Eurasia Specialized Veterinary Publication

International Journal of Veterinary Research and Allied Science
ISSN:3062-357X

2021, Volume 1, Issue 1, Page No: 84-98

Available online at: www.esvpub.com/

Rumen-Derived Yeasts Outperform Saccharomyces cerevisiae in Biomass and Cellulase Production: Isolation of Pichia

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ABSTRACT

Saccharomyces cerevisiae is a yeast strain frequently incorporated into ruminant feed formulations to enhance nutritional value. Despite its widespread use, this species demonstrates weak biomass formation when cultivated with carbon substrates and exhibits minimal cellulase enzyme synthesis. The current investigation proposed that native ruminal yeasts may proliferate more efficiently and generate cellulases capable of degrading fibrous material. Therefore, the study aimed to isolate, characterize, and evaluate yeasts obtained from the rumen content of Holstein Friesian steers with respect to their biomass yield and cellulolytic capacity. Fermentation media were optimized using sugarcane molasses as the carbon source and urea as the nitrogen source. Two fistulated crossbred Holstein Friesian steers, each averaging 350 ± 20 kg body weight, served as the donors of ruminal fluid for yeast isolation and screening. The research comprised two experimental phases. The first utilized a 12 × 3 × 3 factorial arrangement within a completely randomized design to analyze biomass accumulation and carboxymethyl cellulase (CMCase) activity. Factor A represented both the isolated yeast strains and S. cerevisiae; Factor B was molasses (M) concentration; Factor C was urea (U) concentration. In the second phase, selected yeast strains with promising performance were identified and assessed using a 7 × 4 factorial randomized design. Here, Factor A denoted incubation time, and Factor B referred to the isolated yeast codes: H-Khon Kaen University (KKU) 20 (P. kudriavzevii-KKU20), I-KKU20 (C. tropicalis-KKU20), and C-KKU20 (Galactomyces sp.-KKU20). Aerobic culturing yielded 11 morphologically distinct colonies. Two colony types were recorded: irregular (A, B, C, E, J) and oval (D, F, G, H, I, K). Strains grown in molasses–urea formulations of 15% + 3% (M15 + U3), 25% + 1% (M25 + U1), 25% + 3% (M25 + U3), and 25% + 5% (M25 + U5) demonstrated significantly greater biomass yields than others ($p < 0.01$). In total, 11 biomass-forming yeasts were identified in the M25 + U1 treatment. Four isolates capable of producing cellulase enzymes were also detected in M25 + U1 and M25 + U5, while other combinations—M5 + U1, M5 + U3, M5 + U5, M15 + U1, M15 + U3, and M25 + U3—contained 2, 3, 1, 2, 1, and 2 isolates, respectively. The three strains, H-KKU20, I-KKU20, and C-KKU20, were chosen for their high biomass productivity. Identification showed that H-KKU20 and I-KKU20 corresponded to Pichia kudriavzevii-KKU20 and Candida tropicalis-KKU20, respectively, while C-KKU20 belonged to Galactomyces sp.-KKU20. The greatest cell densities were achieved by P. kudriavzevii-KKU20 (9.78 and 10.02 Log cells/mL) and C. tropicalis-KKU20 (9.53 and 9.6 Log cells/mL) after 60 and 72 h of incubation, respectively. S. cerevisiae displayed peak ethanol synthesis at 76.4, 77.8, 78.5, and 78.6 g/L following 36, 48, 60, and 72 h, respectively ($p < 0.01$). The lowest residual reducing sugar concentrations were observed in P. kudriavzevii-KKU20, with 30.6 and 29.8 g/L recorded at 60 and 72 h. In summary, 11 yeast isolates were recovered from rumen fluid, among which P. kudriavzevii-KKU20, C. tropicalis-KKU20, and Galactomyces sp.-KKU20 emerged as the most promising, with P. kudriavzevii-KKU20 yielding superior biomass, enzyme activity, and cell proliferation.

Keywords: Ruminal yeast, Biomass formation, Cellulolytic activity, Yeast isolation, Screening

Received: 08 February 2020
Revised: 18 May 2021
Accepted: 22 May 2021

How to Cite This Article: Kongsiri N, Arunchai P, Namsai A. Rumen-Derived Yeasts Outperform Saccharomyces cerevisiae in Biomass and Cellulase Production: Isolation of Pichia**.** Int J Vet Res Allied Sci. 2021;1(1):84-98. https://doi.org/10.51847/Qi09LNC0u9

Introduction

Microbial additives, especially yeast-based supplements, have become increasingly common in ruminant feeding programs [1]. Such additives not only improve feed efficiency but may also replace antibiotic growth promoters

[2]. Yeasts contribute to rumen stabilization by reducing oxygen levels and fostering anaerobic microbial growth. Upon autolysis, yeast cells release mannooligosaccharides, proteins, amino acid complexes, B-group vitamins, and trace minerals that support ruminal microorganisms and enhance digestive efficiency. They also promote immune responses within the hindgut [3]. These combined effects enhance microbial abundance and activity in the rumen, thereby boosting the animal's productive output [4].

Research indicates that supplementing feed directly with S. cerevisiae improves growth and performance in ruminants [5]. Alternatively, yeast can be propagated on feed substrates such as molasses (carbon source) and urea (nitrogen source) before being used as an additive [6]. Fermented byproducts enriched with yeast have shown beneficial effects comparable to direct supplementation in livestock feeding trials [7].

Application of S. cerevisiae and development of ruminal yeast for fiber improvement

Fermenting agricultural residues such as durian hull and cassava-pulp ethanol waste with *Saccharomyces cerevisiae* has been reported to improve rumen fermentation efficiency, stimulate bacterial growth in the rumen, and increase the average daily gain (ADG) of Brahman × Thai native cattle [8, 9]. Moreover, cassava pulp fermented with *S. cerevisiae* and malic acid was found suitable as a substitute for 50% of the soybean meal (SBM) component in beef cattle concentrate diets [10]. These benefits were largely associated with yeast multiplication and its contribution of amino acids to the host animal [9]. Other researchers have explored the use of *S. cerevisiae* for upgrading the nutritional quality of rice straw (RS) through fermentation.

In Thailand, RS is a plentiful agricultural residue commonly fed as roughage to ruminants [11]. Typically, it contains about 3–4% crude protein and 70.2% neutral detergent fiber (NDF), but its total digestible nutrient (TDN) level is relatively poor [12]. Although numerous methods have been tested to improve RS quality, most efforts focus on increasing its protein content and improving fiber degradation [12]. However, there is limited evidence on the application of *S. cerevisiae* for RS improvement, and few studies have directly examined its effect on fiber fractions [13]. Additionally, *S. cerevisiae* often exhibits low biomass formation when cultured under high-glucose conditions before being used in byproduct fermentation, particularly with RS.

Wardrop *et al*. [14] demonstrated that *S. cerevisiae* generates nearly seven times less biomass than *Kluyveromyces marxianus* in glucose-enriched media. This occurs because high glucose levels drive *S. cerevisiae* toward ethanol production instead of cell growth, as pyruvate dehydrogenase (PDH) activity becomes inhibited [15]. Consequently, fewer yeast-derived nutrients—such as proteins, amino acids, and vitamins—are available to animals. Exploring alternative yeast species could therefore yield more effective nutritional outcomes.

Research on rumen-origin yeasts used as feed additives has shown that isolates from the rumen environment tend to perform better for ruminant nutrition than yeasts obtained elsewhere [16, 17]. Nevertheless, limited data exist on the ability of ruminal yeasts to generate biomass or secrete cellulase enzymes, despite earlier findings that some yeasts can synthesize cellulases [18]. This study was designed to overcome the constraints of *S. cerevisiae* by evaluating the enzymatic behavior and biomass potential of rumen-derived yeasts, aiming to improve RS as a feed resource.

Before employing ruminal yeasts for RS fermentation, it was essential to characterize the strains present, determine their growth preferences, and understand their metabolic profiles. A modified fermentation medium using sugarcane molasses as a carbon source and urea as a nitrogen source was formulated for this purpose. The central hypothesis proposed that ruminal yeast could produce substantial biomass and secrete cellulase enzymes capable of hydrolyzing fiber. Therefore, the objectives were to isolate, identify, and assess yeasts from the rumen fluid of Holstein Friesian steers for their biomass production and cellulolytic efficiency.

Materials and Methods

All animal procedures were performed under the approval of the Institutional Animal Care and Use Committee, Khon Kaen University (KKU), Thailand (protocol number IACUC-KKU 38/62).

Screening and Identification of potential ruminal yeasts

Experimental animals, feeding regimen, and isolation technique

The experiment took place at the Tropical Feed Resources Research and Development Center (TROFREC), Faculty of Agriculture, Khon Kaen University (KKU), Thailand. Two rumen-fistulated Holstein Friesian × native steers, each weighing approximately 350 ± 20 kg, served as donors of ruminal fluid.

Animals were housed individually in separate pens and received rice straw *ad libitum* as roughage together with a concentrate mix supplied at 0.5% of body weight. The concentrate formulation contained 16.0% crude protein (CP) and 75.0% total digestible nutrients (TDN) following NRC (National Research Council) guidelines [19]. Fresh drinking water and mineral blocks were provided continuously. Feeding occurred twice daily at 07:00 and 16:00. Diets were designed to meet nutrient requirements and sustain optimal rumen microbial activity. Animals were maintained on this regimen for seven days before rumen sampling.

In accordance with Sirisan [20], rumen fluid was collected once, on day 7, through the fistula approximately 4 h after the morning feeding. The fluids from both steers were pooled, homogenized, and strained through four layers of cheesecloth. The filtrate was transferred into sterilized bottles, placed immediately in an ice container (maintained at 4 °C), and delivered to the laboratory within 15 min.

In the lab, 1 mL of rumen fluid from each animal was serially diluted (1:10, 1:100, and 1:1000) using 0.85% sodium chloride solution for total yeast enumeration. Each dilution was plated on yeast–malt extract (YM) agar (HiMedia Laboratories Pvt. Ltd., India) and incubated at 39 °C for 72 h. The YM agar, sterilized at 121 °C for 15 min in an autoclave, contained malt extract (3 g/L), yeast extract (3 g/L), peptone (5 g/L), agar (20 g/L), and glucose (10 g/L).

Morphological characterization

Yeast samples were cultured on agar plates by the streak plate method. Colonies obtained were restreaked on new YM agar and incubated for seven days under ambient laboratory conditions. Colony features were studied microscopically at 40× magnification, and isolates were taxonomically differentiated based on their external traits as outlined in [21]. Observations included colony diameter, outline, elevation, surface characteristics, and pigmentation during the purification phase. Individual colonies forming along the streak were selected, transferred for purification, then subcultured in yeast–malt broth (HiMedia Laboratories Pvt. Ltd., Mumbai, India). Cultures were refrigerated at 4 °C and maintained as permanent yeast stocks. All isolates were preliminarily evaluated for their potential to generate biomass and produce cellulase enzymes in liquid media enriched with sugarcane molasses, urea, and carboxymethyl cellulose (CMC).

Measurement of biomass and carboxymethyl cellulase enzyme activity

Experimental setup and media preparation

This experiment was carried out between June and September 2018 at the Fermentation Research Center for Value Added Agricultural Products (FerVAAP), Faculty of Technology, Khon Kaen University, Thailand.

To evaluate growth efficiency under variable nutrient conditions, a fermentation system containing sugarcane molasses (carbon provider) and urea (nitrogen donor) was formulated. The experimental design followed a 12 × 3 × 3 completely randomized arrangement, incorporating twelve yeast strains (eleven from rumen fluid and one control strain), three sugarcane molasses concentrations (50, 100, and 250 g/L), and three urea concentrations (10, 30, and 50 g/L). Each treatment was duplicated.

Media were prepared using sugarcane molasses (Khon Kaen Dairy Cooperative Co., Ltd., Khon Kaen, Thailand), urea (Saengtawee Panit Co., Ltd., Khon Kaen, Thailand), and CMC (10 g/L) (Chemipan Co., Ltd., Bangkok, Thailand) as an additional substrate. All mixtures were autoclaved at 121 °C for 15 min, cooled to room temperature, and acidified to pH 3.5 with 70% H_2SO_4 [10]. The pH was recorded using a glass-electrode pH meter (Hanna Instruments, Inc., Woonsocket, RI, USA) for each yeast–molasses–urea combination.

Each 250 mL Erlenmeyer flask received 100 mL of the prepared medium and was inoculated with 1 mL of yeast suspension (≈10^6 cells/mL) under aseptic conditions. Flasks were sealed with cotton plugs and placed in an orbital shaker–incubator [22].

Every combination of molasses and urea level was tested using 100 mL of media in a 250 mL flask. A single colony from each yeast stock culture (including *S. cerevisiae* as reference) was introduced and incubated at 30 °C and 150 rpm for 72 h. After incubation, 1 mL samples were collected into 1.5 mL tubes (two replicates per condition) for biomass and cellulase assessment. The best-performing yeast strains and optimal molasses–urea concentrations—those giving the greatest cell mass and enzymatic activity—were selected for later analyses, including total cell count, reducing sugar concentration, and ethanol output.

Quantification of biomass and enzyme reaction

Dry cell biomass was determined as outlined by Johnson *et al*. [23]. One milliliter of each culture was centrifuged at 10,000×g for 10 min at room temperature. The sedimented cells were rinsed with distilled water and oven-dried at 105 °C until constant weight. The remaining liquid phase (supernatant) was used for cellulase enzyme analysis. Carboxymethyl cellulase (CMCase) activity was estimated following the DNS colorimetric approach [14]. Briefly, 0.5 mL of the enzyme extract was mixed with 0.5 mL of 1% (w/v) CMC dissolved in 0.05 M citrate–phosphate buffer (pH 4.0) and incubated at 45 °C for 30 min [15]. The reaction was stopped by adding 1.0 mL of DNS reagent and heating in boiling water for 10 min. The resulting color intensity was read at 540 nm with a spectrophotometer.

One enzyme unit (U) corresponded to the amount of enzyme releasing 1 µmol of glucose-equivalent reducing sugar per minute under the described assay settings. Calculations were based on the glucose standard curve, enzyme solution volume (0.5 mL), and the 30 min incubation period [24].

Carboxymethyl cellulase activity (U/mL) = (C × D) ÷ MTV

Where:

C = Amount of glucose liberated by the enzyme (mg)

D = Enzyme dilution coefficient

µM = Molecular weight of glucose (180 µg/µmol)

T = Incubation duration

V = Enzyme volume used

The goal of this experiment was to determine the nutrient formulation that allows the yeast to reach maximum biomass and the highest cellulase performance. Only the most productive medium was used to multiply the yeast culture before RS fermentation in later stages. Three colonies that yielded the largest biomass and strongest cellulase response were chosen. Consequently, data interpretation focused on two metrics—biomass accumulation and enzyme output.

Assessment and genetic profiling of ruminal yeast

DNA characterization of selected yeast samples

Genomic DNA was obtained through a modified version of the boiling lysis method described [20]. Yeast cells were taken with a sterile loop and suspended in 100 µL lysis buffer inside a 1.5 mL tube. The suspension was heated for 15 min in a boiling block or water bath, followed by the addition of 100 µL 2.5 M potassium acetate (pH 7.5). Samples were cooled on ice for 1 h and centrifuged at 14,000 rpm for 5 min. The resulting supernatant was cleaned twice with chloroform: isoamyl alcohol (24:1, v/v). DNA precipitation was carried out with isopropanol for 10 min at 20 °C, then centrifuged for 15 min at 15,000 rpm. Pellets were rinsed sequentially in 70% and 90% ethanol, air-dried for 15–30 min, and dissolved in 30 µL of Milli-Q water.

Amplification of the D1/D2 region of the 26S rDNA used the primer set NL1 (5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′) and NL4 (5′-GGT CCG TGT TTC AAG ACG G-3′) [25]. The reaction mixture (100 µL) consisted of 100 ng template DNA, 2.5 U Taq polymerase, 40 mM of each primer, 20 mM dNTPs, 1.5 mM $MgCl_2$, and 10 mM Tris-HCl. Thermal cycling included pre-denaturation at 94 °C for 5 min, 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2.5 min, concluding with 10 min at 72 °C. PCR amplicons were purified with a QIAquick purification kit following the manufacturer's protocol. Electrophoresis was performed using 0.8% agarose gel in 1× TBE buffer, stained with ethidium bromide (8×10^{-5} µg/mL), and observed under UV illumination.

The nucleotide sequence of the 26S rDNA D1/D2 domain was obtained directly from the PCR products according to the modified protocol of Kurtzman and Robnett [25]. Sequencing reactions employed the NL1 (forward) and NL4 (reverse) primers in conjunction with the ABI PrismTM BigDyeTM Terminator Cycle Sequencing Kit (Applied Biosystems, Stafford, TX, USA), adhering to the supplier's recommendations.

Monitoring of yeast growth, sugar reduction, and ethanol output

The potential yeast isolates were studied under a 7 × 4 factorial design within a completely randomized setup and triplicated runs. Factor A represented incubation times (0, 12, 24, 36, 48, 60, and 72 h), while Factor B included

isolates: H-KKU20 (P. kudriavzevii-KKU20), I-KKU20 (C. tropicalis-KKU20), C-KKU20 (Galactomyces sp.-KKU20), and S. cerevisiae. Media conditions were optimized for maximum growth, total biomass, and CMCase enzyme performance.

At every incubation point (0–72 h), six 1.5 mL microtubes were collected in duplicate—two for microscopic cell counting, two for reducing sugar analysis, and two for ethanol detection. Cell counts were immediately determined using a hemocytometer under a light microscope following Darvishi *et al*. [26].

The reducing sugar concentration was analyzed using the DNS (3,5-dinitrosalicylic acid) procedure [27]. One milliliter of the diluted sample (1 mL sample plus 9 mL distilled water) was combined with 1.0 mL DNS reagent, while the blank contained equal volumes of distilled water and DNS solution. The mixtures were boiled for 15 min, cooled, and diluted with 5 mL distilled water. Absorbance was recorded at 540 nm using a UV–Vis spectrophotometer, and the sugar reduction was calculated using a glucose standard curve and the corresponding dilution factor [28].

For ethanol evaluation, samples were centrifuged at 16,000 rpm for 15 min, and the supernatant was passed through a 0.45 µm syringe filter. Quantification was carried out using an Agilent 7890B gas chromatograph (Agilent Technologies, Santa Clara, CA, USA). Standard aqueous ethanol solutions (0.0–1.0% v/v) were injected in 1–2 µL volumes and analyzed on an HP-5 column (30 m) with these GC settings: oven temperature 40 °C, inlet 150 °C, detector (FID) 300 °C, and airflow 350 mL/min [29].

Statistical evaluation

Biomass yield and carboxymethyl cellulase enzyme activity

The dataset obtained from ruminal yeast cultivated with varying sugarcane molasses and urea concentrations was assessed under a 12 × 3 × 3 factorial design in a completely randomized setup. Statistical evaluation was carried out using the ANOVA procedure in SAS software, with the following analytical model applied:

$$Y_{ijk} = \mu + A_i + B_j + AB_{ij} + C_k + AC_{ik} + BC_{jk} + ABC_{ijk} + \varepsilon_{ijk} \quad (1)$$

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 = 5, 15, and 25%),

AB_{ij} = interaction between yeast strain and molasses,

C_k = urea concentration (k = 1, 3, and 5%),

AC_{ik} = yeast strain × urea interaction,

BC_{jk} = molasses × urea interaction,

ABC_{ijk} = triple interaction among yeast strain, molasses, and urea,

and ε_{ijk} = residual error.

Yeast cell density, ethanol output, and reducing sugar

The experimental data regarding yeast isolates and incubation time were examined under a 7 × 4 factorial arrangement using a completely randomized design. The SAS ANOVA module was employed with the following model:

$$Y_{ij} = \mu + A_i + B_j + AB_{ij} \quad (2)$$

where:

Y_{ijk} = observation,

μ = grand mean,

A_i = incubation time effect (i = 0, 12, 24, 36, 48, 60, and 72 h),

B_j = yeast strain effect (j = H-KKU20 (P. kudriavzevii-KKU20), I-KKU20 (C. tropicalis- C-KKU20 (Galactomyces sp.-KKU20), and S. cerevisiae),

AB_{ij} = interaction between incubation duration and yeast strain,

and ε_{ijk} = error.

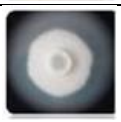
Treatment means were determined using the LSMEANS procedure in SAS. The overall analysis relied on ANOVA and GLM modules (Version 6.0; SAS Institute Inc., Cary, NC, USA) [30]. When F-tests indicated significance, orthogonal polynomial contrasts (single-degree-of-freedom) were applied to assess factor trends. Differences among means were identified using Duncan's Multiple Range Test (DMRT) at a 5% probability level [31].

Results and Discussion

Yeast isolation and morphological assessment

Under aerobic culture, 11 distinct colonies were isolated from rumen samples **(Table 1)**. Yeast colonies developed on YM agar plates were categorized based on elevation, form, texture, and pigmentation. Observations under a 40× light microscope confirmed yeast morphology **(Table 1)**. Two distinct colony forms were found—asymmetric (A, B, C, E, J) and ovoid (D, F, G, H, I, K). Colony elevation varied: raised (A, B), flat (C, D, E, I, J), and convex (F, G, H, K). Smooth textures were recorded for D, E, I, J, while the remainder exhibited rough surfaces. Most colonies were white, except D and J (colorless), and G (turbid).

Table 1. Morphological traits of ruminal yeast isolates from Thai-Holstein-Friesian cattle.

ISOLATE CODE	MORPHOLOGICAL IMAGE	COLONY SHAPE	ELEVATION	SURFACE TEXTURE	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

Biomass formation and enzymatic activity

Influence of sugarcane molasses and urea concentration on biomass after 72 h

Significant interactions among yeast strain, molasses, and urea levels were evident **(Figure 1)**. Biomass accumulation of ruminal yeast ranged from 3.19 to 17.08 g/L across all test media.

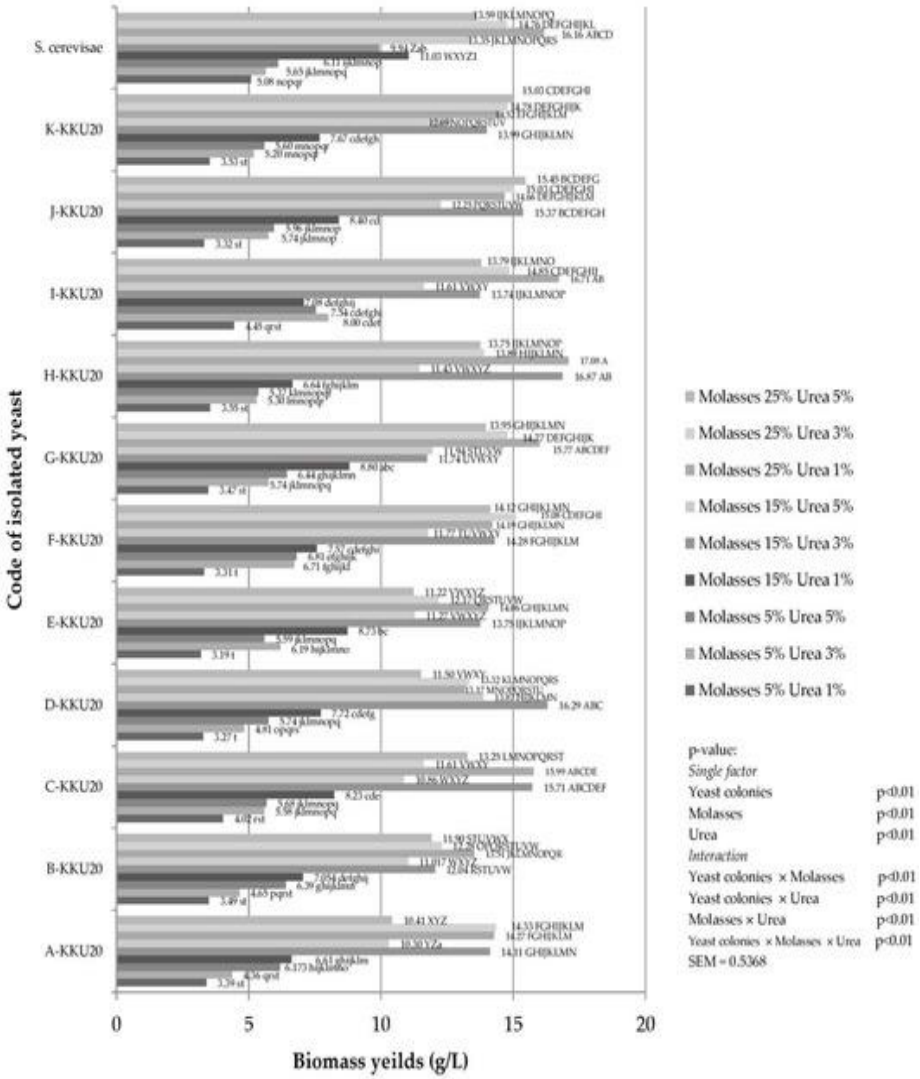


Figure 1. Biomass (horizontal axis) of yeast isolates cultured in varying molasses (5, 15, 25%) and urea (1, 3, 5%) concentrations for 72 h. Bars with different superscripts differ significantly ($p < 0.01$); SEM = standard error of mean; S. cerevisiae = Saccharomyces cerevisiae.

Yeast strains derived from rumen fluid produced notably greater biomass ($p < 0.01$) in media combinations of M15 + U3, M25 + U1, M25 + U3, and M25 + U5. Among 11 isolates, M25 + U1 yielded the highest biomass occurrence, followed by M15 + U3 (8 isolates), M25 + U3 (7 isolates), and M25 + U5 (2 isolates).

Influence of sugarcane molasses and urea levels on cellulase activity in isolated yeast after 72 hours of cultivation

A notable relationship was detected among yeast isolates, molasses percentages, and urea concentrations regarding carboxymethyl cellulase (CMCase) activity **(Figure 2)**. Yeasts obtained from ruminal fluid produced a significant quantity of CMCase in all formulations, except for M15 + U5 ($p < 0.01$). Within the culture medium, four isolates exhibiting elevated cellulase synthesis were identified in M25 + U1 and M25 + U5, while M5 + U1, M5 + U3, M5 + U5, M15 + U1, M15 + U3, and M25 + U3 yielded 2, 3, 1, 2, 1, and 2 isolates, respectively.

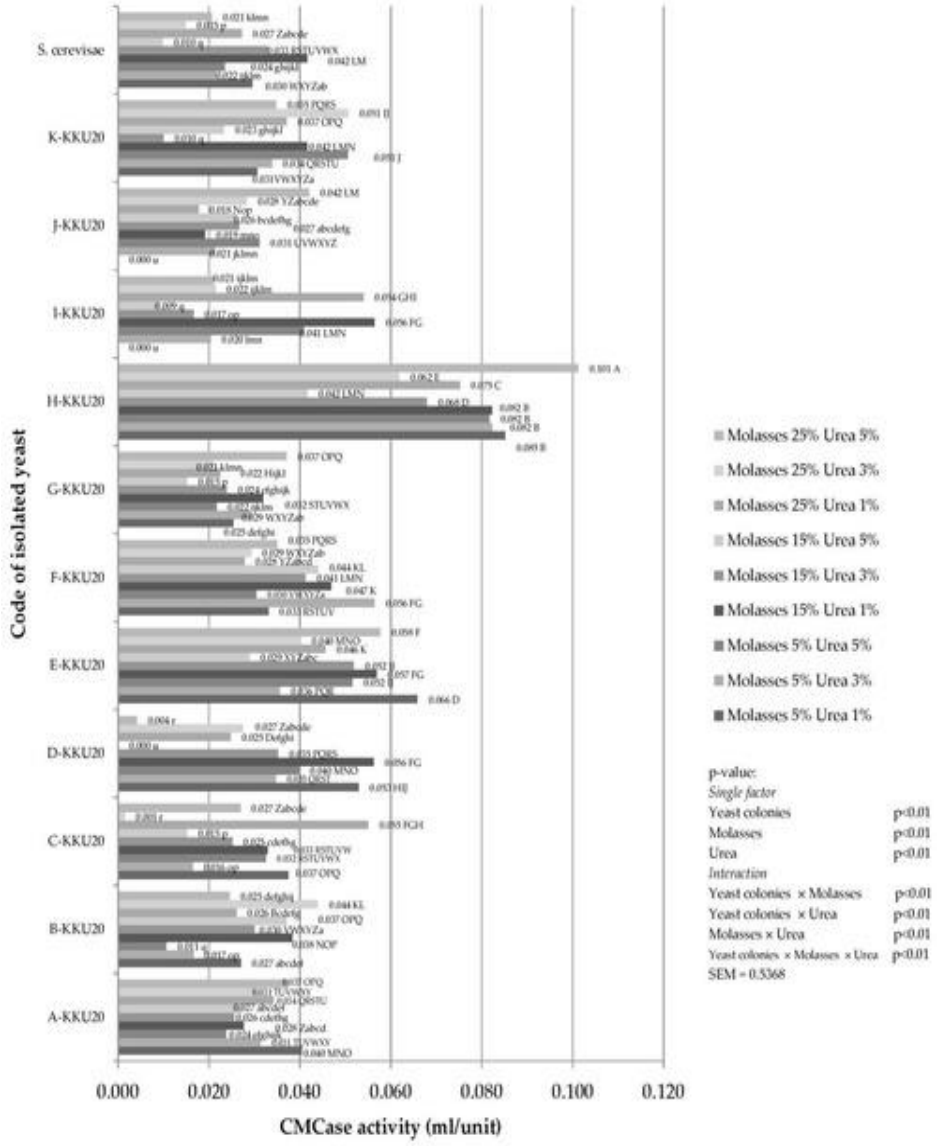


Figure 2. Influence of 1% (w/v) CMC concentration on cellulase enzyme formation in yeast isolates from rumen origin after 72 h. Columns labeled with distinct superscripts (A–Z, a–u) show significant variation ($p < 0.01$). SEM = standard error of the mean; S. cerevisiae = Saccharomyces cerevisiae.

Screening and genetic identification of efficient yeast strains

After comparing performance parameters, a single nutrient medium was chosen to propagate yeast cultures prior to RS fermentation. The mixture M25 + U1 was chosen for its superior propagation ability compared with the other combinations. The three most active isolates—H-KKU20, I-KKU20, and C-KKU20—originating from M25 + U1 were chosen on the basis of CMCase yield. Although M25 + U5 exhibited similar cellulase activity, M25 + U1 was considered more favorable because it required less nitrogen and yielded greater biomass. Within this group, H-KKU20 showed the highest CMCase output. Therefore, isolates H-, I-, and C-KKU20 were advanced for the subsequent assays.

Molecular identification of these isolates was performed by sequencing the 26S rRNA D1/D2 domain [25]. The analysis determined that H-KKU20 and I-KKU20 corresponded to Pichia kudriavzevii-KKU20 and Candida tropicalis-KKU20, respectively. The D1/D2 domain of C-KKU20 shared 99.82% similarity (one base substitution) with the undescribed species Galactomyces sp. HN21-4 (EU651849), formerly Geotrichum sp. HN21-4, and showed closest alignment with Galactomyces geotrichum strain NRRL Y-17569T (NG_054826), differing by 11 substitutions and one gap. Consequently, C-KKU20 was classified as Galactomyces sp.-KKU20 **(Table 2)**.

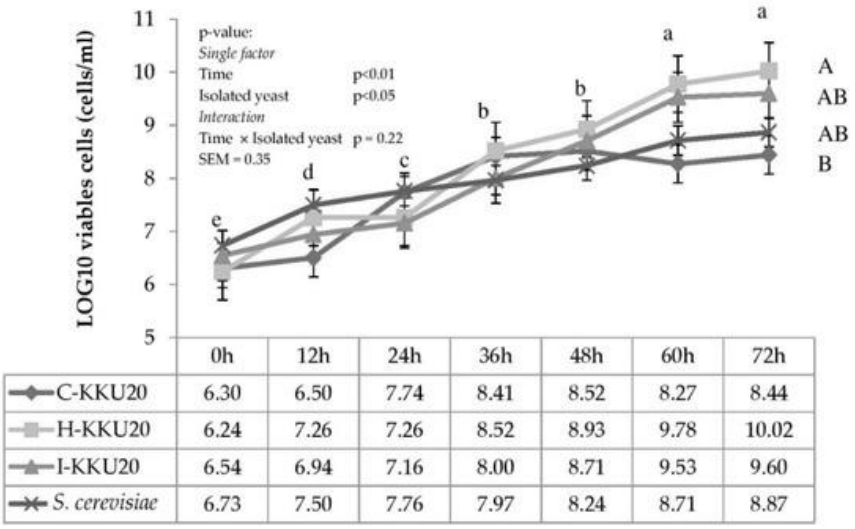
Table 2. Genetic identification of yeast strains derived from ruminal samples.

Isolate	GenBank Accession No.	Closest Match (Accession No.)	Nucleotide Identity (%)	Nucleotide Differences
H-KKU20	MH545928	*Pichia kudriavzevii*	**572/572 (100)**	**0**
I-KKU20	U45749	*Candida tropicalis*	**570/570 (100)**	**0**
C-KKU20	EU651849	*Galactomyces* spp.	**552/553 (99.82)**	**1**

Yeast population dynamics, ethanol yield, and reducing sugar trends

Effects of incubation duration and yeast species on cell growth

Figure 3 illustrates the changes in cell population influenced by incubation period and yeast strain. The two factors did not interact significantly ($p > 0.05$). During 0–72 h of cultivation, total cell counts ranged between 6.24 and 10.02 Log cells/mL. The isolate P. kudriavzevii-KKU20 demonstrated the highest cell density (10.02 Log cells/mL at 72 h), whereas S. cerevisiae exhibited the lowest (8.87 Log cells/mL at 72 h) ($p < 0.05$). Cell viability progressively increased with incubation time, peaking between 60 and 72 h ($p < 0.01$), indicating that the maximum growth phase could be attained within 60 hours.



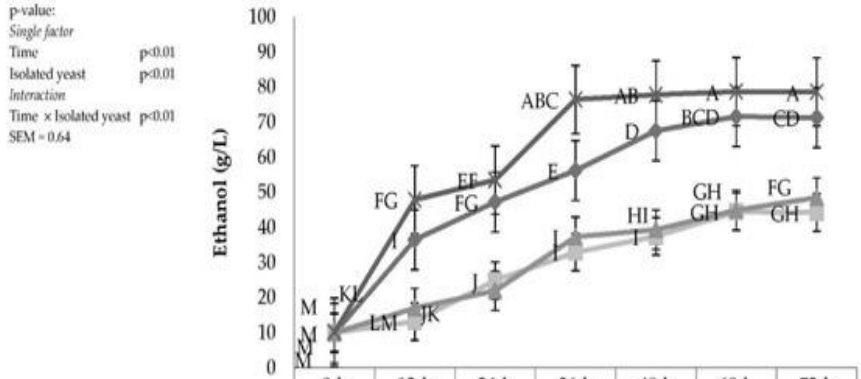
	0h	12h	24h	36h	48h	60h	72h
C-KKU20	6.30	6.50	7.74	8.41	8.52	8.27	8.44
H-KKU20	6.24	7.26	7.26	8.52	8.93	9.78	10.02
I-KKU20	6.54	6.94	7.16	8.00	8.71	9.53	9.60
S. cerevisiae	6.73	7.50	7.76	7.97	8.24	8.71	8.87

Figure 3. Viable yeast population during batch fermentation using 25% sugarcane molasses supplemented with 1% urea and 1% CMCase, carried out in a shaking incubator at 30 °C and 150 rpm for 72 h. ^{A,B} Superscripts with different letters indicate significant variation among yeast species ($p < 0.05$), while $^{a–e}$ represent significant changes over time ($p < 0.01$). SEM = standard error of mean; S. cerevisiae = Saccharomyces cerevisiae.

Impact of fermentation duration and yeast isolates on ethanol output

Ethanol formation was assessed in Galactomyces sp.-KKU20, C. tropicalis-KKU20, S. cerevisiae, and P. kudriavzevii-KKU20 **(Figure 4)**. The interaction between incubation length and yeast strain was significant ($p < 0.01$). Across the 0–72 h period, ethanol levels ranged from 9.76 to 78.6 g/L. The highest ethanol yield occurred in S. cerevisiae, producing 76.4, 77.8, 78.5, and 78.6 g/L at 36, 48, 60, and 72 h, respectively ($p < 0.01$).

Conversely, the lowest ethanol formation was seen in P. kudriavzevii-KKU20 (32.9, 37.4, 44.4, and 44.2 g/L) and C. tropicalis-KKU20 (37.3, 39.3, 44.9, and 48.5 g/L) during the same intervals ($p < 0.01$).

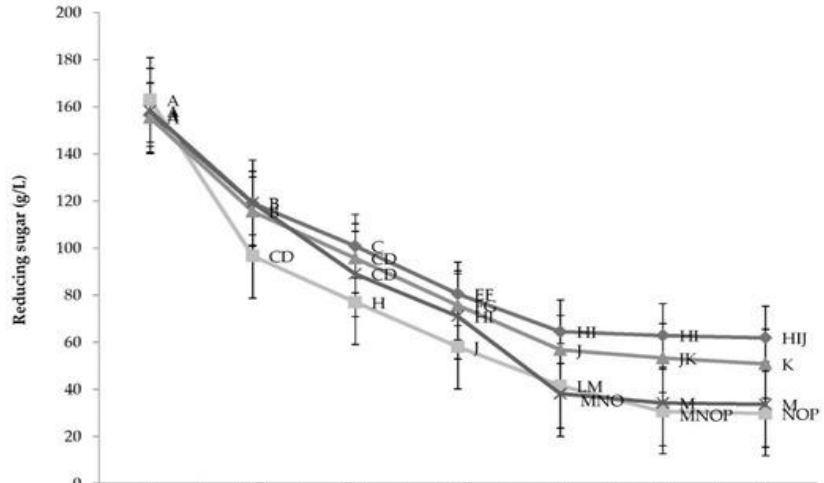


	0-h	12-h	24-h	36-h	48-h	60-h	72-h
C-KKU20	9.76	36.42	47.18	56.16	67.50	71.54	71.24
H-KKU20	9.97	13.13	24.78	32.87	37.43	44.43	44.19
I-KKU20	9.86	16.96	21.92	37.26	39.31	44.86	48.47
S. cerevisiae	10.06	47.85	53.47	76.41	77.77	78.65	78.56

Figure 4. Ethanol synthesis by four distinct yeast isolates in 25% sugarcane molasses enriched with 1% urea and 1% CMC under shaking incubation at 30 °C and 150 rpm for 72 h. $^{A–M}$ Different superscript letters represent significant interaction between time and yeast type ($p < 0.01$). SEM = standard error of mean; S. cerevisiae = Saccharomyces cerevisiae.

Effect of fermentation time and Yeast isolates on reducing sugar content

The combined influence of incubation duration and yeast strain on reducing sugar was significant ($p > 0.05$) **(Figure 5)**. The concentration of reducing sugars decreased from 162.9 to 29.8 g/L over 72 h. The smallest residual sugar was detected in P. kudriavzevii-KKU20, which showed 30.6 and 29.8 g/L at 60 and 72 h, respectively. In contrast, Galactomyces sp.-KKU20 and S. cerevisiae exhibited the greatest sugar reduction at 60 and 72 h of fermentation, respectively.



	0h	12h	24h	36h	48h	60h	72h
C-KKU20	156.7	119.1	100.8	80.5	64.5	62.9	61.9
H-KKU20	162.9	96.6	77.0	58.1	41.5	30.6	29.8
I-KKU20	155.3	115.5	95.7	75.7	56.8	53.3	50.9
S. cerevisiae	158.2	119.3	89.0	71.0	38.2	34.2	33.6

Figure 5. Reduction in sugar levels by four yeast isolates in 25% sugarcane molasses containing 1% urea and 1% CMC at 30 °C and 150 rpm for 72 h. $^{A–P}$ Superscripts with unique letters denote significant interactions between incubation period and yeast strain ($p < 0.01$). SEM = standard error of mean; S. cerevisiae = Saccharomyces cerevisiae.

Discussion

Isolation and identification of yeast from rumen samples

Yeast isolates displayed morphological variations, including ovoid (6 of 11), flat (5 of 11), convex (4 of 11), and rough (7 of 11) appearances. White colonies were most frequent (9 of 11). Similar morphological traits were reported by Marrero *et al*. [32], who described rumen-derived yeasts as smooth, slightly convex, and cream to white in tone. Variations in yeast type may arise from differing feed sources, roughage-to-concentrate ratios (R:C), or animal species. Marrero *et al*. [33] also identified pink colonies from dairy cattle, later classified as Levica strain 18 (L18). Therefore, the morphology of rumen yeasts can vary widely depending on environmental and biological factors.

Biomass formation and cellulase enzymatic function of yeast isolates

Yeast proliferation requires a sufficient nutrient supply, particularly soluble carbohydrates and nitrogen, to sustain cell metabolism and biomass accumulation [34]. According to Paserakung *et al*. [35], increasing molasses levels from 8% to 16% led to the maximum cell mass of Trichosporon asahii, reaching 25.9%. Similarly, Johnson, Singh, Saini, Adhikari, Sista, and Yadav [23] evaluated Rhodotorula glutinis IIP-30 using single carbon substrates such as molasses, glucose, and sucrose under nitrogen-restricted conditions. Their observations revealed an 87.8% increase in biomass when molasses was used, suggesting it is a superior carbon source for T. asahii compared to other sugars [6]. In the present experiment, the use of 25% molasses with 1% urea produced the highest ruminal yeast biomass at 29.2%, indicating that an optimal combination of these components enhances growth. Nevertheless, since only molasses was employed as the carbon base, future research should evaluate additional carbon inputs.

Manikandan and Viruthagiri [36] emphasized that nitrogen availability, its concentration, and the carbon-to-nitrogen (C:N) proportion significantly affect biomass yield. Danesi *et al*. [37] showed that the use of sugarcane blackstrap molasses combined with yeast extract at a 10:1 C:N ratio yielded the most substantial yeast mass. Likewise, Sokchea *et al*. [38] reported a peak biomass value of 7.57 g/L at the same C:N ratio. In comparison, the current study maintained a higher C:N ratio (25:1), which resulted in a biomass level of 17.07 g/L—likely due to the surplus carbon content. Achieving a balanced C:N ratio supports yeast multiplication, as demonstrated in previous work where the inclusion of urea and yeast in molasses media elevated yeast density and improved livestock performance. Boonnop, Wanapat, Nontaso, and Wanapat [6] observed that protein levels in cassava chips increased from 3.4% to 32.5% (93.5% as true protein), while lysine content rose from 3.8% to 8.5%. Experiments using cassava root by Khampa, Chuelong, Kosonkittiumporn, and Khejornsart [7] reported protein augmentation up to 36.1% following yeast fermentation, whereas Khampa *et al*. [39] documented a lower enhancement of 19.2%.

In this investigation, cellulase secretion from the isolated yeast ranged between 0.020 and 0.075 units/mL—an outcome not previously reported, which could contribute to fiber hydrolysis in the rumen. Though cellulase synthesis by ruminal yeasts has not been widely explored, yeasts from environmental sources have exhibited such enzymatic potential. Sarawan [22] found that Candida glabrata, Candida natalensis, and Kluyveromyces africanus (from Jasminum adenophyllum) secreted cellulases within 0.004–0.08 units/mL in yeast extract peptone dextrose broth containing 1% CMC. Therefore, yeast-derived cellulases might facilitate cellulose degradation during feed digestion. Functionally, cellulase catalyzes the cleavage of β-1,4-glycosidic bonds within cellulose chains [40]. Among these enzymes, cellobiohydrolases (CBHs; EC 3.2.1.91) play a major role in cellulose depolymerization [41], with CBH1 (Cel7A) and CBH2 (Cel6A) commonly expressed in yeast strains [42].

Classification of the yeast isolates

Three rumen-derived yeasts were identified as Pichia kudriavzevii-KKU20, Candida tropicalis-KKU20, and Galactomyces sp.-KKU20. The abbreviation “KKU” designates Khon Kaen University, the institution where isolation occurred, while “20” indicates the year 2020. Comparable isolates (P. kudriavzevii, C. tropicalis, and Galactomyces sp.) have been reported previously [43–46], though their physiological attributes remained unexamined. This study provides the first account of these strains’ characteristics, including their capacity for biomass synthesis, cellulase production, growth kinetics, ethanol output, and sugar utilization. Furthermore, these isolates were determined to be Crabtree-negative. In particular, P. kudriavzevii-KKU20 and C. tropicalis-KKU20

exhibited efficient conversion of carbon substrates into biomass, making them promising for development as microbial protein sources in ruminant feed formulations.

Population density, ethanol output, and sugar depletion of selected ruminal yeasts

Among the evaluated isolates, Pichia kudriavzevii-KKU20 exhibited superior cell proliferation in the culture medium. Its cell density peaked at 10.02 Log cells/mL after 72 hours of aerobic incubation. Being a Crabtree-negative yeast, P. kudriavzevii-KKU20 demonstrates higher propagation efficiency since it actively transports glucose via an inducible, high-affinity proton symport mechanism, ensuring consistent growth under aerobic environments [47].

Conversely, Saccharomyces cerevisiae generated significant ethanol quantities when cultivated under high-carbon aerobic conditions. After 72 hours, ethanol concentration reached 78.6 g/L—greater than that produced by other isolates. The elevated ethanol synthesis arises because, at high sugar levels, the pyruvate dehydrogenase complex becomes suppressed, while pyruvate decarboxylase activity increases three- to fourfold, channeling sugar conversion toward ethanol even in oxygen-rich conditions [48, 49]. The 25% molasses concentration likely enhanced ethanol productivity of S. cerevisiae relative to the other yeast species.

Sugar utilization and biomass development in yeast strains

The rate at which yeasts metabolize sugars corresponds closely to their growth dynamics and differs across species [50]. Yeasts isolated from the rumen displayed a more pronounced growth curve than Crabtree-positive species such as S. cerevisiae. In this study, Pichia kudriavzevii-KKU20 utilized 133.1 g of sugar between 0 and 72 h of incubation, whereas S. cerevisiae consumed only 124.6 g. Van Urk *et al*. [51] demonstrated that S. cerevisiae mutants lacking pyruvate decarboxylase exhibit slower sugar consumption compared with Crabtree-negative yeasts, highlighting the pivotal role of pyruvate decarboxylase in controlling glycolytic flux.

Different yeast taxa also vary in their capacity for biomass formation. According to Van Urk, Voll, Scheffers, and Van Dijken [51], S. cerevisiae tends to have reduced growth under high-glucose aerobic conditions. In contrast, Wardrop, Liti, Cardinali, and Walker [14] found that Kluyveromyces marxianus generated approximately sevenfold higher biomass than S. cerevisiae in glucose-enriched media. Under aerobic metabolism, oxygen serves as the terminal electron acceptor. Yeasts with complete oxidative pathways, such as P. kudriavzevii-KKU20 and Candida tropicalis-KKU20, channel energy toward biomass synthesis with minimal ethanol formation—characteristics typical of Crabtree-negative species. Conversely, S. cerevisiae, a Crabtree-positive yeast, favors fermentative metabolism and releases larger quantities of ethanol [48]. This explains why P. kudriavzevii-KKU20 and C. tropicalis-KKU20 achieved higher biomass yields than S. cerevisiae.

The true nutritional significance of these rumen-derived yeasts lies in their superior biomass output compared to the 11 other isolates and S. cerevisiae. The observed biomass accumulation and viable cell counts indicate potential for developing yeast-based feeds enriched in protein, amino acids, and vitamins. Utilizing diverse yeast species could therefore yield valuable outcomes. Moreover, the cellulase activity detected in these isolates suggests a role in enhancing the nutritive quality of roughages such as rice straw (RS), thereby increasing feed value.

Conclusion

Isolation and screening of ruminal yeasts identified 11 distinct strains. Among them, Pichia kudriavzevii-KKU20, Candida tropicalis-KKU20, and Galactomyces sp.-KKU20 were the most promising, producing substantial biomass and notable cellulase activity. Optimal growth occurred in media containing 25 % sugarcane molasses and 1 % urea at pH 3.5 with agitation at 150 rpm. Under these optimized parameters, P. kudriavzevii-KKU20 demonstrated exceptional potential for biomass generation, cellulase secretion, and cell proliferation. Further assessment of this strain's performance in fiber degradation and biomass enrichment is recommended to determine its capacity to enhance the nutritional quality of feed for ruminant livestock.

Acknowledgments: None

Conflict of Interest: None

Financial Support: None

Ethics Statement: None

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