPD broth medium containing (*w/v*): yeast extract (10 g/L), peptone (20 g/L) and glucose (20 g/L). The cultures were incubated at 37 °C for 24 h and maintained at 4 °C on agar slants and sub-cultured fortnightly. The culture was further maintained on YPD slants. The glycerol stocks were prepared and stored at −80 °C. The cultures were also maintained in YPD agar plates (yeast extract 10 g/L, peptone 20 g/L, dextrose 20 g/L and agar 15 g/L).

Materials

Galactose and all other chemicals used in the present study were procured from Sigma. The cheese whey was provided by National Dairy Research Institute, Experimental dairy plant, Karnal, India. Further lactic acid (1 N) was used to lower the pH of the whey for the precipitation of protein. The precipitated proteins were separated using filter paper, and the obtained lactose-rich whey was used as the raw material for further studies. The whey was passed through an ultra-filter followed by reverse osmosis to achieve a lactose concentration of up to 150 g/L (Saini et al. 2017b). The hydrolysis of the lactose into glucose and galactose was carried out by commercially available β-galactosidase (2600 units/g) procured from Sigma (G3665).

Minimal media for cultivation in shake flask and bioreactor

The minimal medium was prepared in double-distilled water with the addition of $(NH_4)_2SO_4$, 5.0 g; KH_2PO_4, 3.0 g; $MgSO_4{\cdot}7H_2O$, 0.5 g; trace elements (EDTA, 15 mg; $ZnSO_4{\cdot}7H_2O$, 4.5 mg; $MnCl_2{\cdot}2H_2O$, 0.84 mg; $CoCl_2{\cdot}6H_2O$, 0.3 mg; $CuSO_4{\cdot}5H_2O$, 0.3 mg; $Na_2{\cdot}MoO_4{\cdot}2H_2O$, 0.4 mg; $CaCl_2{\cdot}2H_2O$, 4.5 mg; $FeSO_4{\cdot}7H_2O$, 3.0 mg; H_3BO_3, 1.0 mg; KI, 0.1 mg). Soon after autoclaving, the media was cooled up to 30 °C and filtered vitamin solution (1.0 mg; pyridoxine HCl, 1.0 mg, D-biotin, 0.05 mg; nicotinic acid, calcium pantothenate, 1.0 mg; 1.0 mg; myo-inositol, 25 mg; thiamine HCl,; and para-aminobenzoic acid, 0.20 mg to a final concentration of 1 L) was added as described by Verduyn et al. 1992. The sterilized solution of the sugar was also added with different concentrations varying from 10 to 100 g/L of galactose.

Screening of galactose utilization of different strains

The various strains used in the present study were screened for the galactose utilization using the minimal media containing galactose as the sole carbon source. The cultivation was carried out in 500-mL baffled shake flask containing 250 mL of minimal medium (pH 5.0). The cultures were activated from frozen glycerol stock in YPD plates and were grown at 30 °C. The colonies that appeared

after 48 h were used to inoculate 10 ml of media. This was used to grow the pre-cultures in 500-mL Erlenmeyer flasks at 150 rpm. The cultivation on galactose was carried out in triplicate for checking the reproducibility of the experiment.

Cultivation of *K. marxianus* 6C17 in 3-L bioreactor

The aerobic batch cultivation was carried out in a 3-L bioreactor (New Brunswick, BioFlo115, USA) with 2L working volume at the stirrer speed of 150 rpm with air flow rate of 1 vvm (vessel volume per minute).The dissolved oxygen present in the fermentation medium was measured by Mettler Toledo polarographic electrode and kept greater than 50% of saturation with air throughout the fermentation run. The pH of the cultivation medium was kept at 5.0 by automatic addition of 1M KOH and 1N H_2SO_4. Lastly, cold water (4 °C) was circulated through the outlet condenser for minimizing the loss of ethanol.

Mixed sugar and whey fermentation

For determining the utilization of mixed sugar during fermentation, YP medium (10 g/L yeast extract and 20 g/L peptone) was supplemented with a variable concentration (10, 20 and 50 g/L) of sugar (galactose, glucose, lactose, and fructose) as a substrate for *K. marxianus*. Cultivation was carried out from flasks with cells grown for 10 h under shaking conditions such that the initial O.D in bioreactor maintained was 0.015 (Abs.600) at different temperatures (30, 37 and 42 °C) during the batch mode. Similarly, the hydrolyzed concentrated whey (25–75 g/L of glucose and galactose) fermentation was carried out with initial 10-h pre-grown cells previously inoculated in whey media by *K. marxianus.*

Determination of dry cell biomass of culture

The cell biomass of the samples was measured using 10 mL of the culture media filtered on pre-weighed nitrocellulose filters (pore size, 0.22 µ, Millipore). The filter was washed and dried in the oven. The dry weight of biomass was measured using analytical balance (Shimadzu, Japan).

Determination of physiological parameters during exponential phase

The maximum specific growth rate (µmax) was measured by the slope of the linear region of the plot between ln (X) versus time. The biomass yield (Yx/s) was measured as the slope of a line on an X versus S plot considering the exponential growth phase only, where X is the cell concentration and S represents the sugar concentration (Fonesca et al. 2013). Further, the specific rate of substrate consumption (µS) was measured using following equation:

$$\mu S = \mu \max / Yx/s$$

where µS denotes specific rate of substrate consumption (grams per gram DW per hour); µmax represents maximum specific growth rate (per hour); $Y_{X/S}$ represents biomass yield on different substrates (grams DW per gram); DW represents dry cell weight.

Determination of sugar and extracellular metabolites using HPLC

The sugar present in the fermentation medium and whey was detected by HPLC (LC20AD, Shimadzu, Japan) using SCX H^+ column (TOSOH, Japan). The samples were processed immediately, and 20 µL of the sample was injected into HPLC column for minimizing the loss of ethanol. The mobile phase used for the separation of the metabolites was 5 mM sulfuric acid with a flow rate of 0.8 mL/min at 35 °C (CTO-10AS). The compounds (galactose, glucose, lactose, fructose, ethanol, glycerol and acetic acid) present were detected by refractive index detector (RID10A).

Results

The bioprocess for the development and construction of efficient cell factory ensure robust industrial strain that can convert effectively the raw material into the desired product. The *K. marxianus* cell metabolizes a wide variety of substances as a carbon source and uses them as precursors of anabolic reaction and other valuable compounds. Galactose is a valuable component of various industrial media metabolized by *K. marxianus* and its utilization depend on the strain and metabolic physiology of the strain. Therefore, the objective was to study the physiology of industrially important *K. marxianus* strain on galactose as a sole source of carbon. The *K. marxianus* has been observed as a rapidly evolving strain and depicts a high degree of genetic polymorphism as reported by other authors (Belloch et al. 2002); therefore, in the present study all the experiments were carried out by sub-culturing the original single colony revived from the frozen glycerol stock.

Screening of the culture in shake flask

The initial screening of five strains of *K. marxianus* was performed using synthetic minimal media containing galactose as a sole carbon source. Physiology of all the

strains was measured during the exponential growth phase and is represented in Table 1. The cultures were screened on the basis of galactose utilization for a period of 48 h. From the comparative analysis of all the five strains cultivated on galactose at 30 °C, *K. marxianus* 6C23 offered the least substrate utilization ($\mu s = 0.58 \pm 0.011$). In addition, the strain showed least ethanol production as a metabolite. However, 6C17 strain of *K. marxianus* presented the highest specific galactose consumption rate and also achieved a maximum ethanol yield of Yp/s $= 0.33 \pm 0.03$ (Fig. 1). This strain also depicted the lower biomass concentration ($X_{max} = 3.73$ g DW/L) and other extracellular metabolite production on galactose as compared to other strains. This may be due to the diversion of the galactose sugar more toward the fermentative pathway rather than the respiration. Ethanol formation by *K. marxianus* 6C17 was also accompanied by the production of fewer metabolites such as acetic acid and glycerol. These metabolites were also generated during fermentation in other yeast strain such as *S. cerevisiae*. So after the screening of the highest galactose utilizing strain, remaining part of the study was conducted on this strain.

Galactose utilization in 3-L bioreactor on minimal media

Availability of the sugar and other key nutrients dictates the physiology and growth rate of the yeast cell factory. So in the present study, we measured the kinetic parameters during galactose utilization using a fermentative strain of *K. marxianus* 6C17. The physiology of the strain on galactose was determined in batch cultivation using galactose as the sole carbon source in minimal defined media. Firstly, we analyzed the difference obtained in parameters such as specific growth rate, sugar uptake rate and ethanol yield (Table 2).To understand the underlying changes that take place during the uptake of galactose due to temperature, the kinetic parameters were measured at three different temperatures (30, 37 and 42 °C). Cultivation

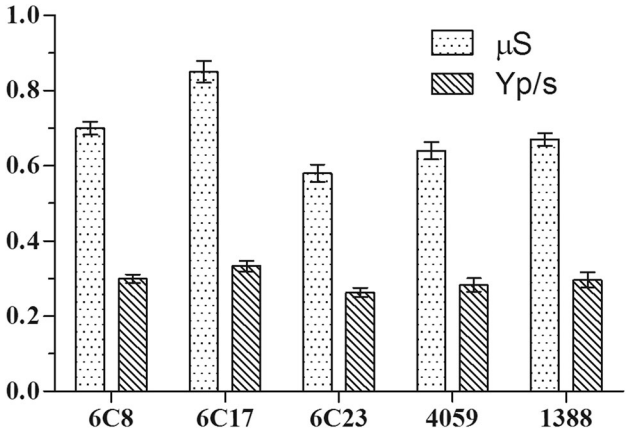

Fig. 1 Comparative galactose utilization rate (μS) and max ethanol yield (Max.$Y_{P/S}$) of five different strains of *K. marxianus* during cultivation on minimal media (at 30 °C and 150 rpm)

in all the experiments was performed in triplicate for measuring the reproducibility of the result as performed by another group of researcher (Rodrussamee et al. 2011).

Table 2 represents growth kinetics, sugar utilization and extracellular metabolites produced during fermentation. The maximum specific growth rate measured on galactose (50 g/L) at 30 °C was 0.32 ± 0.009 h^{-1}. The maximum specific growth rate upsurged with an increase in temperature, and a maximum of 0.34 ± 0.03 was obtained at 37 °C using 50 g/L of galactose. Another group of researcher also observed a similar behavior in *K. marxianus* growing faster at 37 °C as compared to 30 °C (Rocha et al. 2011) but in contrast to Fonseca et al. 2013 who observed a less efficient uptake of galactose by *K. marxianus* CBS 6556. A higher growth rate at elevated temperature offers an industrial advantage, as it saves the extra cooling cost provided during fermentation (Hensing et al. 1995).This difference that was observed in growth rate may be rather due to a greater physiological difference observed between the *K. marxianus* strains and not due to experimental errors. Further, we observed that growth on galactose at 42 °C (0.33 ± 0.01) was found to be similar to that

Table 1 Physiological parameters during exponential growth of various strains of *K. marxianus* on galactose as sole carbon source kept at the temperature of 30 °C

Strain	C-source	S_0 (g/L)	T (°C)	μ_{max} (h^{-1})	$Y_{X/S}$ (g DW/g)	μ_S (g g/DW/h)	Max. $Y_{P/S}$ (g/g)	X_{max} (g DW/L)
MTCC-4059	GAL	20	30	0.29 ± 0.05	0.45 ± 0.011	0.64 ± 0.021	0.29 ± 0.03	4.44
MTCC 1388	GAL	20	30	0.28 ± 0.02	0.42 ± 0.012	0.67 ± 0.013	0.30 ± 0.02	3.98
6C17	GAL	20	30	0.33 ± 0.04	0.39 ± 0.009	0.85 ± 0.017	0.33 ± 0.03	3.73
6C8	GAL	20	30	0.29 ± 0.06	0.41 ± 0.006	0.70 ± 0.018	0.30 ± 0.01	3.89
6C23	GAL	20	30	0.26 ± 0.05	0.45 ± 0.014	0.58 ± 0.011	0.27 ± 0.009	4.21

GAL galactose, *S0* initial substrate concentration, *T* temperature, *μmax* maximum specific growth rate, *μS* specific substrate consumption rate, *Xmax* maximum biomass concentration, $Y_{X/S}$ biomass yield on substrate, *Max. Yp/s* maximum ethanol yield, ± SD from three independent experiments

Table 2 Physiological parameters during exponential growth of *K. marxianus* 6C17 on minimal media containing different concentrations of galactose at different temperatures

C-source	S_0 (g/L)	T (°C)	μ_{max} (h^{-1})	$Y_{X/S}$ (g DW/g)	μ_S (g g/DW/h)	Yp/s	X_{max} (g DW/L)
GAL	10	30	0.29 ± 0.007	0.42 ± 0.007	0.69 ± 0.014	0.32 ± 0.007	3.44
GAL	10	37	0.31 ± 0.009	0.39 ± 0.009	0.79 ± 0.015	0.32 ± 0.01	3.58
GAL	10	42	0.30 ± 0.02	0.38 ± 0.005	0.79 ± 0.019	0.30 ± 0.02	3.48
GAL	20	30	0.32 ± 0.03	0.41 ± 0.004	0.75 ± 0.017	0.33 ± 0.008	4.06
GAL	20	37	0.32 ± 0.02	0.41 ± 0.007	0.78 ± 0.011	0.34 ± 0.02	3.73
GAL	20	42	0.33 ± 0.009	0.40 ± 0.009	0.83 ± 0.09	0.31 ± 0.01	3.56
GAL	50	30	0.32 ± 0.009	0.42 ± 0.011	0.76 ± 0.011	0.31 ± 0.01	4.37
GAL	50	37	0.34 ± 0.03	0.39 ± 0.008	0.87 ± 0.08	0.34 ± 0.02	3.82
GAL	50	42	0.33 ± 0.01	0.38 ± 0.003	0.87 ± 0.021	0.32 ± 0.03	3.44

GAL galactose, *S0* initial substrate concentration, *T* temperature, *μmax* maximum specific growth rate, *μS* specific substrate consumption rate, *Xmax* maximum biomass concentration, $Y_{X/S}$ biomass yield on substrate, *Max. Yp/s* maximum ethanol yield, ± SD from three independent experiments

observed at 37 °C, and this behavior of the strain might be due to the thermotolerant nature of the strain.

The formation of extracellular metabolites was also measured, and the main metabolites produced during the cultivation were ethanol and glycerol. Figure 2a shows that at 37 °C the maximum ethanol titer observed was 17.12 g/L using 50 g/L of galactose, whereas a maximum biomass of 3.82 g/L was obtained. Analysis of biomass yield showed that it is comparably less than that observed by another group of researchers during cultivation of *K. marxianus* on galactose (Foncesa et al. 2013). The decreased biomass yield may be diverted toward ethanol production. The formation of ethanol confirms the behavior of *K. marxianus* 6C17 toward fermentative pathway. This movement of energy obtained from galactose toward ethanol as the main metabolite might be due to the increased carbon flux toward pyruvate (Castrillo and Ugalde 1993). Moreover, it was observed that there is an increase in ethanol titer with a rise in temperature up to 37 °C. Further, an increase in temperature to 42 °C leads to a small decline in ethanol concentration (15.9 g/L). The other metabolites like glycerol and acetate were also detected as observed by other authors (Hensing et al. 1994). The formation of glycerol was higher at an elevated temperature as it might be related to the protective role toward the cell.

Galactose utilization in 3-L bioreactor on YP galactose media

The potential of *K. marxianus* 6C17 for the utilization of galactose was then monitored in YP galactose media under

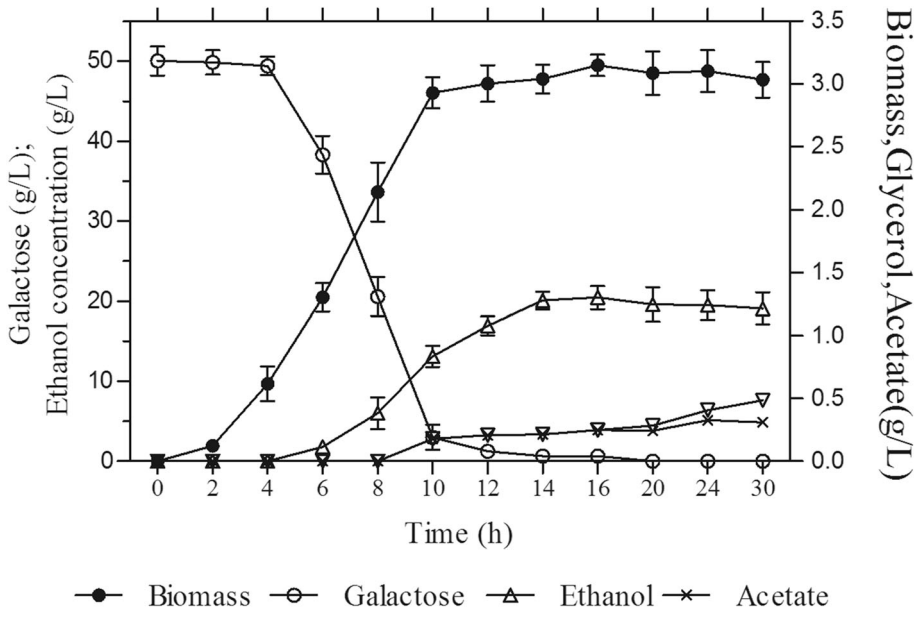

Fig. 2 Kinetics of galactose utilization with metabolites profile of *K. marxianus* 6C17 during cultivation on **a** minimal media containing 50 g/L of galactose as carbon source and **b** YPG media containing 50 g/L of galactose, 20 g/L of peptone, 10 g/L of yeast extract (37 °C and 150 rpm)

fixed agitation conditions (150 rpm) at different temperatures. Table 3 shows that the galactose consumption rate in complex media increases with a rise in temperature from 30 to 37 °C. The value of μS indicates that the cell substrate consumption is dependent on the temperature used for the cultivation of the strain. The maximum galactose consumption rate was observed at 37 °C (μS = 0.87) while comparing with μS of 0.80 at 30 °C using 50 g/L of galactose. So, there seems an interesting trend that specific galactose consumption rate increases when cells are cultivated at a higher temperature; however, this is in contrast with that observed by Fonseca et al. 2013, who observed a decrease in substrate consumption rate of *K. marxianus* CBS 6556, with an increase in temperature for glucose and galactose. There is no specific reason observed for this behavior in fermentative yeast, but there might be a role of a different type of physiology and media used for cultivation.

From the comparative analysis of the experiments, we observed an increase in ethanol yield during cultivation at the higher temperature. The maximum ethanol concentration observed at 37 °C was 19.75 g/L and was significantly higher from the set of experiments carried out at 30 °C where a maximum of 17.81 g/L of ethanol was measured using 50 g/L of galactose (Fig. 2b). The specific growth rate and the biomass yield also dropped with an increase in galactose concentration (μmax = 0.33 h^{-1} using 10 g/L of galactose at 37 °C) when compared with media containing 50 g/L of galactose (μmax = 0.35 h^{-1}). Moreover, due to the thermotolerant nature of the yeast strain, the ethanol yield was further found to be higher at 37 °C compared to 30 °C. Further increase in temperature decreases the ethanol yield. A similar role of temperature was also reported by other group of researcher (Furlan et al. 1994).

Mixture of glucose and galactose as carbon source

In the present set of experiments, the media contained a mixture of sugars and as a result yeast cell regulated the response according to the phenomenon of catabolite repression. We investigated the physiological growth in a mixture of galactose and glucose using *K. marxianus* 6C17 and two different growth phases in correspondence to two sugars (Glu and Gal) were observed. The first growth phase was observed on glucose until 10 h followed by galactose. Considering the presence of two distinct phases on glucose and galactose, various kinetic parameters such as the biomass yield, specific growth rate, and specific sugar consumption rate were measured individually for each phase. Table 4 represents these parameters along with other kinetic parameters using different concentrations of sugar mixture at different temperatures. The values obtained indicated that glucose exerted a control over galactose utilization, and therefore a sequential substrate consumption is observed in Fig. 3a.

The genetic capability of *K. marxianus* to utilize the galactose in the presence of glucose is controlled by glucose repression. This catabolite repression system governs the response of *K. marxianus* toward the availability of glucose and galactose present in media and further affects a multitude of sugar fermentation genes and the genes of the Leloir pathway. GAL genes responsible for galactose utilization are induced only after glucose that is present in the media gets exhausted to a level where repression system is relieved. A short lag phase of nearly 45 min was observed after glucose exhaustion when *K. marxianus* 6C17 was cultivated at 37 °C. This phase levels toward the production of necessary galactose metabolizing genes. Further shorter lag phase also denotes that the necessary enzyme machinery to utilize the galactose was induced before the

Table 3 Physiological parameters during exponential growth of *K. marxianus* 6C17 on YP galactose media containing different concentrations of galactose at different temperatures

C-source	S_0 (g/L)	T (°C)	μ_{max} (h^{-1})	$Y_{X/S}$ (g DW/g)	μ_S (g g/DW/h)	Yp/s	X_{max} (g DW/L)
GAL	10	30	0.32 ± 0.02	0.43 ± 0.003	0.74 ± 0.017	0.34 ± 0.01	3.04
GAL	10	37	0.33 ± 0.05	0.41 ± 0.006	0.80 ± 0.09	0.36 ± 0.02	3.18
GAL	10	42	0.33 ± 0.01	0.40 ± 0.005	0.82 ± 0.021	0.34 ± 0.02	3.08
GAL	20	30	0.32 ± 0.03	0.42 ± 0.009	0.76 ± 0.05	0.34 ± 0.01	3.96
GAL	20	37	0.34 ± 0.08	0.40 ± 0.011	0.85 ± 0.011	0.37 ± 0.03	3.13
GAL	20	42	0.33 ± 0.02	0.39 ± 0.007	0.87 ± 0.014	0.35 ± 0.02	3.01
GAL	50	30	0.33 ± 0.02	0.41 ± 0.008	0.80 ± 0.014	0.36 ± 0.02	3.97
GAL	50	37	0.35 ± 0.06	0.40 ± 0.013	0.87 ± 0.011	0.39 ± 0.009	3.17
GAL	50	42	0.34 ± 0.07	0.39 ± 0.008	0.87 ± 0.019	0.36 ± 0.006	3.11

GAL galactose, *S0* initial substrate concentration, *T* temperature, *μmax* maximum specific growth rate, *μS* specific substrate consumption rate, *Xmax* maximum biomass concentration, $Y_{X/S}$ biomass yield on substrate, *Max. Yp/s* maximum ethanol yield, ± SD from three independent experiments

Table 4 Physiological parameters during exponential growth of *K. marxianus* 6C17 on mixture of glucose and galactose kept at different temperatures

C-source	S_0 (g/L)	T (°C)	μ_{max} (h^{-1})	$Y_{X/S}$ (g DW/g)	μ_S (g g/DW/h)	Yp/s	X_{max} (g DW/L)
GLU,GAL	10,10	30	0.37,0.30	0.43,0.39	0.86,0.76	0.30 ± 0.02	4.14
GLU,GAL	10,10	37	0.38,0.32	0.42,0.40	0.90,0.80	0.31 ± 0.01	4.19
GLU,GAL	10,10	42	0.36,0.32	0.40,0.39	0.90,0.82	0.31 ± 0.008	4.10
GLU,GAL	20,20	30	0.35,0.29	0.42,0.38	0.83,0.76	0.30 ± 0.01	4.43
GLU,GAL	20,20	37	0.40,0.34	0.42,0.40	0.95,0.85	0.32 ± 0.02	4.39
GLU,GAL	20,20	42	0.36,0.32	0.39,0.38	0.90,0.84	0.31 ± 0.02	3.98
GLU,GAL	50,50	30	0.36,0.29	0.41,0.38	0.87,0.76	0.30 ± 0.01	4.38
GLU,GAL	50,50	37	0.41,0.34	0.43,0.40	0.95,0.83	0.32 ± 0.03	3.98
GLU,GAL	50,50	42	0.37,0.32	0.42,0.38	0.88,0.84	0.31 ± 0.02	4.06

GLC glucose, *GAL* galactose, *S0* initial substrate concentration, *T* temperature, *μmax* maximum specific growth rate, *μS* specific substrate consumption rate, *Xmax* maximum biomass concentration, $Y_{X/S}$ biomass yield on substrate, *Max. Yp/s* maximum ethanol yield, ± SD from three independent experiments

exhaustion of glucose. This similar behavior has also been observed in many wild varieties of *S. cerevisiae* strains (Escalante-Chonga et al. 2015). The 6C17 strain preferentially utilizes the glucose over galactose as this sugar enters directly in glycolytic pathway and further glucose affects the transport of galactose inside the cell as both sugars share a common transport system. We further observed a decline in growth rate ($\mu max = 0.30\ h^{-1}$) at a lower concentration of glucose (2 g/L) and when shifting toward galactose. The regulatory system, therefore, achieves the prioritization by using glucose as chief carbon source and reducing the uptake of galactose.

Further, the role of increasing the galactose and glucose concentration was also measured at three different temperatures and the level of oxygen throughout the experiment was maintained higher than 50% saturation. An increase in the concentration of each substrate from 10 to 50 g/L was accompanied by a direct increase in specific growth rate (μ) from 0.38 to 0.412 h^{-1} for glucose and 0.32–0.34 h^{-1} for galactose. The biomass yield was also found to be higher with an increase in the concentration of sugar under completely aerobic conditions. The ethanol titer of 6.2 g/L was obtained and was maintained throughout the fermentation process (20 g/L of mixed sugar concentration). An increase in sugar concentration directly influenced the ethanol production, and an increase in ethanol level was obtained with 31.88 g/L from 100 g/L of mixed sugar concentration.

Mixture of lactose and galactose as carbon source

To understand the underlying changes in the physiology of *K. marxianus* 6C17 strain when a mixture of lactose and galactose was present as the carbon sources, we calculated the various growth parameters under similar conditions used for the mixture of glucose and galactose (Table 5). The behavior of different strains of yeast differs in response toward the presence of different sugars in the environment in a manner that has been dependent on the evolutionary pressure maintained on that strain. Thus, in the present study, the strain was observed to be growing on both sugars in a simultaneous manner in initial hours and later the utilization of the lactose has been found to be increased as compared to galactose. This is in contrary to what was observed by Fonseca et al. (2013) who observed a lactose preference over galactose for strain *K. marxianus* CBS6556, and no simultaneous utilization of the sugar was observed by them. This behavior may be due to the different natural physiology of the yeast strain. Further, sometimes when a number of sugars are present in the media, the sensing of sugar is important in a manner that cells feel better to have multiple sugar utilization instead of single sugar. This behavior is further beneficial in a natural environment as the sugar concentration varies in a frequent manner and utilization of both sugars offer survival benefits.

The specific rate of substrate consumption (μS) at 30 °C presented different values for galactose and lactose, and the values obtained were higher for the lactose as compared to galactose for all temperature used in the present study. This was probably due to the fact that lactose acts as the main substrate present in the natural environment for the *K. marxianus* and further the *K. marxianus* strain is evolved to utilize lactose as its chief carbon source. Moreover, ethanol concentration of 6.9 ± 0.45 g/L using 20 g/L of sugar (10 g/L of each sugar, lactose and galactose at 37 °C) was also observed as the strain depicted fermentative metabolism. The ethanol production further improved with an increase in the concentration of the individual sugar, and maximum of 34.4 ± 0.2 g/L of ethanol was obtained using 100 g/L of mixed sugar at 37 °C (Fig. 3b).

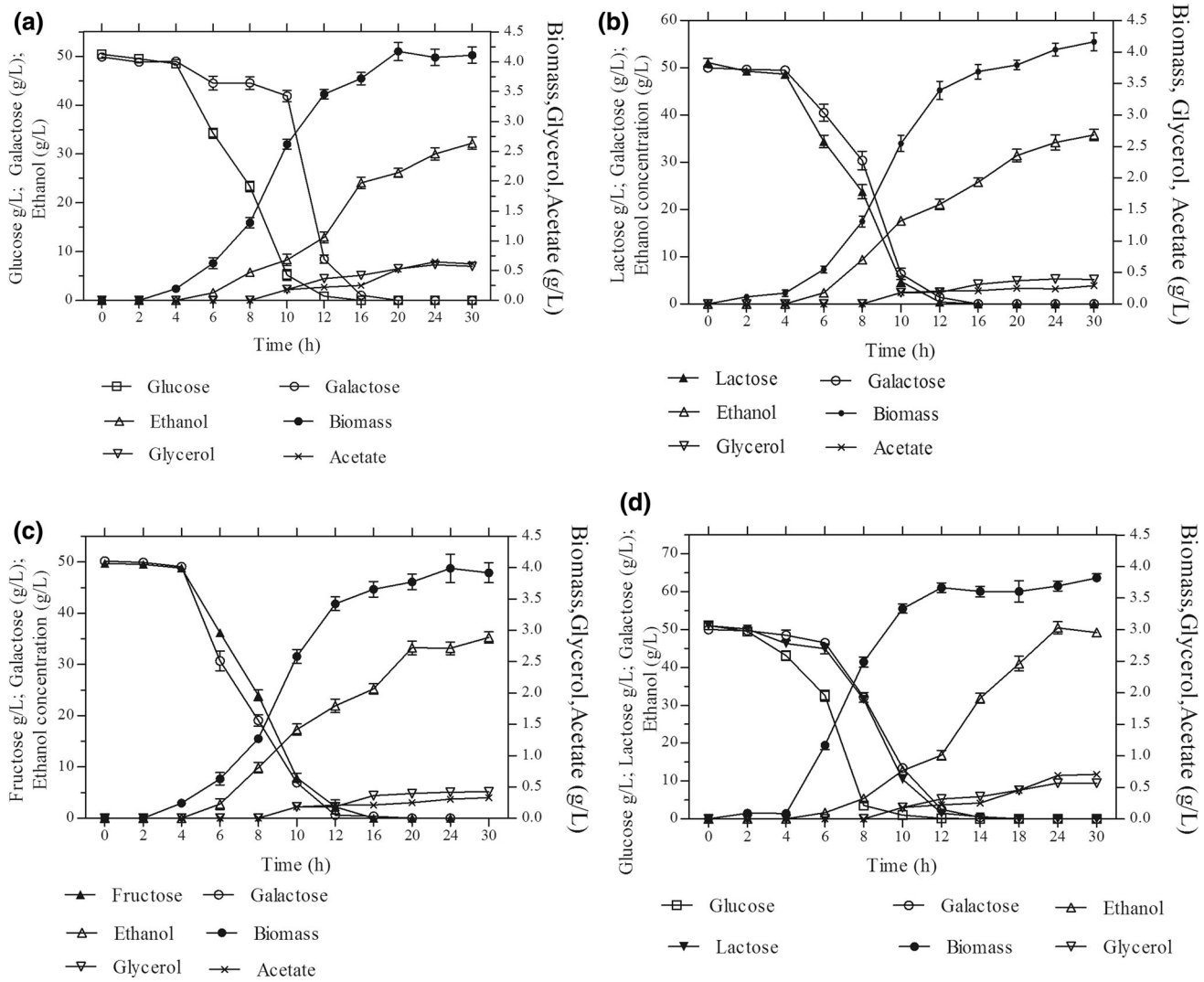

Fig. 3 Kinetics of mixed sugar utilization with metabolites profile of *K. marxianus* 6C17 during cultivation on media containing 50 g/L of each sugar **a** galactose and glucose, **b** galactose and lactose, **c** galactose and fructose, **d** galactose, glucose and lactose (peptone 20 g/L, yeast extract 10 g/L, 37 °C, and 150 rpm)

Mixture of galactose and fructose as carbon source

When the cultivation was carried out on two sugars, galactose and fructose, the utilization profile of both the sugars depicts similar behavior. Figure 3c represents that both the sugars were utilized in the similar fashion which shows that both galactose and fructose were imported inside the cell from different transport systems and further shows that there is no regulatory mechanism which prevents the utilization of other sugar when both were present in the media. Gasnier (1987) also reported proton-sugar symporters specific for fructose in *K. marxianus*. The kinetic parameters were measured at different temperatures, and it was found that both sugars have similar utilization rate (μSGal, μSFRU = 0.32) (Table 6). The main metabolite observed was ethanol, and another metabolite produced during fermentation was mainly glycerol. The concentration of sugar present initially plays a major role in the synthesis of extracellular metabolites except for ethanol. The ethanol was detected even at a concentration of 10 g/L of each sugar, but the other metabolites were not detected at this concentration. The increase in the concentration of sugar increases the amount of ethanol and a maximum of 34.4 g/L of ethanol was produced at 37 °C using 100 g/L of both sugar (Fig. 3c).

Mixture of glucose, galactose and lactose as carbon source

The study was conducted keeping in view the fact that some natural substrates used for fermentation have a mixture of sugar in media. So, a triple-sugar utilization was

Table 5 Physiological parameters during exponential growth of *K. marxianus* 6C17 on mixture of galactose and lactose kept at different temperatures

C-source	S_0 (g/L)	T (°C)	μ_{max} (h^{-1})	$Y_{X/S}$ (g DW/g)	μ_S (g g/DW/h)	Yp/s	X_{max} (g DW/L)
GAL,LAC	10,10	30	0.32 ± 0.04	0.42 ± 0.011	0.76 ± 0.011	0.31 ± 0.01	4.28
GAL,LAC	10,10	37	0.34 ± 0.02	0.41 ± 0.009	0.82 ± 0.08	0.34 ± 0.01	3.79
GAL,LAC	10,10	42	0.35 ± 0.01	0.40 ± 0.008	0.87 ± 0.011	0.32 ± 0.04	3.81
GAL,LAC	20,20	30	0.32 ± 0.05	0.43 ± 0.005	0.74 ± 0.015	0.32 ± 0.02	4.33
GAL,LAC	20,20	37	0.36 ± 0.03	0.41 ± 0.008	0.88 ± 0.017	0.34 ± 0.03	3.99
GAL,LAC	20,20	42	0.34 ± 0.01	0.40 ± 0.003	0.85 ± 0.02	0.34 ± 0.006	3.95
GAL,LAC	50,50	30	0.33 ± 0.06	0.40 ± 0.005	0.82 ± 0.005	0.31 ± 0.003	4.18
GAL,LAC	50,50	37	0.36 ± 0.02	0.41 ± 0.004	0.88 ± 0.017	0.34 ± 0.01	4.11
GAL,LAC	50,50	42	0.36 ± 0.04	0.40 ± 0.012	0.90 ± 0.009	0.32 ± 0.02	4.17

GLC glucose, *GAL* galactose, *S0* initial substrate concentration, *T* temperature, *μmax* maximum specific growth rate, *μS* specific substrate consumption rate, *Xmax* maximum biomass concentration, $Y_{X/S}$ biomass yield on substrate, *Max. Yp/s* maximum ethanol yield, ± SD from three independent experiments

Table 6 Physiological parameters during exponential growth of *K. marxianus* 6C17 on mixture of galactose and fructose kept at different temperatures

C-source	S_0 (g/L)	T (°C)	μ_{max} (h^{-1})	$Y_{X/S}$ (g DW/g)	μ_S (g g/DW/h)	Yp/s	X_{max} (g DW/L)
GAL,FRU	10,10	30	0.28 ± 0.03	0.41 ± 0.005	0.68 ± 0.017	0.30 ± 0.02	4.58
GAL,FRU	10,10	37	0.31 ± 0.01	0.42 ± 0.003	0.74 ± 0.014	0.32 ± 0.03	4.29
GAL,FRU	10,10	42	0.32 ± 0.04	0.40 ± 0.004	0.80 ± 0.011	0.32 ± 0.005	4.22
GAL,FRU	20,20	30	0.30 ± 0.01	0.40 ± 0.006	0.75 ± 0.019	0.30 ± 0.03	4.73
GAL,FRU	20,20	37	0.32 ± 0.04	0.42 ± 0.005	0.76 ± 0.02	0.32 ± 0.01	4.19
GAL,FRU	20,20	42	0.33 ± 0.01	0.40 ± 0.004	0.82 ± 0.06	0.31 ± 0.01	4.05
GAL,FRU	50,50	30	0.32 ± 0.05	0.42 ± 0.003	0.76 ± 0.02	0.31 ± 0.006	4.78
GAL,FRU	50,50	37	0.33 ± 0.02	0.40 ± 0.007	0.82 ± 0.04	0.34 ± 0.009	4.01
GAL,FRU	50,50	42	0.34 ± 0.01	0.38 ± 0.002	0.89 ± 0.03	0.33 ± 0.007	4.27

FRU fructose, *GAL* galactose, *S0* initial substrate concentration, *T* temperature, *μmax* maximum specific growth rate, *μS* specific substrate consumption rate, *Xmax* maximum biomass concentration, $Y_{X/S}$ biomass yield on substrate, *Max. Yp/s* maximum ethanol yield, ± SD from three independent experiments

conducted and it was observed that during the logarithmic phase the glucose was the first sugar to be utilized and the remaining sugar catabolism was blocked due to repression. The simultaneous utilization of both galactose and lactose was observed after consumption of glucose. The consumption started after 8 h, and utilization of the both sugars was finished within 14 h. Table 7 represents similar values obtained for the specific rate of sugar consumption on galactose and lactose, but the μS at 37 °C for glucose was found higher as compared to lactose and galactose (μSGLU = 0.93, μSGal = μSLac = 0.80); this might be a consequence of the higher substrate consumption rate of monosaccharides as compared to disaccharides as glucose can directly enter into glycolytic pathway and will be metabolized faster as compared to lactose, which requires Leloir pathway genes and shunt the galactose into the glycolytic pathway. Therefore, decreased uptake of the sugar is observed as compared to glucose. Regarding the production of ethanol, *K. marxianus* 6C17 strain produced a higher amount of ethanol at two temperature conditions conducted during this study (37 and 42 °C). Figure 3d represents the ethanol production and the substrate utilization pattern of mixed sugars at 37 °C. A maximum of 49.86 g/L of ethanol was produced during fermentation using 150 g/L of mixed sugar at 37 °C.

Galactose utilization in 3-L bioreactor on hydrolyzed whey media

Considering the role of whey in ethanol production, *K. marxianus* 6C17 was cultivated in deproteinized whey media containing different concentrations of lactose. We have tried to find out the growth conditions in whey as the life habituated by yeast cell in cheese whey often differs as

Table 7 Physiological parameters during exponential growth of *K. marxianus* 6C17 on mixture of glucose, galactose and lactose kept at different temperatures

C-source	S_0 (g/L)	T (°C)	μ_{max} (h^{-1})	$Y_{X/S}$ (g DW/g)	μ_S (g g/DW/h)	Yp/s	X_{max} (g DW/L)
GLU,GAL,LAC	10,10,10	30	0.38,0.31	0.44,0.42	0.86,0.73	0.29 ± 0.01	4.22
GLU,GAL,LAC	10,10,10	37	0.39,0.34	0.43,0.42	0.90,0.80	0.31 ± 0.02	4.09
GLU,GAL,LAC	10,10,10	42	0.37,0.33	0.42,0.42	0.88,0.78	0.32 ± 0.009	4.10
GLU,GAL,LAC	20,20,20	30	0.36,0.30	0.44,0.42	0.81,0.71	0.32 ± 0.003	4.68
GLU,GAL,LAC	20,20,20	37	0.40,0.35	0.43,0.42	0.93,0.83	0.33 ± 0.03	4.79
GLU,GAL,LAC	20,20,20	42	0.39,0.34	0.42,0.43	0.92,0.79	0.33 ± 0.01	4.26
GLU,GAL,LAC	50,50,50	30	0.36,0.30	0.42,0.40	0.85,0.75	0.30 ± 0.007	4.88
GLU,GAL,LAC	50,50,50	37	0.40,0.34	0.43,0.42	0.93,0.80	0.33 ± 0.02	4.07
GLU,GAL,LAC	50,50,50	42	0.39,0.32	0.45,0.40	0.86,0.80	0.32 ± 0.01	4.19

GLC glucose, *LAC* lactose, *GAL* galactose, *S0* initial substrate concentration, *T* temperature, *μmax* maximum specific growth rate, *μS* specific substrate consumption rate, *Xmax* maximum biomass concentration, $Y_{X/S}$ biomass yield on substrate, *Max. Yp/s* maximum ethanol yield, ± SD from three independent experiments

compared to that observed in a laboratory. This is due to exposure of cells to highly nutritious media having an excess amount of all the basic components required as building blocks inside the cell. For measuring the utilization of individual sugar in whey, lactose was hydrolyzed into its components, glucose, and galactose, using β-galactosidase and further, the strain was used to measure the uptake of sugar. *K. marxianus* is evolved to be reliant on relatively less nutrient components in its natural habitat, and therefore appropriate responses should be mounted toward the utilization of mixed sugar. Further, the understanding of the relation between the natural environment present in whey media with intracellular sugar sensing and its utilization system will result in the generation of the hypothesis regarding the correct physiology and the molecular mechanism dealing with sugar utilization by *K. marxianus* (Saini et al. 2017c). With the use of hydrolyzed whey, we have attempted to illustrate the physiology of dairy yeast *K. marxianus* 6C17 strain in its original niche environment.

The growth physiology on whey revealed a slight difference in behavior, in relation to that observed in synthetic media containing a mixture of glucose and galactose. Firstly, during the initial uptake of sugar, galactose was also consumed to some extent along with glucose and later on galactose utilization was stopped. Therefore, two distinct phases were observed. The exact reason for this observation is not clear, but there may be chances that cells during the initial uptake were unable to differentiate between the two monosaccharides as both the sugars share similar transporter. Table 8 represents the kinetic parameters measured at different temperatures (30, 37 and 42 °C), and the maximum ethanol production rate was observed at 37 °C (Fig. 4). The specific growth rate observed for glucose was higher as compared to that of galactose (μmax Glu = 0.37 h^{-1} and μmax Gal = 0.30 h^{-1} containing 50 g/L of each sugar at 37 °C). The growth rate of yeast cell was found to be slower on deproteinized whey media compared to the synthetic YPG media, and this might be due to the presence of lesser nutrients and therefore exhibit a slower proliferation profile on both galactose and glucose. The galactose and the glucose contents of the hydrolyzed media were found to be 49.8 and 51.0 g/L, respectively. The major observation was that the main metabolite ethanol was generated during the cultivation and a maximum of 31.38 g/L of ethanol was observed during the run. The other main extracellular metabolite observed during the cultivation of whey media was acetic acid and glycerol.

Discussion

Although *K. lactis* is regarded as the model organism in the *Kluyveromyces* genus, role of *K. marxianus* has received attention due to its large industrial potential for the production of ethanol from whey (Fonseca et al., 2008; Guimaraes et al. 2010). *K. marxianus* has been isolated from different habitats accounting for the presence of their great variable metabolic diversity. Moreover, *K. marxianus* has been found to be adapted to environments containing lactose and galactose and therefore the present study was undertaken to study physiology on galactose. The yeast cell fitness is governed by a number of cues starting with its ability to integrate the number of signals regarding sugar availability from its natural environment, which are used further to coordinate and metabolize the necessary sugar under the control of regulatory networks. Galactose is the major sugar constituent after the breakdown of lactose as well as constituents of other substrates like hemicellulose

Table 8 Physiological parameters during exponential growth of *K. marxianus* 6C17 in hydrolyzed and concentrated whey kept at different temperatures

C-source	S_0 (g/L)	T (°C)	μ_{max} (h^{-1})	$Y_{X/S}$ (g DW/g)	μ_S (g g/DW/h)	Yp/s (g/g)	X_{max} (g DW/L)
GLU,GAL	25,25	30	0.35,0.29	0.44,0.39	0.79,0.74	0.28 ± 0.005	4.34
GLU,GAL	25,25	37	0.37,0.30	0.43,0.41	0.86,0.73	0.30 ± 0.02	4.09
GLU,GAL	25,25	42	0.33,0.30	0.41,0.39	0.80,0.76	0.30 ± 0.02	4.10
GLU,GAL	50,50	30	0.34,0.26	0.44,0.39	0.77,0.66	0.28 ± 0.01	4.12
GLU,GAL	50,50	37	0.37,0.30	0.42,0.42	0.88,0.71	0.31 ± 0.006	3.93
GLU,GAL	50,50	42	0.34,0.28	0.42,0.40	0.80,0.70	0.28 ± 0.009	4.03
GLU,GAL	75,75	30	0.30,0.28	0.42,0.43	0.71,0.65	0.28 ± 0.02	4.81
GLU,GAL	75,75	37	0.33,0.27	0.44,0.41	0.75,0.65	0.30 ± 0.01	4.19
GLU,GAL	75,75	42	0.30,0.25	0.44,0.35	0.71,0.83	0.29 ± 0.007	4.09

GLC glucose, *GAL* galactose, *S0* initial substrate concentration, *T* temperature, *μmax* maximum specific growth rate, *μS* specific substrate consumption rate, *Xmax* maximum biomass concentration, $Y_{X/S}$ biomass yield on substrate, *Max. Yp/s* maximum ethanol yield, ± SD from three independent experiments

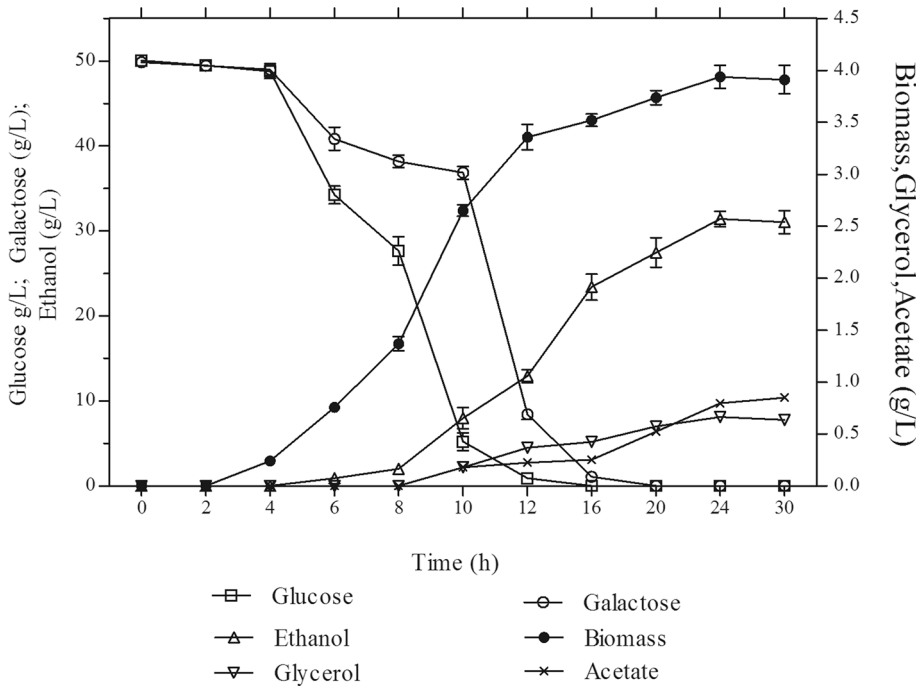

Fig. 4 Kinetics of galactose and glucose utilization with metabolites profile of *K. marxianus* 6C17 during cultivation on hydrolyzed cheese whey containing 50 g/L of glucose and galactose (37 °C and 150 rpm)

and red seaweed. So, in this study, we measured the ability of the *K. marxianus* for the utilization of the galactose as the carbon source. Galactose utilization in *K. marxianus*, as in *S. cerevisiae*, is a galactose-inducible activity.

The initial result of the present study shows the growth and fermentation pattern of *K. marxianus* on galactose and on mixed sugar glucose and galactose. The data represent that glucose exerts a strong repressive effect on galactose utilization. This could be due to two sugars, glucose and galactose, competing for the same sugar transporter which has a high affinity for the glucose. The galactose utilization is higher when galactose is solely present as the carbon source but comparatively less as compared to the glucose. Moreover, the growth rate is comparably slower using galactose when compared to glucose and lactose. So, this may be due to the less affinity of the galactose toward the transporter as compared to glucose and lactose. Furthermore, glucose repression was even observed at a higher temperature of 42 °C and this may be because of *GAL1*, which is one of the proteins responsible for utilization of galactose (Leloir pathway), and main repressor Mig1p. The Mig1p may exert its prominent binding effect on *GAL1* at a higher temperature and thereby prevent the utilization of galactose (Ostergaard et al. 2001, Rodrussamee et al. 2011).

Moreover, the growth rate on galactose was found to be lower as compared to other research groups and the difference observed in growth rate was not due to experimental errors, but rather due to a physiological difference observed between the strains used in different studies. Different isolation sources for different strains and high level of intraspecific polymorphism are major factor responsible for governing the physiology of the *K. marxianus* (Belloch et al. 1998). It should also be mentioned that as reported in the literature that at a lower growth rate, crabtree-positive yeast strain confers more fermentative metabolism also support our data in the present study.

The repression effect of glucose in *K. marxianus* plays a vital role in the utilization of other sugars. When a mixture of galactose and lactose is present, both sugars are simultaneously metabolized, but the rate of lactose utilization is preferentially higher over galactose. Similarly, when galactose and fructose are used simultaneously, utilization of both sugars takes place at a similar rate and this happens because there may be the separate transporter for fructose and galactose, similar to the conclusion observed by de Bruijne et al. (1988) and Fonseca et al. (2013).

Finally, it should be mentioned that galactose is known to be a slow fermenting sugar in the *S. cerevisiae*. Despite some variation in the niches occupied by *K. marxianus* and *S. cerevisiae*, the basic metabolic pathway is highly similar among both divergent species. In contrast to glucose and fructose, which are directly incorporated into glycolysis, galactose needs some additional metabolic steps, before it can be channeled into glycolysis (also known as the Leloir pathway).

From this study, we found that *K. marxianus* 6C17 strain was efficiently able to ferment the galactose into ethanol as the main by-product, thereby showing fermentative behavior. It has been observed that *K. marxianus* shows variability in the utilization of sugar. Strains have been observed which confer more fermentative behavior than those of respiratory. This behavior is in counterpart to *K. lactis* which shows more respiratory mode. This phenomenon of ethanol production is postulated to deliver a competitive advantage in certain industrial application such as the production of ethanol from cheese whey. Moreover, the preference for a fermentative metabolism may represent the ancestral state for the yeast clade, as observed in *S. cerevisiae* and might be similar in *Kluyveromyces* (Merico et al. 2007). Ethanol production has been observed in present study even at a higher temperature of 42 °C but the yield is low compared to 37 °C. Indeed, the production of ethanol benefits greatly from fermentation at high temperature, because this prevents contamination and reduces cooling costs. We found that *K. marxianus* 6C17 grow well on galactose as sole carbon source at 30, 37 and 42 °C with the production of ethanol and does not present any special nutritional requirements.

Kluyveromyces marxianus 6C17 was further found to have a strong ability to produce pyruvate and acetate when exposed to an excess of galactose (> 5%). Considering this further it was observed that the acetate and pyruvate were not utilized until the galactose is exhausted in the media. This may be due to the repressive effect of galactose on pyruvate and acetate. Moreover, it is evident from the present study that the biomass yield on galactose is influenced by the temperature, with higher conversions at lower temperatures (30 °C). Increase in temperature during cultivation up to 42 °C found to have decreased biomass yield. The values of biomass yield obtained in the present work were similar to those reported by other on galactose as well as for different sugars (Fonseca et al. 2013; Fonseca et al. 2007). Since for increasing the productivity using the *K. marxianus*, an approach important from an industrial point of view has been used toward simultaneous utilization of both glucose and galactose. *K. marxianus* offer advantage over the other yeast as it has a shorter lag phase after utilization of glucose when a mixture of glucose and galactose is present as compared to other industrial applicable yeast strains.

Conclusion

Besides providing the insights of physiological behavior regarding the *K. marxianus* the present study was helpful in providing the galactose utilization pattern of *K. marxianus* 6C17 strain and further aids in the intuitive understanding of galactose regulon. In summary, the strain of *K. marxianus* used in the present study aligns with the so-called fermentative Crabtree-positive yeasts in terms of fermentation parameters. Further, the utilization of galactose in converting the sugar into ethanol is useful from the industrial point of view and results obtained provide useful data regarding the future application of *K. marxianus* in processes aimed at the production of whey based ethanol with high ethanol productivities.

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Compliance with ethical standards

Conflict of interest The author declared that they have no conflict of interest.

Ethical statement This article does not contain any studies with human participants or animals performed by any of the authors.

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