Isolation and Characterization of Novel Yeasts From Dairy Cattle Rumen for Potential Use as Feed Additives

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Research Article

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Isolation and characterization of novel yeasts from dairy cattle rumen for potential use as feed additives

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Abstract

The aims of this study were to screen and isolate yeast which is producing high biomass and cellulase enzyme from rumen fluid. Two fistulated Thai crossbred Holstein Friesian steers, averaging 350 ± 20 kg body weight, were used. The experiments were designed by 12 × 3 × 3 factorial arrangement in a completely randomized (CRD). Factor A was isolated yeasts (11 difference characteristics) and *S. cerevisiae*. Factor B was sugarcane molasses (M) concentration (50, 150, and 250 g/L). Factor C was urea (U) concentration (10, 30, and 50 g/L) (experiment 1). To assess biochemical properties, the potential yeast were selected for identified and analyzed. The highest yeast biomass was observed in three yeasts including codes H-KKU20, I-KKU20, and C-KKU20. The highest CMCase activity was observed in yeast code H-KKU20. Identification of isolates H-KKU20 and I-KKU20 revealed that those isolates belonged to *Pichia kudriavzevii*-KKU20 and *Candida tropicalis*-KKU20, while C-KKU20 was identified as *Galactomyces* sp.-KKU20. The *P. kudriavzevii*-KKU20 and *C. tropicalis*-KKU20 provided maximum cell growth. The highest ethanol production was observed in *S. cerevisiae*. The *P. kudriavzevii*-KKU20 yielded the least reducing sugar. *P. kudriavzevii*-KKU20 had higher results than the other yeasts in terms of yeast biomass production, cellulase enzyme activity, and cell number. This species may lead to higher production of yeast cell wall than the traditional species in yeast industry (such as *S. cerevisiae*) or in other applications as ruminant feed additives.

Keywords: Novel rumen yeast, Screening, Isolation, Yeast biomass of yeast, Cellulase enzyme, Dairy cattle

Introduction

The use of yeast in ruminant nutrition has been a common practice for over four decades now, the theoretical modes of action of yeast in the rumen environment have been described

47 extensible by Fonty and Chaucheyras-Durand[1] and other, albeit these are not always consistent.
48 Therefore it could be hypothesized that feeding yeast to ruminant could be growth promoting.
49 The mode of yeast action in the rumen depends on many factors such as diet composition,
50 viability, and strain of yeast[2]. Many studies have suggested a commercial strain such as *S.*
51 *cerevisiae* a single cell protein supplement in animal feed; however, potential feed utilization
52 remains limited, particularly the low of yeast biomass and fibrolytic enzyme production.

53 Previous studies have shown the capability of ruminal yeast strains when supplemented
54 in ruminant diets. Although the study by Sirisan *et al.*[3] successfully isolated yeast strains they
55 didn't evaluate its effects on the rumen pH of dairy cattle, they only hypostatized and suggested
56 that this could be the case and as reported by extensive literature the effect of yeast
57 supplementation on reticulo-ruminal pH has resulted in variable results e.g. Hasunuma *et al*.[4].
58 Moreover, Paserakung *et al*.[5] revealed that *Tricosporon asahii* GSY10, a yeast isolated from
59 the environment, was a potential alternative fat source in ruminant diets. A recent study by
60 Intanoo, *et al*.[6] reported that isolated *Kluyveromyces marxianus* yeast from rumen fluid showed
61 potential as an aflatoxin-detoxifying agent in dairy cattle. In addition, there are some study
62 have been described yeast could be produce cellulase enzymes such as *Tricosporon cutaneum*[7].
63 Sarawan[8] discovered a new yeast species, namely *Candida konsanensis* KKU-FW10, isolated
64 from the *Jasminum adenophyllum* plant. *C. konsanensis* could potentially produce 58.24
65 units/ml of carboxymethyl cellulase (CMC) according to an *in vitro* study. However, to our
66 knowledge there are no reports of existing yeast producing cellulase enzyme and high yeast
67 biomass synthesis in the rumen of ruminants. We hypothesized that rumen fluid with yeast
68 producing cellulase enzyme can occur and also produces a high yeast biomass compared to *S.*
69 *cerevisiae.*

70 Therefore, the aim of this study was to screen and isolate yeast from rumen fluid with
71 an experimental design method. We optimized a fermentation medium containing sugarcane

molasses as a carbon source and urea as a nitrogen source to measure the efficiency of yeast biomass production and cellulase activity.

Results

Isolation and morphological characteristics of yeast isolated from rumen fluid

Isolation was under aerobic conditions, resulting in a total of 11 different colonies, whose colony morphology and microscopic observations are shown in Table 1. The 11 isolates of yeast were grown on YM agar plates and selected for formed appearance, elevation, colony nature, and colony color. The budding stage of the isolated yeast was observed under (40×) microscope and the colonies were confirmed to be yeast as, shown in Table 1. We noted two appearances of colonies including, asymmetric (indicated as A, B, C, E, and J) and ovoid colonies (coded as D, F, G, H, I, and K). In addition, elevations of colonies are indicated as A and B, whereas flat colonies are coded as C, D, E, I, and J, and convex colonies are indicated as F, G, H, and K. Smooth colonies are coded as D, E, I, and J, while other codes correspond to rough. Most colonies in this study showed white color, except codes D and J, which were colourless and code G was turbid.

The yeast biomass production and cellulase activity

Effects of varying concentrations of sugarcane molasses and urea on yeast biomass of isolated yeast

Interactions were observed between yeast strains and sugarcane molasses with urea on yeast biomass production and the results are shown in Figure 1. Yeast biomass production from ruminal isolated yeast was observed from 3.19 to 17.08 g/L in all media solutions. The highest yeast biomass was observed in three yeasts including codes H-KKU20, I-KKU20, and C-KKU20 when inoculated in 250 g/kg molasses with 10 g/kg urea (M25+U1) which provided

97 the highest yeast biomasses level at 15.77, 17.08, and 16.71 g/L, respectively ($P < 0.01$). *S.*
98 *cerevisiae* with M25+U1 produced a yeast biomass of 16.16 g/L ($P < 0.01$).
99
100 *Effects of varying concentrations of sugarcane molasses and urea on cellulase activity*
101 *of isolated yeast*
102 Interactions were observed between yeast strains and sugarcane molasses with urea on
103 carboxymethyl cellulase activity, and the results are shown in Figure 2. From 0 to 0.101
104 units/ml CMCase activity was observed in all media solutions. The highest CMCase activity
105 was observed in yeast code H-KKU20 when inoculated in all media solutions, providing a
106 cellulase activity range from 0.041 to 0.101 units/ml ($P < 0.01$). Yeast code H-KKU20
107 inoculant in 250 g/kg molasses with 30 g/kg urea provided the maximum CMCase activity
108 (0.101 ml/unit).
109
110 *Selection and identification of potential yeast strains*
111 Ruminal yeasts strains H-KKU20, I-KKU20, and C-KKU20 were selected for their
112 ability to produce yeast biomass and their CMCase enzyme synthesis.
113 The newly isolated yeast strain was identified via DNA sequencing [10] using 26S rRNA
114 gene D1/D2 domain. Identification of isolates H-KKU20 and I-KKU20 revealed that those
115 isolates belonged to *Pichia kudriavzevii*-H-KKU20 and *Candida tropicalis*-I-KKU20,
116 respectively. The D1/D2 sequence of C-KKU20 had 99.82% (1 nucleotide substitution)
117 similarity with the undescribed species *Galactomyces* sp. HN21-4 (EU651849) (name changes:
118 *Geotrichum* sp. HN21-4) and was closest to the *Galactomyces geotrichum* strain NRRL Y-
119 17569T (NG_054826), but with 11 nucleotide substitutions and 1 one gap. Based on the
120 sequence of the D1/D2 region, strain C-KKU20 was identified as *Galactomyces* sp.-C-KKU20
121 (Table 2).

Cell counts, ethanol production and reducing sugar by ruminal yeast strains

Effect of incubation time and isolated yeast strains on cell count

Interactions were observed between incubation times, and isolated yeast strains ($P > 0.05$) and cell count are shown in Figure 3. Interactions were observed between incubation time and isolated yeast strains ($P < 0.01$). Yeast cell were counted at 6.24 to 10.02 Log cells/ml at 0 to 72 h of incubation time. Two strains provided maximum cell growth: *P. kudriavzevii*-H-KKU20 (9.78 and 10.02 Log cell/ml) and *C. tropicalis*-I-KKU20 (9.53 and 9.6 Log cells/ml) at 60 and 72 h of incubation time, respectively. Meanwhile, *S. cerevisiae* (8.71 and 8.87 Log cells/ml) and *Galactomyces* sp.-C-KKU20 (8.27 and 8.44 Log cells/ml) displayed the least growth at 60 and 72 h of incubation time, respectively.

Effect of incubation time and isolated yeast strains on ethanol production

Galactomyces sp.-C-KKU20*, C. tropicalis*-I-KKU20, *S. cerevisiae*, and *P. kudriavzevii*-H-KKU20 were evaluated for ethanol production, as shown in Figure 4. Interactions were observed between incubation time and isolated yeast strains ($P < 0.01$). The isolated yeast strains produced about 9.76 to 78.6 g/L ethanol at 0 to 72 h of incubation time. The highest ethanol production was observed in *S. cerevisiae*: 76.4, 77.8, 78.5, and 78.6 g/L at 36, 48, 60, and 72 h of incubation time, respectively ($P < 0.01$). *P. kudriavzevii*-H-KKU20 and *C. tropicalis* –I-KKU20 produced the least of ethanol: about 32.9, 37.4, 44.4, and 44.2 g/L and 37.3, 39.3, 44.9, and 48.5 g/L at 36, 48, 60, and 72 h of incubation time, respectively ($P < 0.01$).

Effect of incubation time and isolated yeast strains on reducing sugar

Interaction effects were observed between incubation time and isolated yeast strains ($P > 0.05$) on reducing sugar (result shown in Figure 5). The isolated yeast yielded reducing sugar

147 between 162.9 and 29.8 g/L at 0 to 72 h of incubation time. The *P. kudriavzevii*-H-KKU20
148 yielded the least reducing sugar about 30.6 and 29.8 g/L at 60 and 72 h of incubation time,
149 respectively. Meanwhile, the two strains that yielded the most reducing sugar were
150 *Galactomyces* sp.-I-KKU20 and *S. cerevisiae* at 60 and 72 h of incubation time, respectively.
151
152 **Discussions**
153 Yeast was reported as a member of the ruminal microbial population, as discovered by
154 Orpin[22]. In this study, yeast was isolated from rumen fluid of Holstein-Friesian dairy cattle.
155 Rumen fluid was cultured under aerobic conditions, which yielded 11 yeast isolates. Similar,
156 with Sirisan[9] that 10 isolated yeasts were present when dairy cattle were fed a high-concentrate
157 diet, while 7 isolates were present when the cattle were fed a mixture of a high proportion of
158 fermented cassava pulp and concentrate. In addition, Intanoo *et al.*[6] fed cattle rice straw,
159 cassava pulp, and distilled yeast sludge. The presence of 7 to 10 yeast isolates was found when
160 cultured under aerobic conditions.
161 In terms of morphology, yeast isolates were ovoid (6 in 11), flat (5 in 11), convex (4 in
162 11), or rough (7 in 11). White colonies (9 in 11) appeared most frequently. The morphology
163 of the yeasts observed in our study were similar to those reported Marrero *et al.*[23], who
164 concluded that the yeast morphologies were slightly convex, smooth, and white- to cream-
165 colored, which is typical of ruminal yeast. However, many factors might cause different types
166 of yeast to be discovered, such as feed sources, R:C ratios, and ruminant species. Marrero *et*
167 *al.*[24] isolated yeast from dairy cattle and found that some isolated yeast could present a pink
168 coloration, which was later discovered to be *Levica* strains 18 (L18). Thus, the morphological
169 characteristics of ruminal yeast colonies can be quite complex.
170 To increase the yeast biomass of yeast, a substrate such as soluble carbohydrate and
171 nitrogen must provide sufficient supplies for the growth of yeast cells[25]. Paserakung *et al.*[5]

reported that increasing molasses concentration from 80 to 160 g/L resulted in the greatest yeast biomass production of 25.9% obtained from *Trichosporon asahii*. In addition, Johnson *et al.*[12], who studied the effects of different single-substrate carbon sources such as molasses, glucose, and sucrose with limited nitrogen sources in media solutions on the yeast biomass production of *Rhodotorula glutinis* IIP-30, found that yeast biomass production increased by 87.8% in molasses treatment. Thus, molasses might be a better potential carbon source for yeast growth than other carbon sources. Our media solution contained molasses 250 g/L with urea 10 g/L, providing a maximum ruminal yeast yeast biomass of 29.2%. This could signify that providing optimum levels of molasses and urea could positively affect yeast biomass production. Manikandan and Viruthagiri[26] reported that the nitrogen source, concentrate of nitrogen, and carbon-/ nitrogen ratio (C:N ratio) also influenced the production of yeast biomass. Danesi *et al.*[27] demonstrated that the use of sugarcane blackstrap molasses and yeast extract at a carbon to nitrogen ratio of 10:1 (C:N is 10) provided the greatest yeast biomass of yeast. In addition, Sokchea *et al.*[28] found that yeast biomass of yeast was highest at 7.57 g/L when the C:N ratio reached 10:1. However, when compare to our study, C:N ratio was higher results about 25:1. This ratio might suitable to produce highest yeast biomass (17.07 g/L).

For yeast, the major factor influencing the production of yeast biomass is not just the supplied carbon and nitrogen. Previous studies have shown that yeast strains have a significant impact on the production of yeast biomass. Van Urk *et al.*[29] reported that *S. cerevisiae* had low potential to proliferate under excessive glucose, even with aerobic conditions. Wardrop *et al.*[30] found that *Kluyveromyces marxianus* provided a higher yeast biomass 7 times greater than *S. cerevisiae* when cultured in a media solution with excessive glucose. Under aerobic conditions, oxygen acts as the final electron acceptor and if yeasts complete metabolism like *P. kudriavzevii*-H-KKU20 and *C. tropicalis*-I-KKU20 as shown in this study, they will be produce high yeast biomass and less alcohol which is called “Crabtree-negative yeast”. In contrast, *S.*

197 *cerevisiae* exhibits alcoholic fermentation and produces high amounts of ethanol, which is
198 called "Crabtree-positive yeast"[31]. The above explanation seems to be the reason that support
199 why *P. kudriavzevii*-H-KKU20 and *C. tropicalis*-I-KKU20 produced greater yeast biomass
200 more than *S. cerevisiae*.

201 In this study, the isolated yeast produced and released cellulase enzymes ranging from
202 0.020 to 0.075 units/ml. This finding can allow us to add this particular property to animal feed.
203 Although to our knowledge studies on the release of cellulase enzymes by yeast from the rumen
204 have not been conducted, studies from natural yeast have demonstrated that yeast could
205 produce cellulase enzymes. Sarawan[8] who revealed that *Candida glabrata*, *Candida natalensis*
206 and *Kluyveromyces africanus* (isolated the from *Jasminum adenophyllum* plant), when cultured
207 in yeast extract peptone dextrose broth with 10 g/kg CMC, have the ability to release cellulase
208 enzymes ranging from 0.004 to 0.08 units/ml. Thus, cellulase enzymes produced by yeasts
209 might be a potential digest feed containing cellulose contents. As for the mechanism by which
210 yeast digests fiber, it is generally known that cellulose is hydrolyzed by the cellulase enzymes
211 system, as it breaks down the β-1,4-glycosidic bonds[32]. Cellobiohydrolases (CBHs, EC
212 3.2.1.91) are important cellulase enzymes found in yeast. CBHs are instrumental in high-
213 performance, natural cellulose hydrolysis[33]. Examples of CBH expressed in yeast include
214 CBH1 (Cel7A) and CBH2 (Cel6A)[34].

215 Our study demonstrated that three yeasts were isolated from rumen fluid: *P. kudriavzevii*-
216 H-KKU20, *C. tropicalis*-I-KKU20, and *Galactomyces* sp.-C-KKU20. The name KKU refers
217 to Khon Kaen University, where the strain was originally isolated, and the number "20" means
218 the year of discovery, 2020. Interestingly, the similar yeast species of *P. kudriavzevii*, *C.*
219 *tropicalis*, and *Galactomyces* sp. had previously been isolated[35-37]. However, qualities of these
220 strains have not been studied, and this is the first report characteristic such as yeast biomass
221 production, cellulase activity, growth patterns, ethanol production, and reducing sugar of yeast

222 from rumen have been studied in them. In addition, the types of potential yeast were Crabtree-
223 negative. Crabtree-negative yeasts *P. kudriavzevii*-H-KKU20 and *C. tropicalis*-I-KKU20 can
224 more rapidly convert available sources of carbon into a solution for yeast biomass. A category
225 of yeast may therefore be used as an alternative to improving feed or use as a feed additive in
226 ruminant animals, rather than the conventional yeast such as *S. cerevisae*.

227 Our results showed that the *Pichia kudriavzevii*-H-KKU20 inoculants in media solution
228 can grow greater than other species. The maximum yeasts growth was 10.02 Log cells/ml in
229 aerobic conditions at 72 h of incubation. *Pichia kudriavzevii*-H-KKU20 was classified as a
230 Crabtree-negative group; the ability to propagate cells is greater than for the Crabtree-positive
231 group because glucose utilization is high. Crabtree-negative yeasts can transport glucose by an
232 inducible high-affinity proton symport mechanism. Aerobic conditions especially provide
233 steady growth conditions[38].

234 The present results clearly indicate that *S. cerevisiae* could produce alcohol when used
235 as an inoculant in a high concentration of carbon source under aerobic conditions. Under
236 aerobic conditions, after 72 h, *S. cerevisiae* produced 78.6 g/L ethanol, which was more than
237 other species produced. The high ethanol production capability of *S. cerevisiae* occurs because
238 when its inoculant in media solution contained high sugar, the pyruvate dehydrogenase
239 complex enzyme was inhibited[31], but the activated pyruvate decarboxylase (about 3-4 times)
240 was activated instead and change sugar to ethanol (although sufficient oxygen)[39]. In the present
241 study, the high level of molasses at 250 g/kg under aerobic conditions could also have allowed
242 *S. cerevisiae* to produce higher ethanol concentration compared to other yeast species.

243 The sugar consumption by yeasts is related to the growth curve and depends on the yeast
244 species[40]. It was indicated that isolated yeast strains from rumen have a higher growth curve
245 than Crabtree-positive yeast such as *S. cerevisiae*. This experiment revealed that *P.*
246 *kudriavzevii*-H-KKU20 consumed more sugar by 133.1 g during 0 to 72 h of incubation than

247 did *S. cerevisiae*, which consumed only by 124.6 g of sugar. Van Urk *et al.*[29] stated that the
248 sugar consumption rate of a pyruvate decarboxylase-deficient mutant of *S. cerevisiae* is much
249 lower than that of Crabtree-negative yeast strains, indicating that pyruvate decarboxylase could
250 have a strong influence on the glycolytic flux.

251 Based on the present study, it could be concluded that screening and isolating yeast from
252 rumen fluid resulted in 11 different characteristics of yeasts. The first new yeasts discovered in
253 the rumen fluid of dairy cattle were *Pichia kudriavzevii*-H-KKU20, *Candida tropicalis*-I-
254 KKU20, and *Galactomyces* sp.-C-KKU20, which could have potential for high production of
255 yeast biomass and cellulase. The maximum growth of isolated yeast was shown in a media
256 solution of 250 g/L of sugarcane molasses and 10 g/L of urea with pH 3.5 and 150 rpm shaking.
257 Under these circumstances, *P. kudriavzevii*-H-KKU20 had higher results than the other yeasts
258 in terms of biomass production, cellulase enzyme activity, and cell number. This species may
259 lead to higher production of yeast cell wall than the traditional species in yeast industry (such
260 as *S. cerevisiae*) or in other applications as ruminant feed additives. However, evaluation of
261 *Pichia kudriavzevii*-H-KKU20's ability in fiber improvement and yeast biomass production
262 needs to be elucidated to obtain increased nutritive value for ruminant animals.

263

264 **Methods**

265 Animals involved in this study were approved by the Animal Ethics Committee of Khon
266 Kaen University (record no. IACUC-KKU 38/62), the certificate that enables designing and
267 carrying out animal experimentation under the Ethics of Animal Experimentation of National
268 Research Council of Thailand. In addition, we confirmed that all methods were performed in
269 accordance with the relevant guidelines and regulations.

270

271 **Experiment 1**

272 **Animals and diet**

273 Experiment 1was conducted at Tropical Feed Resources Research and Development
274 Center (TROFREC), Department of Animal Science, Faculty of Agriculture, Khon Kaen
275 University (KKU), Thailand. Two fistulated-crossbred Holstein Friesian steers, averaging
276 350 ± 20 kg body weight, were used to screen and isolate ruminal yeast.

277 Rumen fluid from the concentrate (crude protein (CP) 160.0 g/kg DM and total
278 digestible nutrient (TDN) 750.0 g/kg DM) fed dairy steers were obtained at 50 g/kg of body
279 weight (BW) in two equal portions at 07.00 and 16.00, and rice straw was fed on an *ad libitum*
280 basis. The animals were held in individual cages and provided clean, fresh water and mineral
281 blocks *ad libitum*. The animals were fed this diet for 7 days before the rumen fluid was
282 obtained.

283

284 **The screening and isolation**

285 Screening and isolation of yeast were done according to Sirisan[9]. Briefly, the ruminal
286 fluid from each fistulated steer was taken via rumen cannula at 4 h after the morning feeding
287 and placed immediately on ice. For the total plate count, 1 ml of ruminal fluid from each animal
288 was diluted to 1:10, 1:100, and 1:1,000. Each ruminal fluid dilution was spread over a yeast-
289 malt (YM) extract agar (HiMedia Laboratories Pvt. Ltd, Mumbai, Maharashtra, India) which
290 was then incubated at 39 °C for 72 h. All of these were dissolved in distilled water and sterilized
291 for 15 min at 121°C by autoclaving. The YM agar consisted of malt extract (3 g/L), yeast extract
292 (3 g/L), peptone (5 g/L), agar (20 g/L), and glucose (10 g/L) and the YM broth was the same
293 formulation without agar. A reference strain of *Saccharomyces cerevisiae* obtained from
294 commercial product (Greathill Co., Ltd., Bangkok, Thailand) was used for comparison.

295

296 **Morphological characterization**

297 Using the streaking method, yeast colonies grown on agar media were picked up and
298 regrown on another YM agar, then incubated for 7 days at room temperature. Yeast colonies
299 were examined under a 40× light microscope. By studying different morphological
300 characteristics[10], the isolated yeast strains were identified. The appearances of the yeast
301 colonies were recorded: size, shape, convexity, surface, and color of colonies for purification.
302 Colonies growing along the points of the streak were picked up, purified, regrown in a YM
303 broth (HiMedia Laboratories Pvt. Ltd, Mumbai, Maharashtra, India), and kept in the
304 refrigerator at 4° C as stock colonies of yeasts. Afterward, all isolates were subjected to an
305 initial assessment to determine their yeast biomass and cellulase production ability by
306 inoculating them in different solutions of sugarcane molasses, urea, and CMC. For further
307 evaluation, the top three strains of isolated yeast showing the largest production of yeast
308 biomass or cellulase were selected.
309
310 **Determination of the yeast biomass and carboxymethyl cellulase activity**
311 *Experimental design and preparation of media solution*
312 The current study was conducted at the Fermentation Research Center for Value Added
313 Agricultural Products (FerVAAP), Department of Biotechnology, Faculty of Technology,
314 Khon Kaen University, Khon Kaen, Thailand, from June 2018 to September 2018. A 12 × 3 ×
315 3 factorial was used in a completely randomized design. Factor A was isolated yeast at A, B,
316 C, D, E, F, G, H, I, J, K codes and *S. cerevisiae*. Factor B was sugarcane molasses concentration
317 at 50, 100, and 250 g/L distilled water. Factor C was urea concentration at 10, 30, and 50 g/L
318 distilled water. Fermentation media were prepared by addition of sugarcane molasses as a
319 carbon source (Khon Kaen Dairy cooperative Co., Ltd., Khon Kaen, Thailand), urea as a
320 nitrogen source (Saengtawee Panit Co., Ltd., Khon Kaen, Thailand), and carboxymethyl

cellulose (CMC) 10 g/L distilled water as a stimulant substrate (Chemipan Co., Ltd., Bangkok, Thailand).

The media solution was autoclaved at 121 °C for 15 min, then a cool media solution of H_2SO_4 70% was added to adjust to a pH of 3.5. The media solution was placed in 250 ml Erlenmeyer flasks. The flasks were filled to 100 ml, and 1 ml of isolated homogenous yeast suspension from rumen (about 10^6 cells per ml) was inoculated into the media solution in an aseptic condition. Flasks were cotton plugged before incubation in an incubator-shaker machine[8].

The different levels of sugarcane molasses and urea were prepared with 100 ml of solution in 250 Erlenmeyer flask by adding a single colony from stock culture of all isolated and *S. cerevisiae* start culture (10^6 cells) to fermentation media. After that, the cultures were inoculated in an incubator shaker at 30 °C at 150 rpm for 72 h. One milliliter of fluid culture was collected at 0, 6, 12, 18, 24, 30, 36, 48, 60, and 72 h of incubation. The pH, cell count, yeast biomass, reducing sugar, cellulase activity, and ethanol production were determined. The optimum conditions for yeast to produce the greatest yeast biomass and cellulase were recorded, then the yeast was collected as stock for further experiments.

To determine the effect of initial pH value on ruminal isolated yeast, initial pH values adjusted to 3.5 were assigned to the cultures Khampa *et al.*[11]. The pH was recorded for each incubation time. The pH level of fermentation media with isolated yeast and that of sugarcane molasses with urea were measured by a glass electrode pH meter (Hanna HI-8424 Portable pH/ORP Meter, Woonsocket, RI, USA).

Yeast biomass production from isolated *strains*

Yeast biomass was determined following Johnson *et al.*[12]. A 1 ml cultured liquid sample was centrifuged at room temperature at 10,000 g for 10 min. Cell pellets were washed with

346 distilled water and then dried at 105℃ until the weight remained constant. Duplicate samples
347 were tested.
348
349 *Estimation of reducing sugar and carboxymethyl cellulase activity of isolated yeast* strains
350 The activity of carboxymethyl cellulase was determined using 3,5-Dinitrosalicylic acid
351 (DNS) reagent by the colorimetric method according to Miller[13]: 0.5 ml of crude supernatant
352 was applied to 0.5 ml of 1% (w/v) CMC solution. This solution was inserted into a 0.05 M
353 citrate phosphate buffer, (pH 4.0) and incubated at 45 °C for 30 min[14]. The enzymatic reaction
354 was interrupted by adding 1.0 ml DNS reagent and putting it in a boiling water bath for 10 min.
355 A spectrophotometer measured the color of the reaction product as 540 nm. One enzyme unit
356 was defined as the quantity of enzyme that hydrolyzed CMC to produce 1 μmol of sugar per
357 minute under the experimental condition. The equation is derived from the glucose-equivalent
358 factor generated in the assay to mmol of glucose, from the volume of the enzyme being tested
359 in the assay (0.5 ml) and the incubation time (30 min) required for generation of the reducing
360 equivalents[15].
361 Carboxymethyl cellulase activity (Unit/ml) = (C x D) ÷ MTV
362 Then: C = Releasing glucose from cellulase (mg)
363 D = Dilution factor of enzyme
364 M = Glucose molecular weight (180 g/mol)
365 T = Time incubation
366 V = Enzyme volume
367
368 **Experiment 2**
369 **Cell count, ethanol production and reducing sugar by ruminal yeast strains**
370 **Experimental design**

Experiment 2 was run after obtained the highest production of yeast biomass and cellulase enzyme. The potential yeast was selected for identified and analyzed as a 7 × 4 factorial use in a completely randomized design including. Factor A was incubation time at 0, 12, 24, 36, 48, 60, and 72 h. Factor B was isolated yeast strains including code H-KKU20 (as *P. kudriavzevii*-KKU20), I-KKU20 (*C. tropicalis*-KKU20), C-KKU20 (as *Galactomyces* sp.-KKU20), and *S. cerevisiae*. Media solutions were prepared following the appropriate solution for potential yeast, yeast biomass, and CMCase activity.

Measurement of yeast cell *growth by direct count technique*

Yeast cell growth was monitored under the different levels of sugarcane molasses and urea media culture, via counting method using a hemocytometer under microscope according to Darvishi *et al.*[16].

Ethanol production from isolated yeasts

Ethanol concentration in the fermentation media (molasses 250 g/L and urea 10 g/L) was determined by an Agilent 7890B gas chromatographer (Agilent Corporation, California, USA). The standard water-based ethanol solutions were prepared from 0.0 -1.0 % (v/v), and 1-2 µl were injected into the gas chromatographer (GC) injection port and then subjected to quantitative ethanol analysis using a capillary columnHP-5 (length 30 m) on a GC apparatus; oven temperature 40 °C constant flow mode with a Flame Ionization Detector (FID) 300 °C, air flow of 350 ml/min, and inlet temperature of 150 °C were used[17].

Reducing sugar from isolated yeasts

The reducing sugar was determined using the DNS method[13]. To measure absorbance, a double beam UV scanning spectrophotometer was used. Reduction of sugar content before

and after fermentation was determined by applying 1.0 ml diluted solution (1 ml sample in 9 ml distilled water) to a test tube with 1.0 ml DNS reagent. A blank was run in parallel with 1.0 ml of distilled water and 1.0 ml of DNS. The tubes were heated for 15 min in a bath of boiling bath water. Using a spectrophotometer, 5 ml of distilled water was applied after the tubes were cooled at room temperature, and absorbance values were noted at 540 nm. Reduction of the sugar concentration was determined from the standard glucose curve and by the dilution factor multiples[18].

Molecular identification of selected ruminal yeast

Isolation of DNA for polymerase chain reaction

DNA isolation was performed by boiling lysis buffer cells according to Maniatis *et al.* [19] with slight modification. A loopful of yeast cells was transferred to a 1.5 ml Eppendorf tube and 100 µl of lysis buffer was added. The cells were suspended in the distilled water and heated at 95°C for 15 min in a metal block bath. After boiling, 100 µl of 2.5 M potassium acetate (pH 7.5) was added and placed on ice for 1 h, then centrifuge for 5 min at 14,000 rpm. Supernatant was extracted from isoamyl alcohol twice with 100 µl of chloroform (24:1 v/v). DNA was precipitated with isopropanol, placed on hold for 10 min at 20 °C, and centrifuged for 15 min at 15,000 rpm. DNA pellets were rinsed with 70% ethanol and 90% ethanol, then dried at room temperature for 15-30 min. The dried DNA was dissolved in 30 µl Milli-Q purified water.

Polymerase chain reaction (PCR) for D1/D2 domain of 26S rDNA

The divergent D1/D2 domain of 26S rDNA was amplified with primers NL-1 (5'- GCA TAT CAA TAA GCG GAG GAA AAG-3') and NL4 (5'-GGT CCG TGT TTC AAG ACG G-3')[10]. Amplification was performed in 100 µl reaction mixture conditioning 100 ng of 2.5 U of Taq polymerase, genomic DNA, 40 mM of each primer, 20 mM of each dNTP, 1.5 mM MgCl2,

and 10 mM Tris-HCl. The reaction was pre-denatured at 94 ° C for 5 min. This was repeated at 94 ° C for 1 min for 30 PCR cycles, annealed at 55 °C for 1 min, and extension at 72 °C for 2.5 min, followed by the final extension at 72 °C for 10 min.. According to manufacturer instructions, the amplified DNA was purified with a QIAquick PCR purification kit. Visualization of purified amplified DNA was accomplished by electrophoresis using 0.8% agarose gel in 1X TBE buffer and stained with ethidium bromide (8×10^{-5} μg/ml) and observed under a UV illuminator.

D1/D2 domain of 26S rDNA sequencing

The nucleotide sequences of the 26S rDNA domain D1/D2 were determined directly using PCR products according to Kurtzman and Robnett[10] with slight modification. Cycle sequencing of the D1/D2 domain was used with the forward primer NL1 (5'-GCA TAT CAA TAA GCG GAG GAA AAG-3') and reverse primer, NL4 (5'-GGT CCG TGT TTC AAG ACG G-3'), by ABI PrismTM BigDye™ Terminator Cycle Sequence Ready Reaction Kit (Applied Biosystems, Stafford, USA) according to the manufacturer's instruction and delivered to National Center for Genetics Engineering and Biotechnology (BIOTECH, Khlong Luang, Pathum Thani, Thailand) for sequencing. The sequences aliment with CLUSTAL X program and were compared with GenBank DNA database (http:// www.ncbi.nlm.nih.gov/)

Statistical analysis

Experiment 1. Data were analyzed as a 12 x 3 x 3 factorial in a completely randomized design. The analysis of variance procedure of SAS program was used for the analysis and the statistical model is as follows:

$$Y_{ijk} = \mu + A_i + B_j + AB_{ij} + C_k + AC_{ik} + BC_{jk} + ABC_{ijk} + \varepsilon_{ijk}$$

Where Y_{ijk} = observation, μ = overall mean, A_i = Yeast strain effect (i = a,b,c,d,e,f,g, h,i,j,k and *S. cerevisiae*), B_j = sugarcane molasses effect (j = 50, 150 and 250g/kg), AB_{ij} = yeast strain effect × sugarcane molasses effect, C_k = urea effect (k = 10, 30 and 50 g/kg), AC_{ik} = yeast strain effect × urea effect, BC_{jk} = sugarcane molasses effect × urea effect, ABC_{ijk} = yeast strain effect × sugarcane molasses × urea effect and ε_{ijk} = error.

Experiment 2. Data were analyzed as a 7 × 4 factorial in a completely randomized design. The ANOVA procedure of SAS program was used for the analysis and the statistical model is as follows:

$$Y_{ij} = \mu + A_i + B_j + AB_{ij}$$

Where Y_{ijk} = observation, μ = overall mean, A_i = Incubation time effect (i = 0, 12, 24, 36, 48, 60 and 72 h), B_j = Isolated yeast strains effect (j = H-KKU20 (as *P. kudriavzevii*-KKU20), I-KKU20 (as *C. tropicalis*-KKU20), C-KKU20 (as *Galactomyces* sp.-KKU20), and *S. cerevisiae*, AB*ij* = incubation time effect × isolated yeast strains effect and εijk = error.

Treatment means were calculated using the Least Square Means (LSMEANS) option of SAS. All experimental design was use with ANOVA by the General Linear Model (GLM) procedures of SAS[20] (Version 6.0; SAS Institute Inc., Cary, NC, USA). When F-tests were significant, single degree of freedom orthogonal polynomial were used to determine trend of factors. The treatment mean differences were determined by Duncan's New Multiple Range Test (DMRT) at P = 0.05[21].

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Author contributions

C. Suntara, A. Cherdthong, V. Leelavatcharamas, and J. Sawaengkeaw: Investigation, Methodology; C. Suntara, A. Cherdthong, M. Wanapat and J. Sawaengkeaw: Data curation, Formal analysis, Software, and Project administration, Conceptualization, Methodology, and Project administration, Funding acquisition; C. Suntara A. Cherdthong, and S. Uriyapongson: Resources, Supervision, Validation; Visualization; C. Suntara: Roles/Writing – original draft; C. Suntara, A. Cherdthong, J. Sawaengkeaw, P. Chanjula and S. Foiklang: Writing – review & editing. All authors have read and agreed to the published version of the manuscript.

Competing interests

The authors declare no conflict of interest.

Figures

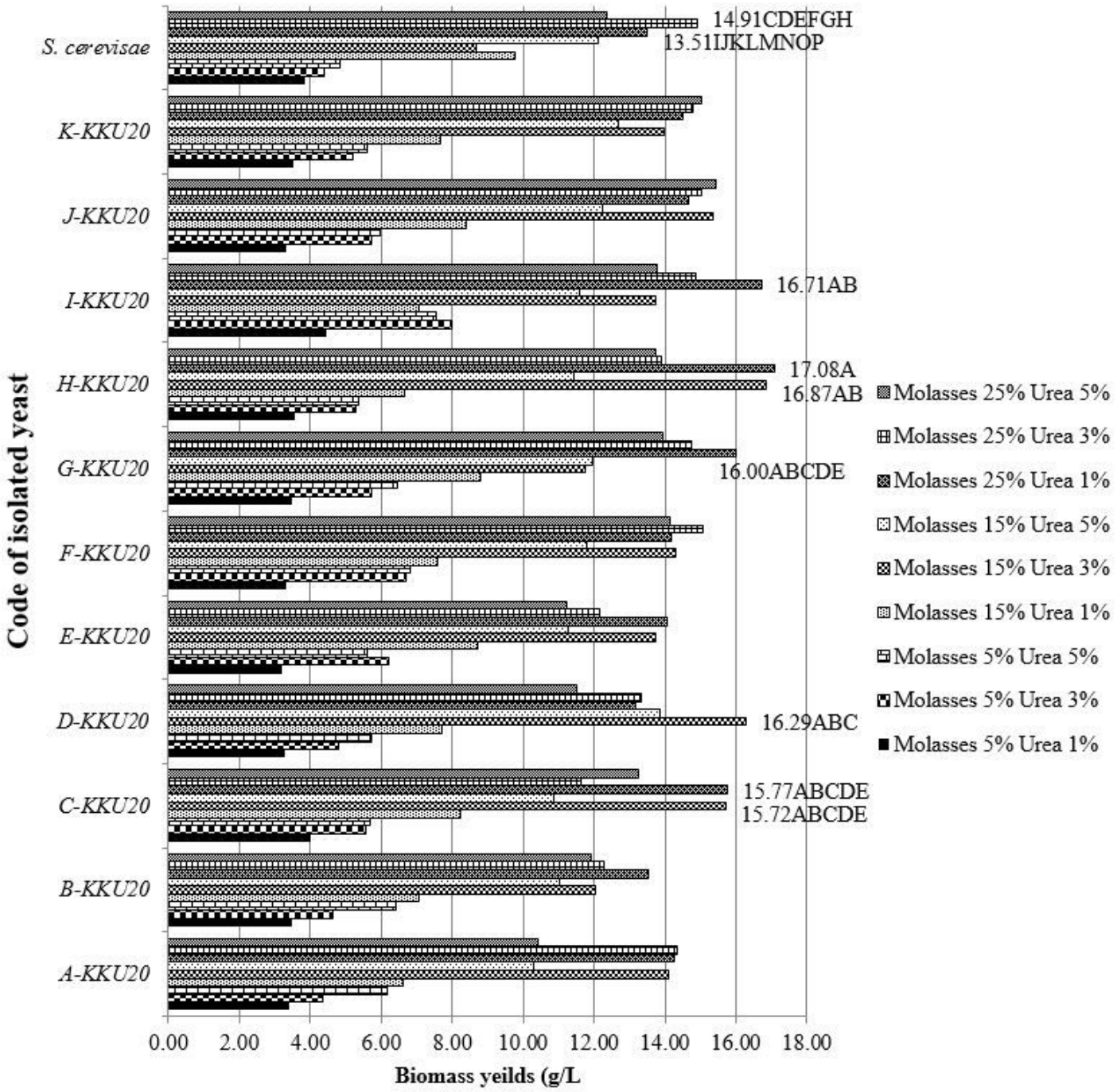

Figure 1

Biomass (horizontal axis) for yeast strains grown in different sugarcane molasses (50, 150 and 250 g/kg) with urea (10, 30 and 50 g/kg) for 72 h. A, B, C, D, E values within a chart show significant different at $P < 0.01$.

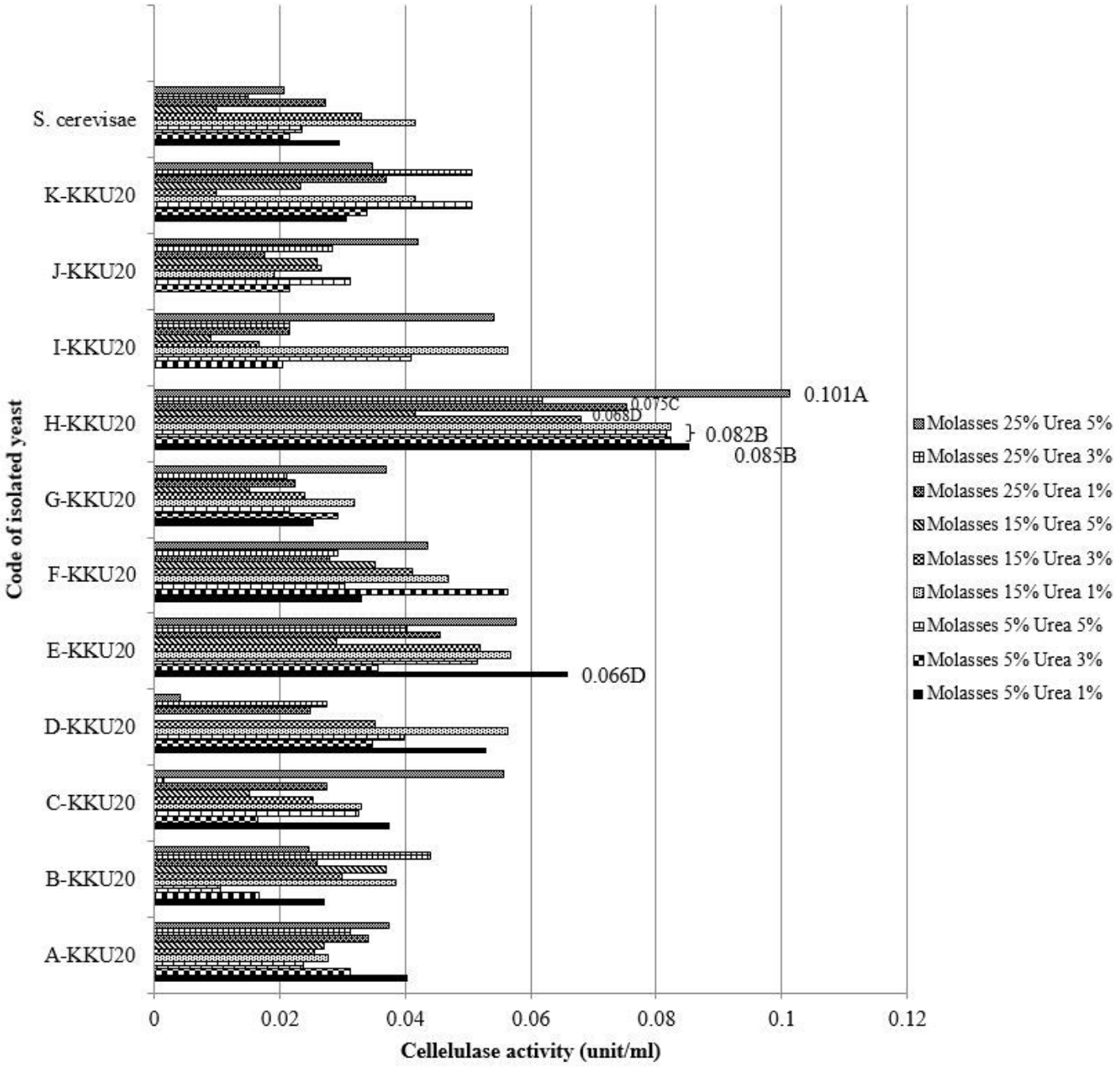

Figure 2

Effect of CMC concentration from 1% (w/v) on cellulase production of each rumen fluids isolates yeast at 72 h. A, B, C, D values within a chart show significant different at $P < 0.01$.

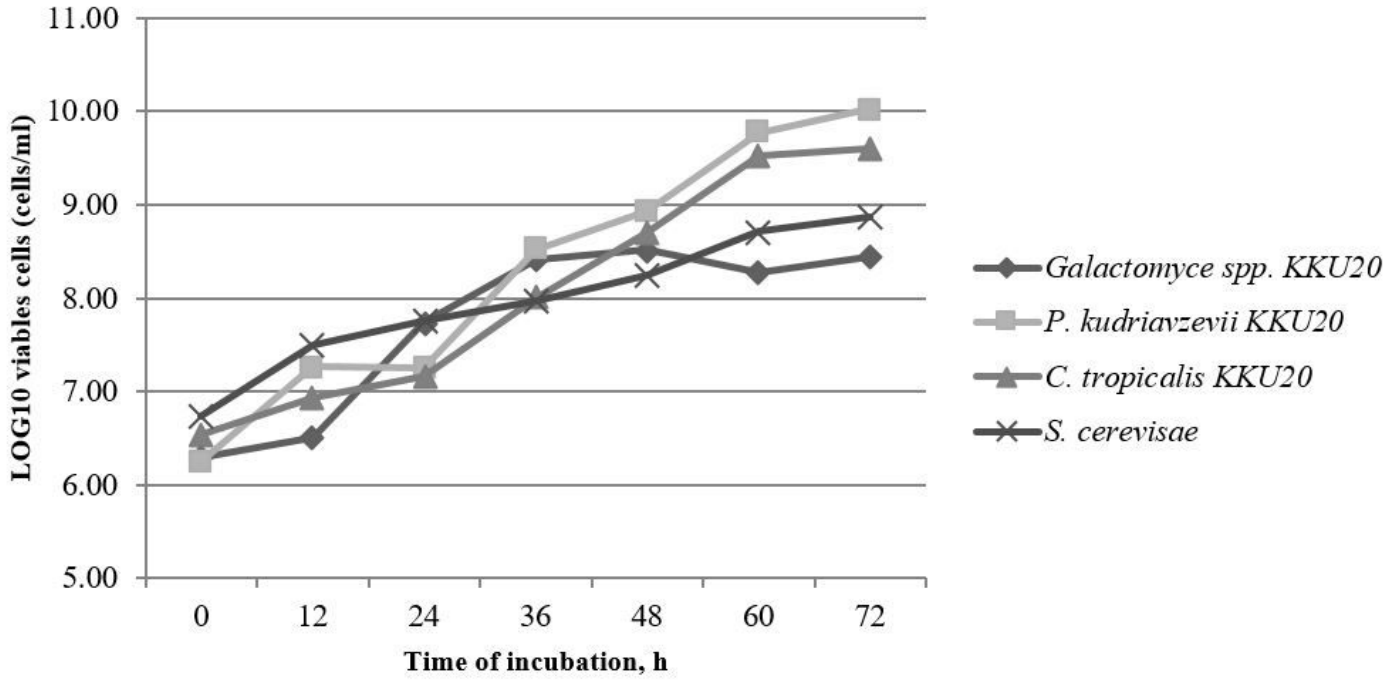

Figure 3

Viable cells count from batch fermentation under sugarcane molasses 250 g/kg with urea 10 g/kg plus 10 g/kg CMCase in the incubator shaker at 30 degrees oC at 150 rpm for 72 h.

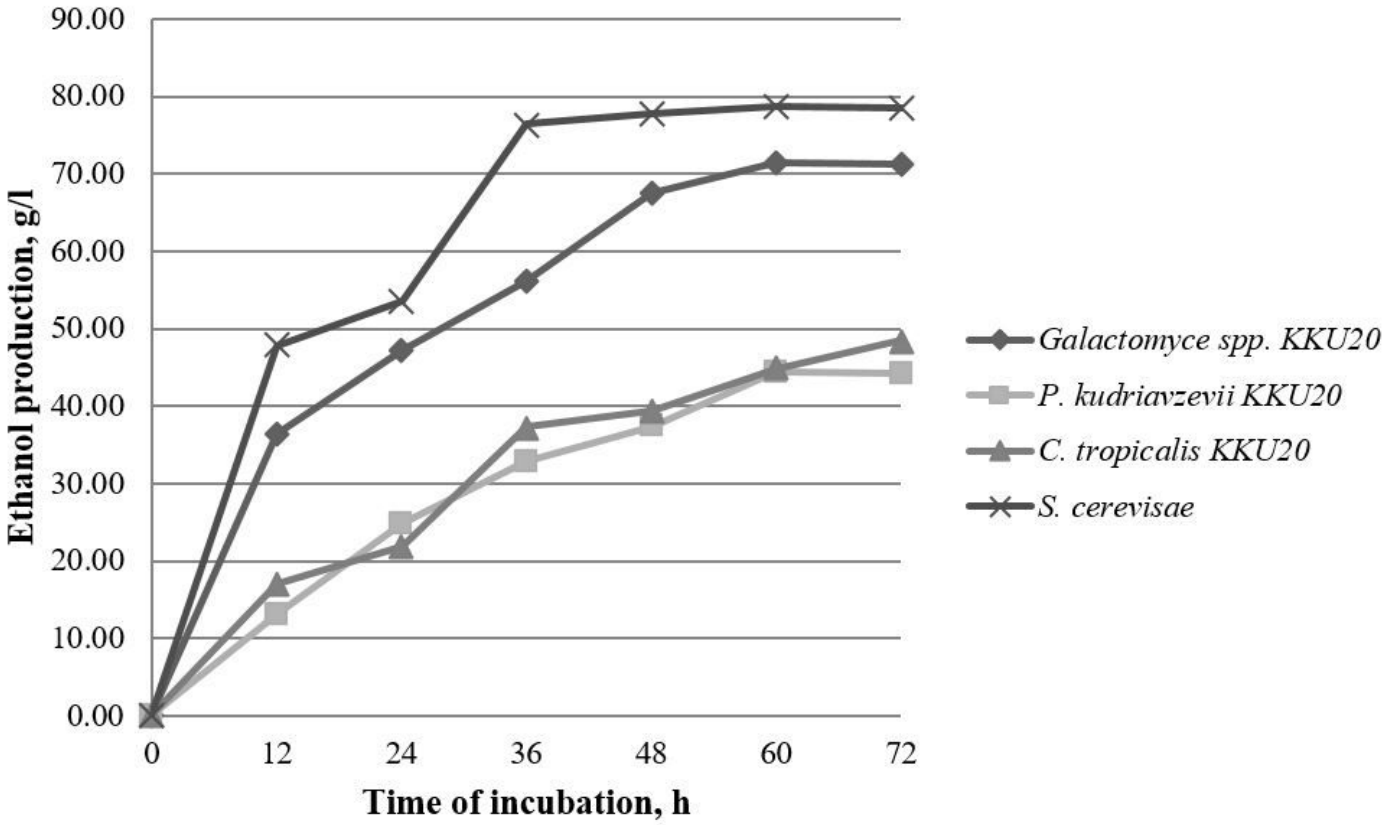

Figure 4

The production of ethanol by four different yeast strains under sugarcane molasses 250 g/kg with urea 10 g/kg plus 10 g/kg CMC in the incubator shaker at 30 degrees oC at 150 rpm for 72 h.

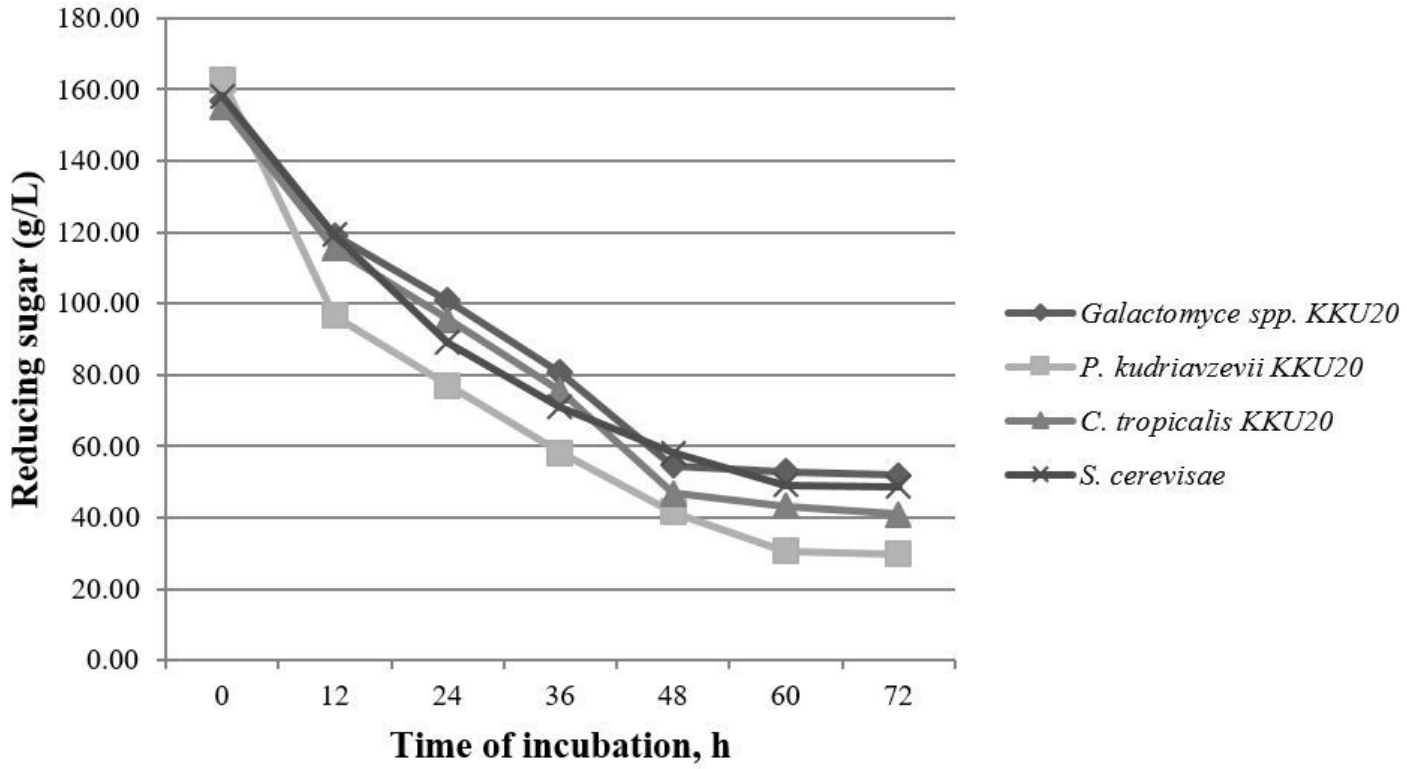

Figure 5

Sugar utilization by four different yeast strains under sugarcane molasses 250 g/kg with urea 10 g/kg plus 10 g/kg CMC in the incubator shaker at 30 oC at 150 rpm for 72 h.

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