

Isolation and characterization of novel yeasts from rumen fluids for potential use  
as additives for ruminant feeding

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**Abstracts:** We hypothesized that rumen fluid with yeast producing cellulase enzyme can occur and also produces a high biomass compared to *S. cerevisiae*. Therefore, the aim of this study was to screen and isolate yeast from rumen fluids with 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 biomass production and cellulase activity. Two fistulated-crossbred Holstein Friesian steers, averaging  $350 \pm 20$  kg body weight, were used to screen and isolate ruminal yeast. The two experiments were designed. A  $12 \times 3 \times 3$  factorial was used in a completely randomized design to determine biomass and carboxymethyl cellulase activity. Factor A was isolated yeasts and *S. cerevisiae*. Factor B was sugarcane molasses (M) concentration. Factor C was urea (U) concentration. Potential yeast was selected for identified and analyzed as a  $4 \times 3$  factorial use in a completely randomized design including. Factor A was incubation times. Factor B was isolated yeast strains including code H-KKU20 (as *P. kudriavzevii*-KKU20), I-KKU20 (*C. tropicalis*-KKU20), and C-KKU20 (as *Galactomyces sp.*-KKU20). Isolation was under aerobic conditions, resulting in a total of 11 different colonies. We noted two appearances of colonies including, asymmetric colonies of isolated yeast (indicated as A, B, C, E, and J) and ovoid colonies (coded as D, F, G, H, I, and K). The highest biomass was observed in three yeasts including codes H, I, and C-KKU20 when inoculated in 25% molasses with 1% urea (M25+U1) ( $p < 0.01$ ). The highest CMC<sub>ase</sub> activity was observed in yeast code H-KKU20 when inoculated in all media solutions ( $p < 0.01$ ). Ruminal yeasts strains H-KKU20, I-KKU20, and C-KKU20 were selected for their ability to produce biomass and their CMC<sub>ase</sub> enzyme synthesis. 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. Two strains provided maximum cell growth: *P. kudriavzevii*-KKU20 (9.78 and 10.02 Log cell/ml) and *C. tropicalis*-KKU20 (9.53

and 9.6 Log cells/ml) at 60 and 72 h of incubation time, respectively. 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 ( $p < 0.01$ ). The *P. kudriavzevii*-KKU20 yielded the least reducing sugar about 30.6 and 29.8 g/L at 60 and 72 h of incubation time, respectively. It could be concluded that screening and isolating yeast from rumen fluids resulted in 11 different characteristics of yeasts. The first novel yeasts discovered in the rumen fluid of cattle were *Pichia kudriavzevii*-KKU20, *Candida tropicalis*-KKU20, and *Galactomyces* sp.-KKU20. *P. kudriavzevii*-KKU20 had higher results than the other yeasts in terms of biomass production, cellulase enzyme activity, and cell number.

**Keywords:** Novel rumen yeast, Screening, Isolation, Biomass of yeast, Cellulase enzyme

## 1. Introduction

The use of microbial additives such as yeast cultures has recently become common practice in ruminant nutrition [1]. In addition, the introduction of yeasts in the ruminant diet could replace antibiotic use for growth promoters [2]. It is also well known that feeding yeasts promotes microbial growth and oxygen scavenging in rumen, providing more optimal conditions for anaerobic microorganisms [3]. The mode of yeast action in the rumen depends on many factors such as diet composition, viability, and strain of yeast [4]. Many studies have suggested a commercial strain such as *S. cerevisiae* a single cell protein supplement in animal feed; however, potential feed utilization remains limited, particularly low biomass and fibrolytic enzyme production.

Previous studies have shown the capability of ruminal yeast strains when supplemented in ruminant diets. Sirisan et al. [5] found that isolated *Kodamaea ohmeri*, yeast from rumen fluids can convert lactic acid to energy, which affects optimum ruminal pH and decreases acidosis in dairy cattle. Moreover, Paserakung et al. [6] revealed that *Tricosporon*

*asahii* GSY10, a yeast isolated from the environment, was a potential alternative fat source in ruminant diets. A recent study by Intanoo et al. [7] reported that isolated *Kluyveromyces marxianus* yeast from rumen fluids showed potential as an aflatoxin-detoxifying agent in dairy cattle. In addition, there are some study have been described yeast could be produce cellulase enzymes such as *Tricosporon cutaneum* [8]. Sarawan [9] discovered a new yeast species, namely *Candida konsanensis* KKU-FW10, isolated from the *Jasminum adenophyllum* plant. *C. konsanensis* could potentially produce 58.24 units/ml of carboxymethyl cellulase (CMC) according to an *in vitro* study. However, there are no reports of existing yeast producing cellulase enzyme and high biomass synthesis in the rumen of ruminants. We hypothesized that rumen fluid with yeast producing cellulase enzyme can occur and also produces a high biomass compared to *S. cerevisiae*.

Therefore, the aim of this study was to screen and isolate yeast from rumen fluids with 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 biomass production and cellulase activity.

## 2. Materials and methods

All procedures involving animals in the metabolism studies were approved by the Institutional Animal Care and Use Committee of Khon Kaen University (KKU) (record no. IACUC-KKU 38/62).

### 2.1. Screening and selection potential yeast in the rumen

#### 2.1.1 Animals and diet

The study was conducted at Tropical Feed Resources Research and Development Center (TROFREC), Department of Animal Science, Faculty of Agriculture, Khon Kaen

University (KKU), Thailand. Two fistulated-crossbred Holstein Friesian steers, averaging  $350 \pm 20$  kg body weight, were used to screen and isolate ruminal yeast.

Rumen fluids from the concentrate (16.0% crude protein (CP) and 75.0% total digestible nutrient (TDN)) fed dairy steers were obtained at 0.5% of body weight (BW) in two equal portions at 07.00 and 16.00, and rice straw was fed on an *ad libitum* basis. The animals were held in individual cages and provided clean, fresh water and mineral blocks as free option. The animals were fed this diet for 7 days before the rumen fluid was obtained.

### 2.1.2 The screening and isolation

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

### 2.1.3 Morphological characterization

Using the streaking method, yeast colonies grown on agar media were picked up and regrown on another YM agar, then incubated for 7 days at room temperature. Yeast colonies were examined under a 40× light microscope. By studying different morphological

characteristics [10], the isolated yeast strains were identified. The appearance of the yeast colonies were recorded: size, shape, convexity, surface, and color of colonies for purification. Colonies growing along the points of the streak were picked up, purified, regrown in a YM broth (HiMedia Laboratories Pvt. Ltd, India), and kept in the refrigerator at 4° C as stock colonies of yeasts. Afterward, all isolates were subjected to an initial assessment to determine their biomass and cellulase production ability by inoculating them in different solutions of sugarcane molasses, urea, and CMC. The top three isolates of yeast showing the least effects from high biomass productivity or cellulase were chosen for further assessment.

#### 2.1.4 Determination of the biomass and carboxymethyl cellulase activity

##### Experimental design and preparation of media solution

The current study was conducted at the Fermentation Research Center for Value Added Agricultural Products (FerVAAP), Department of Biotechnology, Faculty of Technology, Khon Kaen University, Khon Kaen, Thailand, from June 2018 to September 2018. A  $12 \times 3 \times 3$  factorial was used in a completely randomized design. Factor A was isolated yeast at A, B, C, D, E, F, G, H, I, J, K codes and *S. cerevisiae*. Factor B was sugarcane molasses concentration at 50, 100, and 250 g/l distilled water. Factor C was urea concentration at 10, 30, and 50 g/L distilled water. Fermentation media were prepared by addition of sugarcane molasses as a carbon source (Khon Kaen Dairy cooperative Co., Ltd., Khon Kaen, Thailand), urea as a 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<sub>2</sub>SO<sub>4</sub> 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 [11].

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 counts, biomass, reducing sugar, cellulase activity, and ethanol production were determined. The optimum conditions for yeast to produce the greatest 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 [12]. 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, USA).

#### Biomass production from isolated strains

Biomass was determined following Johnson et al. [13]. A 1 ml cultured liquid sample was centrifuged at room temperature at 10,000 g for 10 min. Cell pellets were washed with distilled water and then dried at 105 °C until the weight remained constant. Duplicate samples were tested.

Estimation of reducing sugar and carboxymethyl cellulase activity of isolated yeast strains

The activity of carboxymethyl cellulase was determined using DNS reagent by the colorimetric method according to Miller [14]: 0.5 ml of crude supernatant was applied to 0.5 ml of 1% (w/v) CMC solution. This solution was inserted into a 0.05 M citrate phosphate buffer, (pH 4.0) and incubated at 45 °C for 30 min [15]. The enzymatic reaction was interrupted by adding 1.0 ml DNS reagent and putting it in a boiling water bath for 10 min. A spectrophotometer measured the color of the reaction product as 540 nm. One enzyme unit was defined as the quantity of enzyme that hydrolyzed CMC to produce 1  $\mu$ mol of sugar per minute under the experimental condition. The equation is derived from the glucose-equivalent factor generated in the assay to mmol of glucose, from the volume of the enzyme being tested in the assay (0.5 ml) and the incubation time (30 min) required for generation of the reducing equivalents [13].

$$\text{Carboxymethyl cellulase activity (Unit/ml)} = (C \times D) \div \text{MTV}$$

Then: C = Releasing glucose from cellulase (mg)

D = Dilution factor of enzyme

$\mu$ M = Glucose molecular weight (180  $\mu$ g/ $\mu$ mol)

T = Time incubation

V = Enzyme volume

## 2.2 Study of potential ruminal yeast and identification

### 2.2.1 Experimental design

Potential yeast was selected for identified and analyzed as a 4  $\times$  3 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), and C-KKU20 (as *Galactomyces*

sp.-KKU20). Media solutions were prepared following the appropriate solution for potential yeast, biomass, and CMC<sub>ase</sub> activity.

### 2.2.2 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. [17].

### 2.2.3 Ethanol production from isolated yeasts

Ethanol concentration in the fermentation media (25% molasses and 1% urea) was determined by an Agilent 7890B gas chromatography (Agilent Corporation, California, USA). The standard water-based ethanol solutions were prepared from 0.0 -1.0 % (v/v), and 1-2  $\mu$ l were injected into the gas chromatography (GC) injection port and then subjected to quantitative ethanol analysis using a capillary column HP-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 [18].

### 2.2.4 Reducing sugar from isolated yeasts

The reducing sugar was determined using the 3,5-Dinitrosalicylic acid (DNS) method [14]. 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 [19].

## 2.2.5 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 and Fritsch methods [20] with slight modification. A loopful of yeast cells was transferred to a 1.5 ml Eppendorf tube and 100  $\mu$ l of lysis buffer was added. The cell suspensions were boiled for 15 min in a water or metal block bath. After boiling, 100  $\mu$ l 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  $\mu$ l 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  $\mu$ l 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') [21]. Amplification was performed in 100  $\mu$ l 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 MgCl<sub>2</sub>, 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 ( $8 \times 10^{-5}$  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 [21] 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 Prism™ BigDye™ Terminator Cycle Sequence Ready Reaction Kit (Applied Biosystems, Stafford, USA) according to the manufacturer's instruction.

## 2.3 Statistical analysis

### 2.3.1 Dry biomass and carboxymethyl cellulase activity

Data for isolated yeast from rumen with different of sugarcane molasses and urea 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,  $\mu$  = 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 = 5, 15 and 25%),  $AB_{ij}$  = yeast strain effect × sugarcane molasses effect,  $C_k$  = urea effect (k = 1, 3 and 5%),  $AC_{ik}$  = yeast strain effect × urea effect,  $BC_{jk}$  = sugarcane molasses effect × urea effect,  $ABC_{ijk}$  = yeast strain effect × sugarcane molasses × urea effect and  $\varepsilon_{ijk}$  = error.

### 2.3.2 Cell counts, ethanol production and reducing sugar

Data for isolated yeast strains and incubation time were analyzed as a  $4 \times 3$  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,  $\mu$  = 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) and C-KKU20 (as *Galactomyces* sp.-KKU20),  $AB_{ij}$  = incubation time effect  $\times$  isolated yeast strains effect and  $\epsilon_{ijk}$  = 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 [22] (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  $p = 0.05$  [23].

## 3. Results

### 3.1 Isolation and morphological characteristics of yeast isolated from rumen fluids

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 $\times$ ) microscope and the colonies were confirmed to be yeast as, shown in Table 1. We noted two appearances of colonies including, asymmetric colonies of isolated yeast (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 colorless: code G was turbid.

**Table 1.** Colonies morphology of ruminal yeast strains of Thai-Holstein-Friesian

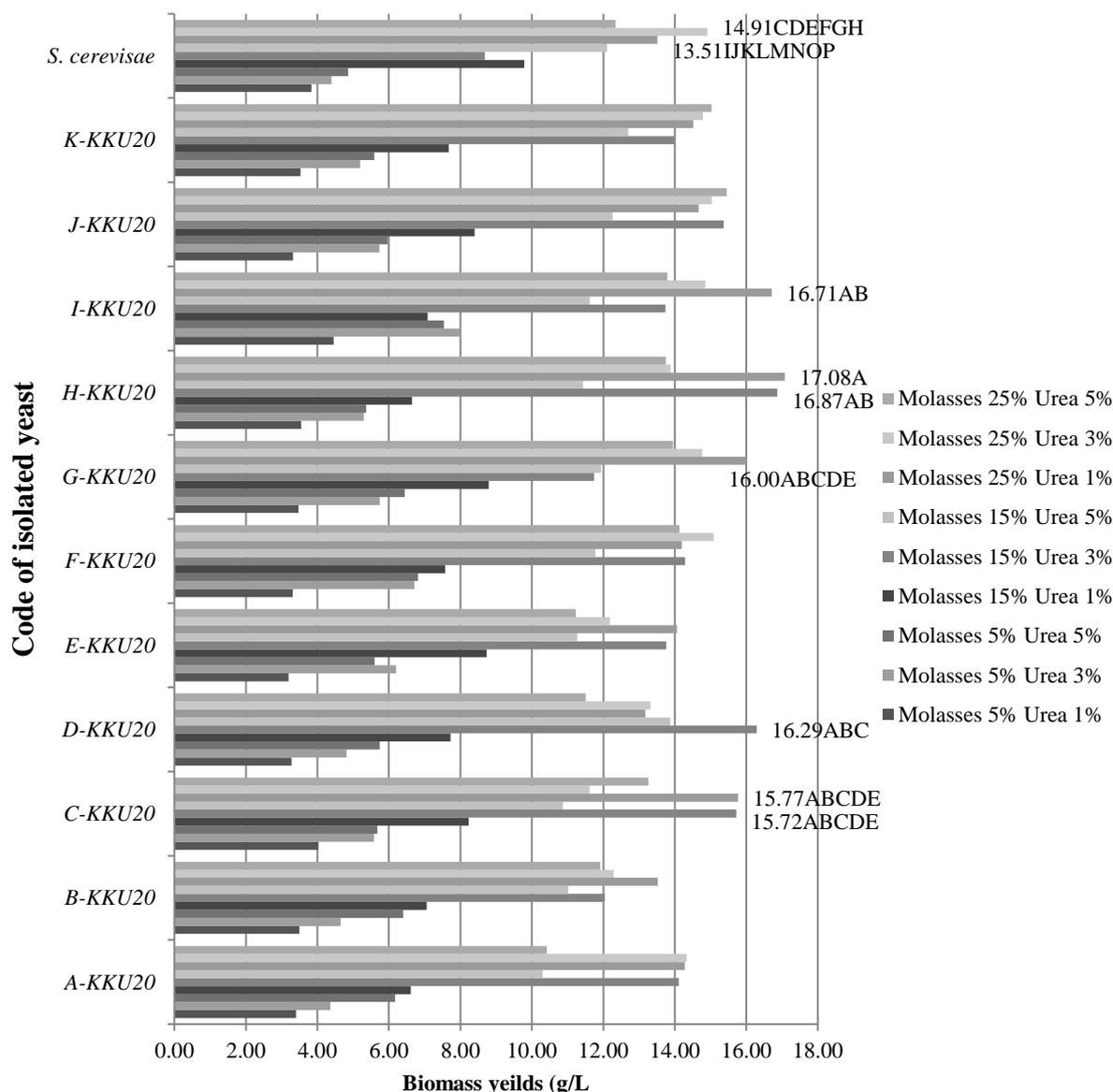
| Code name | Picture                                                                             | Appearance   | Elevation | Colony nature    | Colony color |
|-----------|-------------------------------------------------------------------------------------|--------------|-----------|------------------|--------------|
| A-KKU20   |    | Asymmetrical | Raised    | Rough            | White        |
| B-KKU20   |    | Asymmetrical | Raised    | Rough            | White        |
| C-KKU20   |   | Asymmetrical | Flat      | Rough            | White        |
| D-KKU20   |  | Ovoid        | Flat      | Smooth and shiny | colorless    |
| E-KKU20   |  | Asymmetrical | Flat      | Rough            | White        |
| F-KKU20   |  | Ovoid        | Convex    | Smooth           | White        |
| G-KKU20   |  | Ovoid        | Convex    | Rough            | Turbid       |
| H-KKU20   |  | Ovoid        | Convex    | Rough            | White        |
| I-KKU20   |  | Ovoid        | Flat      | Smooth           | White        |
| J-KKU20   |  | Asymmetrical | Flat      | Smooth           | Colorless    |

|         |                                                                                   |       |        |       |       |
|---------|-----------------------------------------------------------------------------------|-------|--------|-------|-------|
| K-KKU20 |  | Ovoid | Convex | Rough | White |
|---------|-----------------------------------------------------------------------------------|-------|--------|-------|-------|

### 3.2 The biomass production and cellulase activity.

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

Interactions were observed between yeast strains and sugarcane molasses with urea on biomass production and the results are shown in Figure 1. Dry biomass production from ruminal isolated yeast was observed from 3.19 to 17.08 g/l in all media solutions. The highest biomass was observed in three yeasts including codes H, I, and C-KKU20 when inoculated in 25% molasses with 1% urea (M25+U1) which provided highest biomasses at 15.77, 17.08, and 16.71, respectively ( $p < 0.01$ ). *S. cerevisiae* with M25+U1 produced a biomass of 16.16 g/l ( $p < 0.01$ ).

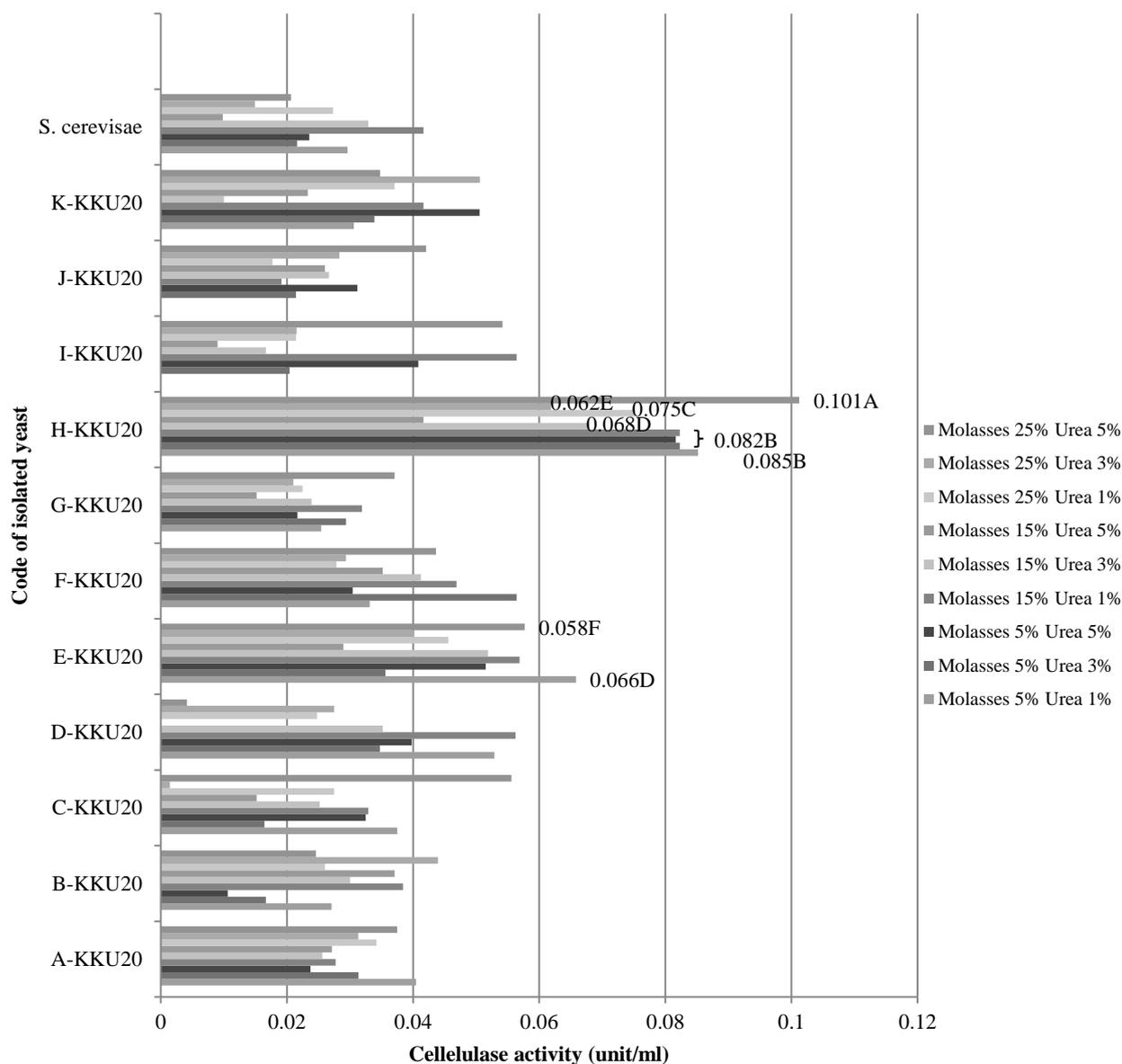


**Figure 1.** Biomass (horizontal axis) for yeast strains grown in different sugarcane molasses (5, 15 and 25%) with urea (1, 3 and 5%) for 72 h.

### 3.2.2. Effects of varying concentrations of sugarcane molasses and urea on cellulase activity of isolated yeast

Interactions were observed between yeast strains and sugarcane molasses with urea on carboxymethyl cellulase activity, and the results are shown in Figure 2. From 0 to 0.101 units/ml CMCase activity was observed in all media solutions. The highest CMCase activity was observed in yeast code H-KKU20 when inoculated in all media solutions, providing a

cellulase activity range from 0.041 to 0.101 units/ml ( $p < 0.01$ ). Yeast code H-KKU20 inoculant in 25% molasses with 3% urea provided the maximum CMCCase activity (0.101 ml/unit).



**Figure 2.** Effect of CMC concentration from 1% (w/v) on cellulase production of each rumen fluids isolates yeast at 72 h

### 3.3 Selection and identification of potential yeast strains

The capability to produce biomass and CMCCase enzyme in various levels of sugarcane molasses and urea are two main criteria to select strains in this study. Ruminal yeasts strains H-KKU20, I-KKU20, and C-KKU20 were selected for their ability to produce biomass and

their CMCase enzyme synthesis. Therefore, the top three isolated yeasts were subjected to identification for these abilities.

The newly isolated yeast strain was identified via DNA sequencing [19] using 26S rRNA gene D1/D2 domain. Identification of isolates H-KKU20 and I-KKU20 revealed that those isolates belonged to *Pichia kudriavzevii*-KKU20 and *Candida tropicalis*-KKU20. The D1/D2 sequence of C-KKU20 had 99.82% (1 nucleotide substitution) similarity with the undescribed species *Galactomyces* sp. HN21-4 (EU651849) (name changes: *Geotrichum* sp. HN21-4) and was closest to the *Galactomyces geotrichum* strain NRRL Y-17569T (NG\_054826), but with 11 nucleotide substitutions and 1 one gap. Based on the sequence of the D1/D2 region, strain C-KKU20 was identified as *Galactomyces* sp.-KKU20 (Table 2).

**Table 2.** Identification of isolated yeast from rumen fluids

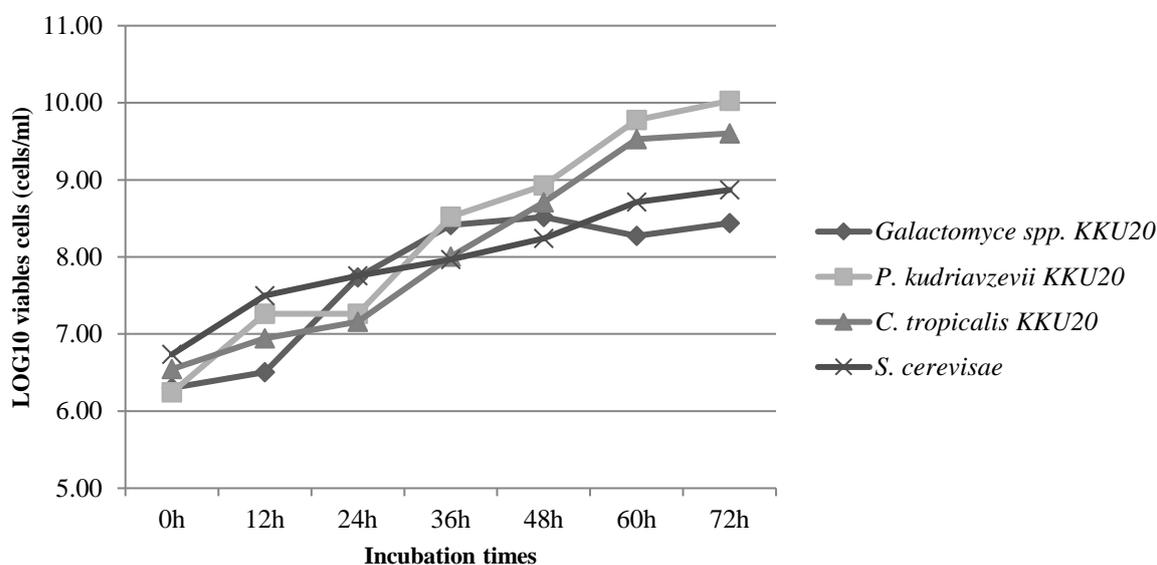
| Isolates | Gene bank Accession No. | Nearest Species with Accession No. | Nucleotide Identity (%) | No. of Nucleotide Differences |
|----------|-------------------------|------------------------------------|-------------------------|-------------------------------|
| H-KKU20  | MH545928                | <i>Pichia kudriavzevii</i>         | 572/572 (100)           | 0                             |
| I-KKU20  | U45749                  | <i>Candida tropicalis</i>          | 570/570 100             | 0                             |
| C-KKU20  | EU651849                | <i>Galactomyce</i> spp.            | 552/553 (99.82)         | 1                             |

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

#### 3.4.1 Effect of incubation time and isolated yeast strains on cell counts

Interactions were observed between incubation times, and isolated yeast strains ( $p > 0.05$ ) and cell counts 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*-KKU20 (9.78 and 10.02 Log cell/ml) and *C. tropicalis*-KKU20 (9.53 and 9.6 Log cells/ml) at 60 and 72 h of incubation time, respectively. Meanwhile, *S. cerevisiae* (8.71

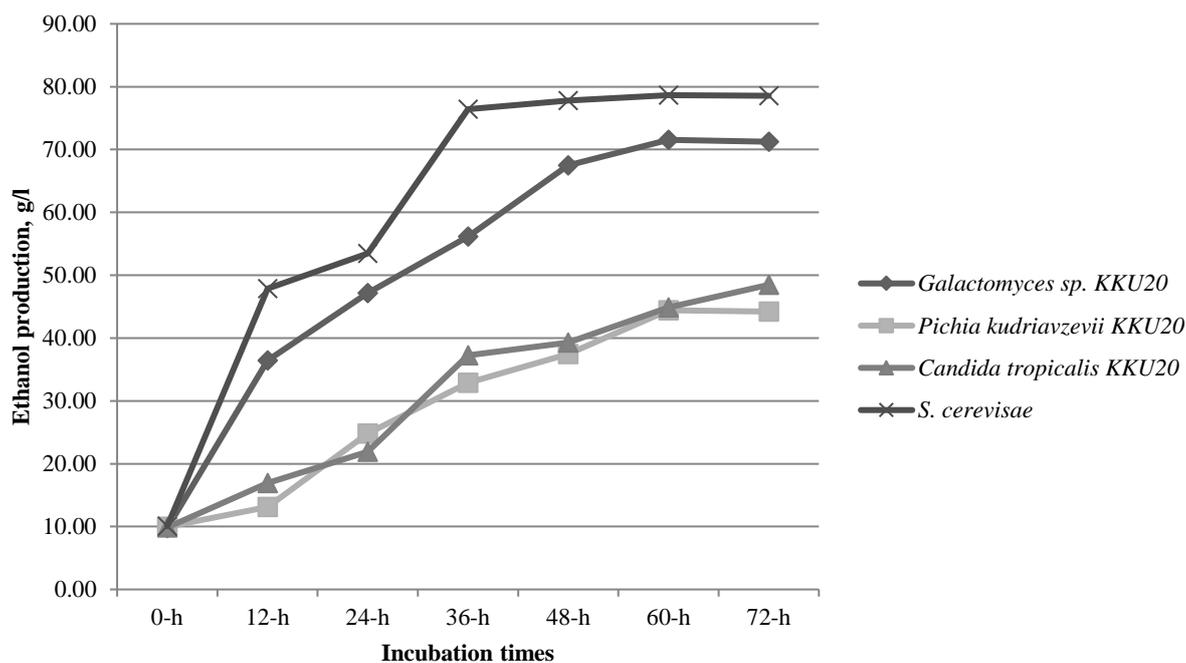
and 8.87 Log cells/ml) and *Galactomyces* sp.-KKU20 (8.27 and 8.44 Log cells/ml) displayed the least growth at 60 and 72 h of incubation time, respectively.



**Figure 3.** Viable cells count from batch fermentation under sugarcane molasses 25% with urea 1% plus 1% CMCcase in the incubator shaker at 30 degrees °C at 150 rpm for 72 h.

### 3.4.2 Effect of incubation time and isolated yeast strains on ethanol production

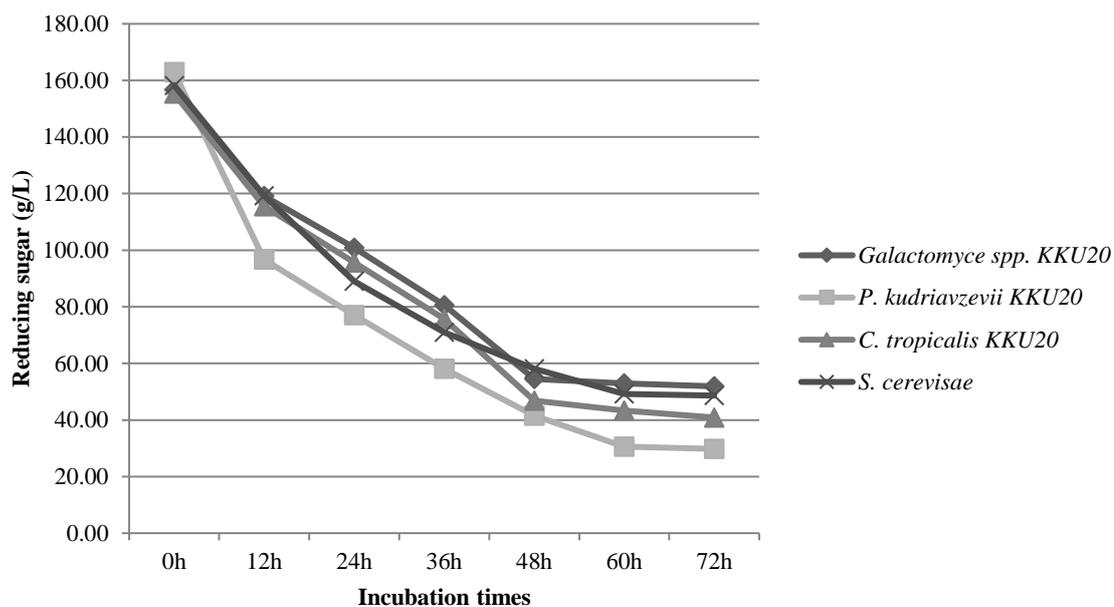
*Galactomyces* sp.-KKU20, *C. tropicalis*-KKU20, *S. cerevisiae*, and *P. kudriavzevii*-KKU20 were evaluated for ethanol production, as shown in Figure 4. Interactions were observed between incubation time and isolated yeast strains ( $p < 0.01$ ). The isolated yeast strains produced about 9.76 to 78.6 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*-KKU20 and *C. tropicalis*-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 ( $p < 0.01$ ).



**Figure 4.** The production of ethanol by four different yeast strains under sugarcane molasses 25% with urea 1% plus 1% CMC in the incubator shaker at 30 degrees °C at 150 rpm for 72 h.

### 3.4.3 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 between 162.9 and 29.8 g/L at 0 to 72 h of incubation time. The *P. kudriavzevii*-KKU20 yielded the least reducing sugar—about 30.6 and 29.8 g/L at 60 and 72 h of incubation time, respectively. Meanwhile, the two strains that yielded the most reducing sugar were *Galactomyces sp.*-KKU20 and *S. cerevisiae* at 60 and 72 h of incubation time, respectively.



**Figure 5.** Sugar utilization by four different yeast strains under sugarcane molasses 25% with urea 1% plus 1% CMC in the incubator shaker at 30 degrees °C at 150 rpm for 72 h.

## 4. Discussions

### 4.1 Isolation of yeast from rumen fluids

Yeast was reported as a member of the ruminal microbial population, as discovered by Orpin [24]. In this study, yeast was isolated from rumen fluids of Holstein-Friesian dairy cattle, which received concentrate (16.0% CP and 75.0% TDN) at 0.5% of BW with rice straw as roughage. It could be assumed that yeasts were isolated from the representative animals, which received a low concentrate diet for maintenance. Rumen fluids were cultured under aerobic conditions, which yielded 11 yeast isolates. Similar, with Sirisan et al. [5] that 10 isolated yeasts were present when dairy cattle were fed a high-concentrate diet, while 7 isolates were present when the cattle were fed a mixture of a high proportion of fermented cassava pulp and concentrate. In addition, Intanoo et al. [7] fed cattle rice straw, cassava pulp, and distilled yeast sludge. The presence of 7 to 10 yeast isolates was found when cultured under aerobic conditions.

In terms of morphology, yeast isolates were ovoid (6 in 11), flat (5 in 11), convex (4 in 11), or rough (7 in 11). White colonies (9 in 11) appeared most frequently. Yeast was detected similar to a previous experiment by Marrero et al. [25], who concluded that the yeast morphologies were slightly convex, smooth, and white- to cream-colored, which is typical of ruminal yeast. However, many factors might cause different types of yeast to be discovered, such as feed sources, R:C ratios, and ruminant species. Marrero et al. [26] isolated yeast from dairy cattle and found that some isolated yeast could present a pink coloration, which was later discovered to be *Levica* strains 18 (L18). Thus, the morphological characteristics of ruminal yeast colonies can be quite complex.

#### 4.2 The biomass production and cellulase enzyme activity of isolated yeasts

To increase the biomass of yeast, a substrate such as soluble carbohydrate and nitrogen must provide sufficient supplies for the growth of yeast cells [27]. Paserakung et al. [6] reported that increasing molasses concentration from 8% to 16% resulted in the greatest biomass production of 25.9% obtained from *Trichosporon asahii*. In addition, Johnson et al. [13], 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 biomass production of *Rhodotorula glutinis* IIP-30, found that 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 25% molasses with 1% urea, providing a maximum ruminal yeast biomass of 29.2%. This could signify that providing optimum levels of molasses and urea could positively affect biomass production. Manikandan and Viruthagiri [28] reported that the nitrogen source, concentrate of nitrogen, and carbon-/nitrogen ratio (C:N ratio) also influenced the production of biomass. Danesi et al. [29] 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 biomass of yeast. In addition, Sokchea et al. [28] found that 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 biomass (17.07 g/L).

In addition, different of yeast species might be involved with biomass production. Van Urk et al. [31] reported that *S. cerevisiae* had low potential to proliferate under excessive glucose, even with aerobic conditions. Wardrop et al. [32] found that *Kluyveromyces marxianus* provided a higher 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*-KKU20 and *C. tropicalis*-KKU20 as shown in this study, they will be produce high biomass and less alcohol which is called “Crabtree-negative yeast”. In contrast, *S. cerevisiae* exhibits alcoholic fermentation and produces high amounts of ethanol, which is called “Crabtree-positive yeast” [33]. The above explanation seems to be the reason that support why *P. kudriavzevii*-KKU20 and *C. tropicalis*-KKU20 produced greater biomass more than *S. cerevisiae*.

In this study, the isolated yeast produced and released cellulase enzymes ranging from 0.020 to 0.075 units/ml. This result was first discovered, which might be support breaking down fiber content in the rumen. Although studies on the release of cellulase enzymes by yeast from the rumen have not been conducted, studies from natural yeast have demonstrated that yeast could produce cellulase enzymes. Sarawan [11] who revealed that *Candida glabrata*, *Candida natalensis* and *Kluyveromyces africanus* (isolated the from *Jasminum adenophyllum* plant), when cultured in yeast extract peptone dextrose broth with 1% CMC, have the ability to release cellulase enzymes ranging from 0.004 to 0.08 units/ml. Thus, cellulase enzymes produced by yeasts might be a potential digest feed containing cellulose contents. As for the mechanism by which yeast digests fiber, it is generally known that

cellulose is hydrolyzed by the cellulase enzymes system, as it breaks down the  $\beta$ -1,4-glycosidic bonds [34]. Cellobiohydrolases (CBHs, EC 3.2.1.91) are important cellulase enzymes found in yeast. CBHs are instrumental in high-performance, natural cellulose hydrolysis [35]. Examples of CBH expressed in yeast include CBH1 (Cel7A) and CBH2 (Cel6A) [36].

#### 4.3 Identification of isolated yeasts

Our study demonstrated that three yeasts were isolated from rumen fluids: *P. kudriavzevii*-KKU20, *C. tropicalis*-KKU20, and *Galactomyces* sp.-KKU20. The name KKU refers to Khon Kaen University, where the strain was originally isolated, and the number “20” means the year of discovery, 2020. Interestingly, the similar yeast species of *P. kudriavzevii*, *C. tropicalis*, and *Galactomyces* sp. had previously been isolated [37, 39, 40]. However, qualities of these strains have not been studied, and this is the first report characteristic such as biomass production, cellulase activity, growth patterns, ethanol production, and reducing sugar of yeast from rumen have been studied in them. In addition, the types of potential yeast were Crabtree-negative. Crabtree-negative yeasts *P. kudriavzevii*-KKU20 and *C. tropicalis*-KKU20 can more rapidly convert available sources of carbon into a solution for yeast biomass. This trend has led to consideration of ruminant nutrition as a candidate for the development of protein sources in feed.

#### 4.4 Cell counts, ethanol production and reducing sugar of selected ruminal yeasts

Our results showed that the *Pichia kudriavzevii*-KKU20 inoculants in media solution can grow greater than other species. The maximum yeasts growth was 10.02 Log cells/ml in aerobic conditions at 72 h of incubation. *Pichia kudriavzevii*-KKU20 was classified as a Crabtree-negative group; the ability to propagate cells is greater than for the Crabtree-positive

group because glucose utilization is high. Crabtree-negative yeasts can transport glucose by an inducible high-affinity proton symport mechanism. Aerobic conditions especially provide steady growth conditions [41].

The present results clearly indicate that *S. cerevisiae* could produce alcohol when used as an inoculant in a high concentration of carbon source under aerobic conditions. Under aerobic conditions, after 72 h, *S. cerevisiae* produced 78.6 g/L ethanol, which was more than other species produced. The high ethanol production capability of *S. cerevisiae* occurs because when its inoculant in media solution contained high sugar, the pyruvate dehydrogenase complex enzyme was inhibited [33], but the activated pyruvate decarboxylase (about 3-4 times) was activated instead and change sugar to ethanol (although sufficient oxygen) [42]. In the present study, the high level of molasses at 25% under aerobic conditions could also have allowed *S. cerevisiae* to produce higher ethanol concentration compared to other yeast species.

The sugar consumption by yeasts is related to the growth curve and depends on the yeast species [43]. It was indicated that isolated yeast strains from rumen have a higher growth curve than Crabtree-positive yeast such as *S. cerevisiae*. This experiment revealed that *P. kudriavzevii*-KKU20 consumed more sugar by 133.1 g during 0 to 72 h of incubation than did *S. cerevisiae*, which consumed only by 124.6 g of sugar. Van Urk et al. [29] stated that the sugar consumption rate of a pyruvate decarboxylase-deficient mutant of *S. cerevisiae* is much lower than that of Crabtree-negative yeast strains, indicating that pyruvate decarboxylase could have a strong influence on the glycolytic flux.

## 5. Conclusions

Based on the present study, it could be concluded that screening and isolating yeast from rumen fluids resulted in 11 different characteristics of yeasts. The first new yeasts discovered

in the rumen fluid of cattle were *Pichia kudriavzevii*-KKU20, *Candida tropicalis*-KKU20, and *Galactomyces* sp.-KKU20, which could have potential for high production of biomass and cellulase. The maximum growth of isolated yeast was shown in a media solution of 25% sugarcane molasses and 1% urea with pH 3.5 and 150 rpm shaking. Under these circumstances, *P. kudriavzevii*-KKU20 had higher results than the other yeasts in terms of biomass production, cellulase enzyme activity, and cell number. However, evaluation of *Pichia kudriavzevii*-KKU20's ability in fiber improvement and yeast biomass production needs to be elucidated to obtain increased nutritive value for ruminant animals.

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