

Growth of *Candida Valida* on Ethanol as Sole Carbon Source in Batch Culture: Preliminary Kinetics and Qualitative Detection of Glycogen and Residual Ethanol

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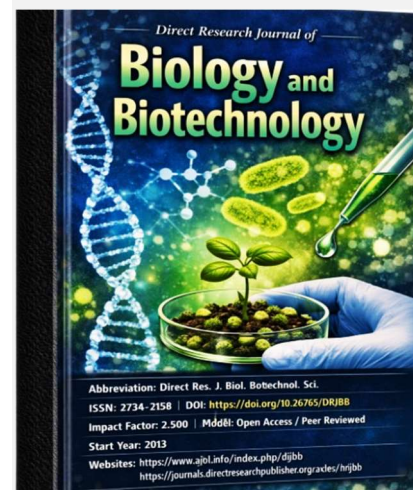
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ABSTRACT

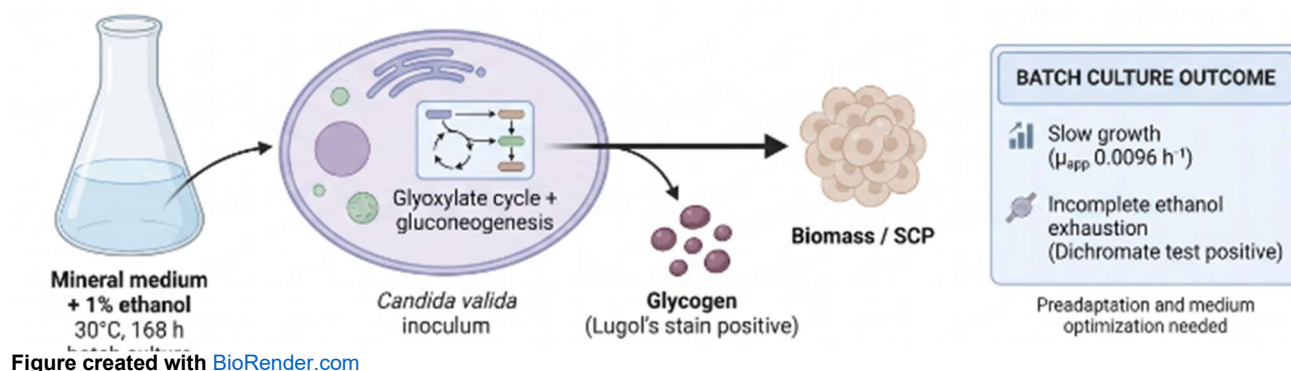
Candida valida (anamorph of *Pichia membranifaciens*) is an ascomycetous yeast capable of using ethanol as a sole carbon and energy source via the glyoxylate cycle and gluconeogenesis. Ethanol is an attractive feedstock for single-cell protein (SCP) production because it can be derived from low-emission sources. This study aimed to determine the apparent growth kinetics of *C. valida* on ethanol in batch culture, to detect intracellular glycogen qualitatively, and to assess residual ethanol qualitatively after cultivation. Batch cultivation was performed in mineral medium containing 1% (v/v) ethanol at 30 °C over 168 h. Cell concentration was determined using a Goryaev–Thom counting chamber, intracellular glycogen was visualized with Lugol's iodine stain, and residual ethanol was detected qualitatively by acidified dichromate oxidation. The initial cell density rose from 1.10×10^6 to 5.50×10^6 cells mL⁻¹, a 4.6-fold increase (approximately 2.2 doublings). The apparent net specific growth rate over the entire 168-h period, calculated as a single interval, was 0.0096 ± 0.0008 h⁻¹ (mean $\pm$ SD, $n = 2$ chamber loadings from one flask), with a 95% confidence interval of 0.0023 – 0.0170 h⁻¹. This value is approximately 8 to 16-fold lower than the 0.08 – 0.15 h⁻¹ reported for well-adapted *C. valida* cultures on ethanol. The estimated biomass-to-supplied-ethanol ratio was 0.028 g g⁻¹, indicating incomplete substrate utilization and suboptimal growth conditions. Brown intracellular granules were observed after Lugol staining in both the zero-time sample and the 168-h sample; however, because glycogen was present in the inoculum, this result does not demonstrate net accumulation during cultivation. Acidified dichromate produced a lighter green color in the post-cultivation filtrate compared with the zero-time sample, suggesting partial ethanol consumption. The data confirm that *C. valida* can grow on ethanol under the conditions tested, but the kinetics were severely suboptimal and the findings are limited by the absence of a growth curve, biological replication, and quantitative substrate and product measurements. Future work should incorporate frequent sampling, pre-adapted inocula, quantitative ethanol and biomass analysis, and vitamin supplementation to establish reliable kinetic parameters for SCP process development.



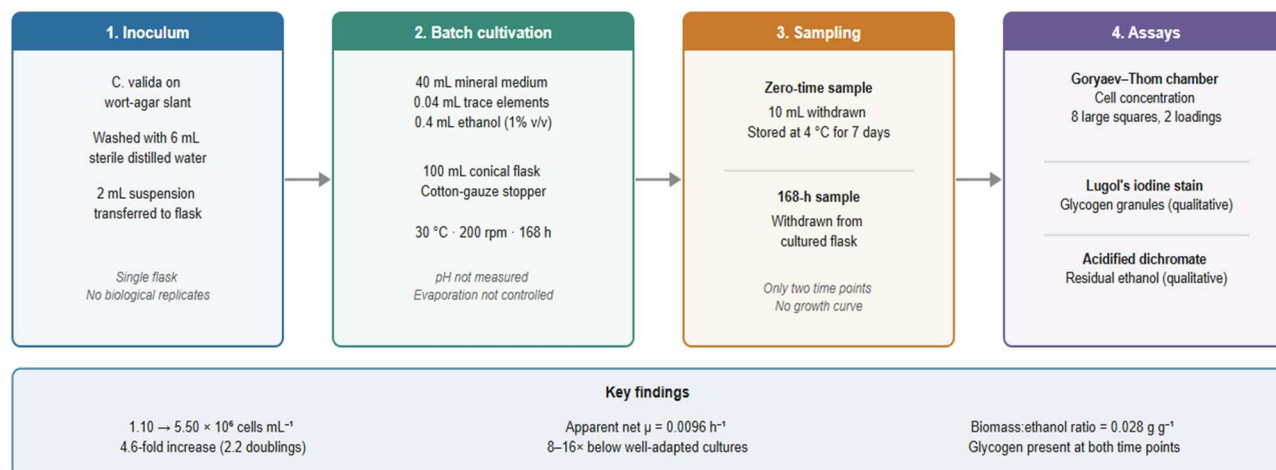
Keywords: *Candida valida*; *Pichia membranifaciens*; ethanol; batch growth; specific growth rate; glycogen; single-cell protein

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Graphical abstract:

Batch cultivation of *Candida valida* on ethanol

Mineral medium, 1% (v/v) ethanol, 30 °C, 200 rpm, 168 h



Batch cultivation of *Candida valida* on ethanol as sole carbon source. The strain was transferred from a wort-agar slant into mineral medium containing 1% (v/v) ethanol and incubated at 30 °C and 200 rpm for 168 h in a single flask without biological replication. Cell concentration was determined using a Goryaev–Thom counting chamber, intracellular glycogen was detected qualitatively with Lugol's iodine stain, and residual ethanol was detected qualitatively by acidified dichromate oxidation. Cell concentration increased 4.6-fold over 168 h, corresponding to an apparent net specific growth rate of 0.0096 h⁻¹, which is 8- to 16-fold below values reported for well-adapted *C. valida* cultures on ethanol. The biomass-to-supplied-ethanol ratio was 0.028 g g⁻¹. Glycogen granules were present at both time points; because glycogen was also present in the inoculum, net accumulation during cultivation was not demonstrated. The dichromate test is qualitative and does not distinguish microbial ethanol consumption from evaporative loss.

INTRODUCTION

The kinetics of microbial growth in batch culture provides the quantitative basis for bioprocess design, enabling the estimation of cellular proliferation and substrate consumption rates. During balanced exponential growth, the increase in biomass concentration (X) over time (t) follows first-order kinetics, and the growth yield ($Y_{X/S}$) reflects the efficiency of nutrient conversion into biomass. Both parameters depend on temperature, pH, medium composition, and the metabolic pathways of the microbe (Monod, 1949; Pirt, 1975).

Ethanol is a nonconventional carbon source for yeast cultivation. In Crabtree-negative species such as *C. valida*, ethanol is oxidized to acetaldehyde by alcohol dehydrogenase and then to acetate by aldehyde dehydrogenase before entering the glyoxylate cycle and gluconeogenesis for biomass synthesis. In the absence of glucose, this pathway allows ethanol to be assimilated without catabolite repression. Unlike *Saccharomyces cerevisiae*, which ferments sugars to ethanol even under aerobic conditions (the Crabtree effect), Crabtree-negative yeasts preferentially oxidize ethanol, making them attractive for single-cell protein (SCP) production and waste valorization (Dashko *et al.*, 2014). Transcriptional regulation of nonfermentable carbon utilization in budding yeast provides the genetic basis for this preference, and the induction of the glyoxylate cycle and gluconeogenic enzymes is tightly controlled in response to carbon source availability (Turcotte *et al.*, 2010).

Candida valida (Leberle) van Uden & H. R. Buckley is an ascomycetous yeast that is the anamorph of *Pichia membranifaciens* (Index Fungorum, 2024; National Center for Biotechnology Information, 2024; Catalogue of Life, 2024). It was originally isolated from soil, water, and decomposing plant material and is known for its ability to use ethanol as a sole carbon and energy source under aerobic conditions. In batch culture with ethanol, *C. valida* exhibits specific growth rates that depend on substrate concentration and temperature; at low ethanol concentrations, the dependence of μ on S follows Monod kinetics (Andreyev *et al.*, 2000). A recent screening of 52 Saccharomycotina yeasts on ethanol reported specific growth rates of 0.12–0.46 h⁻¹ for the fastest strains in shake-flask cultures (Warmerdam *et al.*, 2024). Biomass yields on ethanol for well-adapted yeasts range from 0.56 to 0.73 g g⁻¹, with *Cyberlindnera jadinii* achieving the highest yield and a protein content of 59% (Warmerdam *et al.*, 2024). Ethanol is emerging as a promising feedstock for SCP production because it can be generated from industrial waste gases, CO₂, and agricultural residues, aligning with low-emission biotechnological goals (Warmerdam *et al.*, 2024). *Cyberlindnera jadinii* has received particular attention as an ethanol-grown SCP platform, achieving a maximum biomass yield of 0.83 g g⁻¹ in ethanol-limited chemostat cultures (Vieira-Lara *et al.*, 2024), with protein contents of 54% in chemostat-grown

biomass and 42% in fed-batch-grown biomass (Vieira-Lara *et al.*, 2024). A broader screening of 52 Saccharomycotina yeasts reported a maximum yield of 0.73 g g⁻¹ for *C. jadinii* and a protein content of 59% (Warmerdam *et al.*, 2024). However, *Candida* species are not universally regarded as safe; some are opportunistic pathogens, and their use in food or feed applications requires careful safety assessment. The regulatory status of individual *Candida* species varies, and QPS (Qualified Presumption of Safety) or GRAS (Generally Recognized as Safe) designation depends on the specific strain and intended use. These considerations underscore the need for rigorous strain characterization and safety evaluation in SCP development.

The aim of this study was to determine the apparent growth kinetics of *C. valida* on ethanol as a sole carbon source in batch culture under defined mineral medium conditions. The specific objectives were: (i) to determine cell concentrations at zero time and after 168 h of cultivation using a Goryaev–Thom counting chamber and to estimate the apparent net specific growth rate over the cultivation period; (ii) to detect intracellular glycogen qualitatively using Lugol's iodine staining in both the inoculum and the post-cultivation samples; and (iii) to assess residual ethanol qualitatively in the zero-time and post-cultivation samples using the acidified dichromate oxidation test. While *C. valida* has been studied for ethanol utilization in continuous culture (Andreyev *et al.*, 2000), data on its batch growth kinetics in defined mineral medium, particularly with respect to glycogen storage and residual substrate, remain limited. Recent screening studies have focused on other Saccharomycotina yeasts (Warmerdam *et al.*, 2024), and the behavior of *C. valida* under simple batch conditions without pre-adaptation has not been systematically characterized. We hypothesized that *C. valida* can grow on ethanol as a sole carbon source in mineral medium under batch conditions, and that intracellular glycogen will be detectable in cells after cultivation on ethanol.

MATERIALS AND METHODS

Microorganism and Media Preparation

The yeast strain *Candida valida* was obtained from a stock culture maintained on wort-agar slants. The strain identity was confirmed by the culture collection; however, no culture collection number or molecular identification (e.g., ITS sequencing) was performed in this study. All media and glassware were sterilized by autoclaving at 121 °C and 1 atm for 20 min. The mineral medium stock solution (200 mL) was prepared by dissolving the following mineral salts in distilled water to achieve the indicated concentrations

Table 1: Final medium composition in the cultivation flask.

Component	Final concentration in 40 mL medium
NH ₄ H ₂ PO ₄	10.0 g L ⁻¹
MgSO ₄	0.7 g L ⁻¹
Na ₂ HPO ₄ ·12H ₂ O	0.7 g L ⁻¹
KH ₂ PO ₄	6.80 g L ⁻¹
Trace element solution	1 mL L ⁻¹ (0.04 mL in 40 mL)
Ethanol	1% (v/v) (0.4 mL in 40 mL)

(g L⁻¹): NH₄H₂PO₄, 10.0; MgSO₄, 0.7; Na₂HPO₄·12H₂O, 0.7; KH₂PO₄, 6.80. Subsequently, 40 mL of this stock solution were transferred into a 100-mL conical flask, plugged with a cotton-gauze stopper, and sterilized by autoclaving at 121 °C and 1 atm for 20 min. A trace element stock solution (25 mL) was prepared containing (g L⁻¹): FeSO₄, 12.5; MnSO₄, 12.5; ZnSO₄, 12.5; NaCl, 6.3. Concentrated H₂SO₄ (10 mL L⁻¹) was added, and the volume was brought to 25 mL with distilled water; this solution was sterilized in the same autoclave cycle. After cooling of the autoclaved flask, under a laminar flow system and beside a lighted Bunsen burner, 0.04 mL of trace element solution and 0.4 mL of ethanol were aseptically added to the 40 mL of mineral medium, yielding the complete nutrient medium. The final medium composition in the cultivation flask is summarized in (Table 1). The initial pH was not measured; based on the phosphate and ammonium salts present, the medium is expected to be acidic (approximately pH 4–5).

Inoculation and Cultivation

A cell suspension of *C. valida* was prepared by washing the slant with 6 mL of sterile distilled water. A 2-mL aliquot of this suspension was transferred into the flask containing the complete medium. Immediately after inoculation, a 10-mL zero-time sample was withdrawn, placed in a sterile test tube, tightly capped, and stored at 4 °C for seven days. The culture flask was incubated on a shaker at 200 rpm and 30 °C for 7 days. Only one culture flask was used; no biological replicates were performed. The inoculum density was not measured separately.

Analytical Procedures

Cell Counting

Cell concentration in the zero-time sample (stored at 4 °C for 7 days) and in the post-cultivation sample was determined using a Goryaev–Thom counting chamber. Before loading, the grid lines were examined at 10× magnification to ensure clarity. The chamber and coverslip were cleaned with ethanol, and the coverslip was pressed until Newton's rings appeared. A 10-μL aliquot of cell suspension was loaded, and cells were counted in eight large squares at 40× magnification. All counts were

performed in two chamber loadings, and the mean was calculated. No viability staining was performed; total cell counts therefore include both viable and non-viable cells. The cell concentration (N) was calculated as $N = a \times f$, where a is the average number of cells per large square and $f = 2.5 \times 10^5 \text{ mL}^{-1}$ is the conversion factor for the Goryaev–Thom chamber (depth 0.1 mm, large-square area 1/25 mm²). The apparent net specific growth rate over the entire 168-h period was calculated as $\mu = (\ln N_t - \ln N_0) / t$, where N_0 and N_t are the cell concentrations at zero time and 168 h, respectively, and $t = 168 \text{ h}$. This value represents a net rate across the entire period and does not represent a true specific growth rate, because it mixes lag, growth, and stationary phases.

Glycogen Detection

Both the zero-time sample (stored at 4 °C for 7 days) and the post-cultivation sample were examined for intracellular glycogen. For each sample, 1–3 drops of cell suspension were placed on a clean glass slide, and 1–2 drops of Lugol's solution were added. The mixture was gently stirred, covered with a coverslip, and inspected at 40× magnification for brown-stained intracellular granules. Each sample was prepared and examined in two replicates. Lugol staining is qualitative and does not provide quantitative glycogen data.

Ethanol Detection

Residual ethanol was assessed qualitatively in both the zero-time sample (stored at 4 °C for 7 days) and the post-cultivation sample. For each specimen, 1 mL of culture broth filtered through filter paper was mixed sequentially with 0.2 mL of 2% (w/v) K₂Cr₂O₇ and 1 mL of concentrated H₂SO₄ in a test tube. A color change was noted immediately upon addition of sulfuric acid. Each sample was analyzed in duplicate. No calibration curve or ethanol standards were used; the test is qualitative only and cannot distinguish between microbial ethanol consumption and evaporative loss.

Data Analysis

Cell concentrations were calculated from Goryaev–Thom counting chamber as described above. The apparent net specific growth rate was determined as a single interval

over 168 h. The biomass-to-supplied-ethanol ratio was estimated by converting cell counts to dry biomass using a typical yeast cell dry weight of 40 pg (Łabędź *et al.*, 2017), and dividing the final biomass concentration by the initial ethanol concentration (7.8 g L^{-1}). This ratio is not a true yield coefficient because ethanol consumption was not measured quantitatively. All calculations were performed using Microsoft Excel. Descriptive statistics (mean $\pm$ SD) were computed from two chamber loadings; because these are technical replicates from a single flask, the confidence interval is reported with the explicit caveat that it does not represent biological replication.

RESULTS AND DISCUSSION

Cell Concentration and Growth Kinetics

Microscopic examination of Lugol-stained cells revealed brown intracellular granules in both the zero-time sample (Figure 1A) and the post-cultivation sample (Figure 1B), confirming the presence of glycogen in *C. valida* under the experimental conditions. However, because glycogen was already present in the inoculum, these results do not demonstrate net accumulation during cultivation. Qualitative ethanol detection using acidified dichromate (Figure 1C and 1D) produced a light green color for the zero-time sample and a lighter green color for the post-cultivation filtrate. While this suggests that some ethanol was oxidized during cultivation, the test is uncalibrated and evaporation from the open cotton-plugged flask cannot be excluded. These observations are consistent with the kinetic data (apparent net specific growth rate = 0.0096 h^{-1}) and the low estimated biomass-to-supplied-ethanol ratio ($\approx 0.028 \text{ g g}^{-1}$), collectively indicating suboptimal growth conditions. For the zero-time sample, eight large squares were counted in the Goryaev–Thom counting chamber, yielding 4, 6, 3, 6, 5, 3, 2, and 6 cells, with an average of 4.38 cells per large square. Without dilution ($n = 1$), the initial cell concentration was determined as:

$$N_0 = 4.38 \times 2.5 \times 10^5 = 1.095 \times 10^6 \text{ cells mL}^{-1}.$$

Post-cultivation sample counts from eight large squares gave values of 32, 20, 23, 12, 33, 14, 20, and 22, with an average of 22 cells per large square. Hence:

$$N_t = 22 \times 2.5 \times 10^5 = 5.50 \times 10^6 \text{ cells mL}^{-1}.$$

The cell concentration increased approximately 4.6-fold over the 168-h cultivation period, corresponding to about 2.2 doublings. If the entire period is treated as a single interval, the apparent net specific growth rate is:

$$\mu = (\ln N_t - \ln N_0) / t = (\ln(5.50 \times 10^6) - \ln(1.095 \times 10^6)) /$$

$$168 = (15.52 - 13.91) / 168 = 0.0096 \text{ h}^{-1}.$$

This value represents a net rate across the entire 168-h period and does not represent a true specific growth rate, because it mixes lag, growth, and stationary phases. The culture did not achieve balanced exponential growth during the measured interval. Before interpreting this single number, the raw counting records were revisited. The experiment was designed with two chamber loadings per time point, and by grouping the eight recorded large-square values into the two actual chamber loadings, specific growth rates of 0.00905 and 0.01021 h^{-1} were obtained. Expressed as mean $\pm$ SD ($n = 2$ technical chamber loadings from a single flask), μ becomes $0.0096 \pm 0.0008 \text{ h}^{-1}$, with a 95% confidence interval of 0.0023 – 0.0170 h^{-1} (detailed stepwise derivations are presented in the Supplementary Material). These two loadings were drawn from a single culture flask and are therefore technical rather than biological replicates, which accounts for the width of the interval. The upper confidence limit of 0.0170 h^{-1} corresponds to approximately 5- to 9-fold lower values than the 0.08 – 0.15 h^{-1} range reported for well-adapted *C. valida* cultures on ethanol (Andreyev *et al.*, 2000). Taken together, the central estimate and its upper bound place the observed growth well below the expected range for this species on ethanol.

Biomass-to-Supplied-Ethanol Ratio

Assuming a typical yeast cell dry weight of 40 pg (Łabędź *et al.*, 2017), the final dry biomass concentration was:
 $X = (5.50 \times 10^6 \text{ cells mL}^{-1}) \times (40 \times 10^{-12} \text{ g cell}^{-1}) = 0.22 \text{ g L}^{-1}.$

In the experimental setup, 0.4 mL of ethanol was added to 40 mL of mineral medium, giving a total volume of approximately 40.4 mL. With an absolute ethanol density of $\sim 0.789 \text{ g mL}^{-1}$:

$$\text{Ethanol mass} = 0.4 \text{ mL} \times 0.789 \text{ g mL}^{-1} \approx 0.316 \text{ g}.$$

$$\text{Initial ethanol concentration} = 0.316 \text{ g} / 0.0404 \text{ L} \approx 7.82 \text{ g L}^{-1} \text{ (rounded to } 7.8 \text{ g L}^{-1}\text{)}.$$

Final ethanol concentration was determined qualitatively only; the acidified dichromate test gave a lighter green color. A biomass-to-supplied-ethanol ratio was calculated using the initial ethanol concentration as the denominator, on the assumption that initial biomass was negligible and that the observed biomass originated from the supplied ethanol:

$$\text{Biomass-to-supplied-ethanol ratio} = 0.22 \text{ g L}^{-1} / 7.8 \text{ g L}^{-1} = 0.028 \text{ g g}^{-1}.$$

Because ethanol consumption was not measured

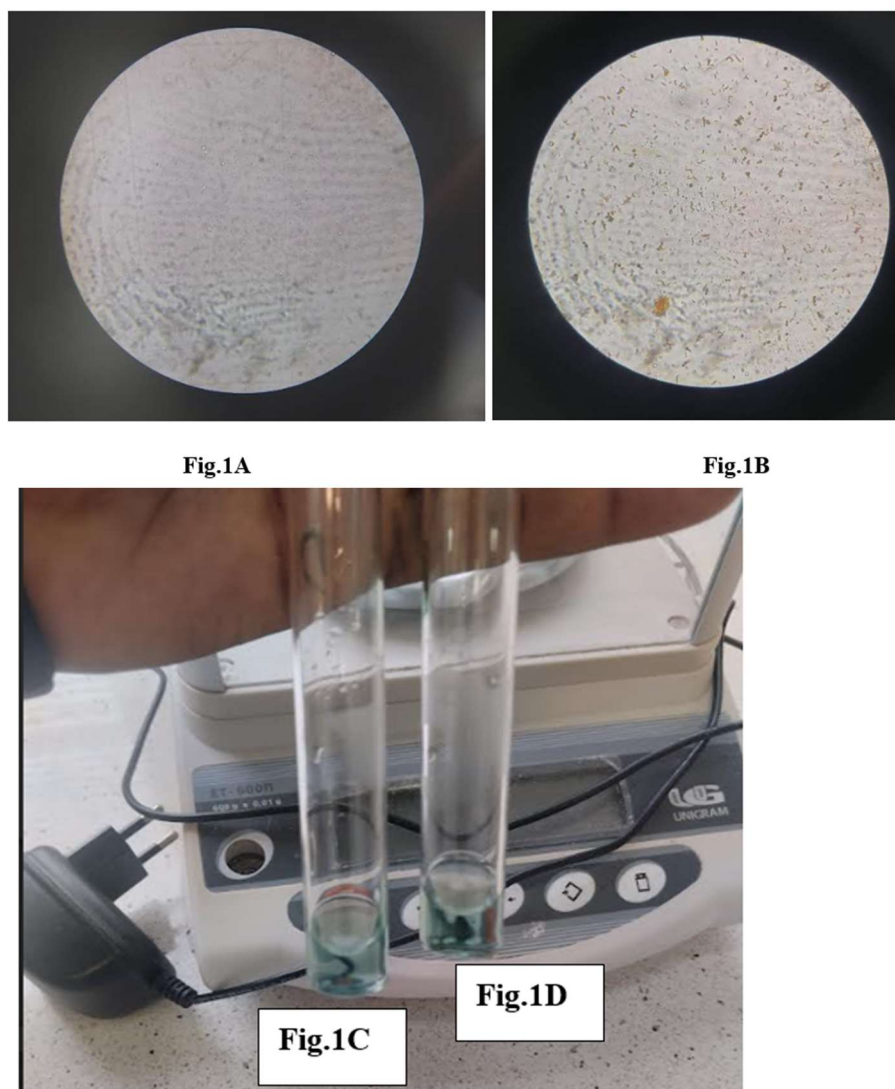


Figure 1. Microscopic and qualitative assays.

quantitatively, this value describes the biomass produced per unit of ethanol supplied rather than a true yield coefficient. Using the change in biomass ($\Delta X = 0.22 - 0.044 = 0.176 \text{ g L}^{-1}$) gives 0.022 g g^{-1} on initial ethanol. Both estimates fall approximately 20- to 26-fold below the $0.56\text{--}0.73 \text{ g g}^{-1}$ reported for well-adapted yeasts on ethanol (Warmerdam *et al.*, 2024), indicating that a large fraction of the supplied carbon was not converted into biomass.

The direct transfer of cells from a rich wort-agar slant to a minimal mineral medium with 1% (v/v) ethanol as the sole carbon source imposes an abrupt nutritional and osmotic shift. Without stepwise adaptation, yeasts commonly exhibit an extended lag before the enzymes of ethanol oxidation and the glyoxylate cycle are fully induced (Turcotte *et al.*, 2010). Ethanol concentrations near 1% can also act together with temperature to stress non-

adapted populations, delaying the onset of exponential division (Aldiguier *et al.*, 2022). A growth-limiting deficiency in the medium, such as biotin or thiamine, is a plausible explanation for the residual ethanol. *C. jadinii* CBS 621 is a thiamine auxotroph, and thiamine supplementation is required for growth of some yeast strains on ethanol (Vieira-Lara *et al.*, 2024). The minimal medium used here contained no added vitamins, which may have limited growth of *C. valida* if the strain has similar auxotrophies. However, these mechanisms were not tested here and remain hypotheses for future work.

Residual Ethanol

Both the zero-time and the 168-h culture filtrates gave a green coloration with acidified dichromate, confirming the presence of ethanol in each sample.

The culture filtrate produced a lighter green than the zero-time sample, indicating that ethanol concentration decreased during cultivation. The test is qualitative and uncalibrated, and evaporative loss from the open cotton-plugged flask at 200 rpm and 30°C over seven days was not controlled; the two samples were also stored differently (4 °C tube versus shaken flask), which may have influenced color intensity. The dichromate result therefore supports partial ethanol depletion without distinguishing between microbial consumption and evaporation. Under well-aerated, nutrient-complete conditions, well-adapted *C. valida* exhausts 1% ethanol within 24–48 h (Andreyev *et al.*, 2000), so the persistence of ethanol after 168 h indicates that substrate utilization was incomplete under the conditions tested.

Glycogen Detection

Brown glycogen granules were visible inside yeast cells from both the zero-time sample and the 168-h culture sample after Lugol staining. This finding is consistent with the well-documented capacity of Crabtree-negative yeasts to channel two-carbon substrates into storage carbohydrates (François & Parrou, 2001; Wilson *et al.*, 2010). The inoculum originated from a sugar-rich wort-

agar slant, where glycogen storage is expected, so glycogen detected at 168 h may reflect either retained reserves or newly synthesized material. Lugol staining is qualitative and does not resolve between these possibilities, nor does it provide evidence of gluconeogenic flux or of a specific physiological phase. Quantitative glycogen measurement across a sampling time course, ideally with a glucose-grown control, would be required to assess accumulation dynamics.

Comparison with Current Studies

The growth parameters obtained here are compared with recent literature in (Table 2). Warmerdam *et al.* (2024) reported specific growth rates of 0.12–0.46 h⁻¹ for the fastest Saccharomycotina yeasts on ethanol in shake-flask culture, and biomass yields of 0.56–0.73 g g⁻¹, with *Cyberlindnera jadinii* reaching the highest yield and a protein content of 59%. Andreyev *et al.* (2000) described Monod-type dependence of μ on ethanol concentration for *C. valida* in pH-auxostat culture. The apparent net specific growth rate of 0.0096 h⁻¹ reported here is approximately 12- to 48-fold lower than the fastest strains of Warmerdam *et al.* (2024), and the biomass-to-supplied-ethanol ratio of 0.028 g g⁻¹ is approximately 20- to 26-fold lower than their reported yields.

Table 2: Comparison of growth parameters for yeasts cultivated on ethanol.

Study	Yeast	Conditions	μ (h ⁻¹)	Y _x /S (g g ⁻¹)
This study	<i>Candida valida</i>	Batch flask, 1% (v/v) ethanol, 30 °C, 168 h	0.0096 (net, single interval)	0.028 (apparent)
Warmerdam <i>et al.</i> (2024)	52 Saccharomycotina strains (fastest 21)	Shake flask, ethanol as sole carbon source	0.12–0.46	Not reported
Warmerdam <i>et al.</i> (2024)	<i>Cyberlindnera jadinii</i>	Chemostat, ethanol-limited	Not reported	0.73 (0.83 max)
Andreyev <i>et al.</i> (2000)	<i>Candida valida</i>	pH-auxostat, ethanol	Monod-type dependence; 0.82 max	0.85 max

Differences in strain adaptation, medium composition, ethanol concentration, and cultivation method all contribute to these discrepancies.

Growth, intracellular glycogen and ethanol utilisation of *Candida valida*

Cell growth: The growth pattern of *Candida valida* in batch culture suggests an increase in cell abundance during the 168-hour incubation, when ethanol was supplied as the sole carbon source. As shown in Table 3, the mean cell count increased from 4.75 and 4.00 cells per large square in the two zero-time loadings to 21.75 and 22.25 calls per large square after 168 hours. This represents an approximately 5.0-fold increase in the mean count (from 4.38 to 22.00 cells per large square), indicating that the culture remained capable of increasing its cell population under the experimental conditions. However, because only

the initial and final time points were reported, the lag, exponential and stationary phases cannot be identified, and the data do not establish a complete growth-kinetic profile.

Intracellular glycogen: Figure 1A and 1B provide complementary qualitative evidence. Brown intracellular granules were observed in Lugol-stained cells at both zero time and 168 hours, suggesting the presence of glycogen-associated material at both sampling points. Nevertheless, the images alone do not show whether glycogen accumulated or was depleted, because no quantitative assessment of staining intensity or glycogen concentration was made.

Ethanol utilization: The acidified dichromate test (Figure 1C and 1D) showed light green coloration in the zero-time sample and a lighter green coloration in the 168-hour culture filtrate. This visual difference may be consistent with a change in the amount of ethanol or other

Table 3: Raw Goryaev–Thom counting chamber counts (cells per large square).

Time point	Loading	Square 1	Square 2	Square 3	Square 4	Mean
0 h	Loading 1	4	6	3	6	4.75
0 h	Loading 2	5	2	4	5	4.00
168 h	Loading 1	32	20	23	12	21.75
168 h	Loading 2	30	17	20	22	22.25

dichromate-reactive substances during incubation. However, the test was qualitative, and the absence of calibration standards and uninoculated evaporation controls prevents reliable quantification, or definitive attribution of the colour change to ethanol consumption by *C. valida*.

Figure 1 (A) Lugol-stained *C. valida* cells from the zero-time sample, showing brown intracellular glycogen granules (40× magnification; scale bar = 10 µm). (B) Lugol-stained cells from the 168-h culture sample, showing brown intracellular granules (40× magnification; scale bar = 10 µm). (C) Acidified dichromate test on the zero-time sample, showing light green coloration. (D) Acidified dichromate test on the 168-h culture filtrate, showing lighter green coloration. The dichromate test is qualitative; no calibration standards or uninoculated evaporation controls were included.

Conclusion

This study measured cell concentration, glycogen presence, and residual ethanol in batch cultures of *Candida valida* grown on 1% (v/v) ethanol in mineral medium over 168 h. Cell concentration increased 4.6-fold, from 1.10×10^6 to 5.50×10^6 cells mL⁻¹, corresponding to an apparent net specific growth rate of 0.0096 h⁻¹ and an estimated biomass-to-supplied-ethanol ratio of 0.028 g g⁻¹, both of which fall well below the ranges reported for well-adapted yeasts growing on ethanol. Glycogen granules were present in both the zero-time and 168-h samples, but because the inoculum was maintained on wort agar, accumulation of glycogen during cultivation was not demonstrated. Residual ethanol was detected in both samples by acidified dichromate, with a lighter color in the 168-h filtrate, although this qualitative test cannot separate microbial consumption from evaporative loss. The principal limitations of the study are the absence of a growth curve, biological replication, viability staining, a no-carbon negative control, quantitative ethanol and biomass measurements, and pH monitoring; in particular, without a no-carbon control, carry-over carbon from the inoculum cannot be excluded as a contributor to the observed growth. Future experiments should sample frequently during the early growth phase, use pre-adapted inocula, supplement the medium with vitamins such as biotin and thiamine, and quantify ethanol, dry biomass, and glycogen in order to establish reliable kinetic parameters for evaluating *C. valida* in ethanol-based single-cell protein processes.

REFERENCES

- Aldiguer, A. S., Alfenore, S., Cameleyre, X., Goma, G., Uribealarea, J. L., Guillouet, S. E., & Molina-Jouve, C. (2004). Synergistic temperature and ethanol effect on *Saccharomyces cerevisiae* dynamic behaviour in ethanol bio-fuel production. *Bioprocess and Biosystems Engineering*, 26(4), 217–222. <https://doi.org/10.1007/s00449-004-0352-6>
- Andreyev, S. V., Minkevich, I. G., & Eroshin, V. K. (2000). The effect of two inhibiting substrates on growth kinetics and cell maintenance of the yeast *Candida valida*. *Process Biochemistry*, 36(3), 209–217. [https://doi.org/10.1016/S0032-9592\(00\)00189-8](https://doi.org/10.1016/S0032-9592(00)00189-8)
- Catalogue of Life. (2024). *Pichia membranifaciens* (Hansen) Kurtzman [Species record 77J3J]. ChecklistBank. <https://www.checklistbank.org/dataset/316441/taxon/77J3J>
- Dashko, S., Zhou, N., Compagno, C., & Piškur, J. (2014). Why, when, and how did yeast evolve alcoholic fermentation? *FEMS Yeast Research*, 14(6), 826–832. <https://doi.org/10.1111/1567-1364.12161>
- Index Fungorum. (2024). *Candida valida* (Leberle) Uden & H.R. Buckley [Nomenclatural record 227217]. Royal Botanic Gardens, Kew. <http://www.indexfungorum.org/Names/NamesRecord.asp?RecordID=227217>
- Łabędź, B., Wańczyk, A., & Rajfur, Z. (2017). Precise mass determination of single cell with cantilever-based microbiosensor system. *PLoS ONE*, 12(11), e0188388. <https://doi.org/10.1371/journal.pone.0188388>
- Monod, J. (1949). The growth of bacterial cultures. *Annual Review of Microbiology*, 3(1), 371–394. <https://doi.org/10.1146/annurev.mi.03.100149.002103>
- National Center for Biotechnology Information. (2024). *Pichia membranifaciens* (taxid 4926) [Taxonomy database record]. NCBI Taxonomy. <https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=4926>
- Pirt, S. J. (1975). *Principles of microbe and cell cultivation*. Blackwell Scientific Publications
- Turcotte, B., Liang, X. B., Robert, F., & Soontornngun, N. (2010). Transcriptional regulation of nonfermentable carbon utilization in budding yeast. *FEMS Yeast Research*, 10(1), 2–13. <https://doi.org/10.1111/j.1567-1364.2009.00555.x>
- Vieira-Lara, M. A., Warmerdam, M., de Hulster, E. A. F., van den Broek, M., Daran, J. M., & Pronk, J. T. (2024). Quantitative physiology and biomass composition of *Cyberlindnera jadinii* in ethanol-grown cultures. *Biotechnology for Biofuels and Bioproducts*, 17(1), 142. <https://doi.org/10.1186/s13068-024-02585-3>
- Warmerdam, M., Vieira-Lara, M. A., Mans, R., Daran, J. M., & Pronk, J. T. (2024). Specific growth rates and growth stoichiometries of *Saccharomycotina* yeasts on ethanol as sole carbon and energy substrate. *FEMS Yeast Research*, 24, foae037. <https://doi.org/10.1093/femsyr/foae037>
- Wilson, W. A., Roach, P. J., Montero, M., Baroja-Fernández, E., Muñoz, F. J., Eydallin, G., Viale, A. M., & Pozueta-Romero, J. (2010). Regulation of glycogen metabolism in yeast and bacteria. *FEMS Microbiology Reviews*, 34(6), 952–985. <https://doi.org/10.1111/j.1574-6976.2010.00220.x>