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Original Research Article

Evaluation of fermentation potential of wild and UV-mutated yeasts screened from traditional *murcha*.

Jeny Subba[1], *Basanta Kumar Rai*[2], *Dil Kumar Limbu*[2], *Sangen Ruma Rai*[1]
[1]*Dharan Multiple Campus, Dharan, Nepal*
[2]*Central Campus of Technology, Tribhuvan University, Hattisar, Dharan, Nepal*
***Corresponding author:** Department of Food Technology, Central Campus of Technology, Tribhuvan University, Hattisar, Dharan, Nepal, *E-mail: basanta_64@yahoo.com*

Abstract:

Murcha (an amylolytic starter) from different parts of Eastern Nepal were screened for fermentative yeasts. The most potential one was UV-mutated (8W lamp at $\lambda = 254$ nm and an intensity of 44.21 Wm^{-2} for 5-50 s) to study the effect of mutation on growth and fermentation properties. Respiratory-deficient mutants (RDMs) that resulted from the mutation were identified by triphenyl tetrazolium chloride (TTC) overlay technique and replica-plated for further isolation. Cell growth, substrate utilization, and ethanol yield of the mutants were compared with normal cells by carrying out fermentation in high-test cane molasses broth of 30 °Bx. An exhaustive screening of the samples resulted in only two *murcha* viz., from Laxmimarga (LM) and Udayapur (UD), having the desirable fermentation properties. UV-mutation study of UD and LM yeasts (both identified as strains of *Saccharomyces cerevisiae*) showed 8-12% survival and ~ 22% RDMs yield of the survived cells. Out of the 8 randomly selected RDMs, only UDm4 (colony No. 4 from UD) showed fermentation properties worth further investigation. Comparison of UD, LM and UDm4 by fermenting molasses (high test) broth of 30°Bx showed the least growth of UDm4 but the highest alcohol yield (9% and 16% more compared to UD and LM, respectively). The present finding indicates that it is possible to improve fermentation properties of feral yeasts from *murcha* by relatively simple UV-mutation approach. Finding the right mutant (the selective screening part), however, may involve considerable time and effort.

Keywords: Feral yeast, *murcha*, respiratory deficient mutants

Introduction

Murcha, an amylolytic starter cake used for cereal-based alcoholic fermentations, harbors feral fermentative yeasts, amylolytic molds and lactic acid bacteria as the essential organisms (KC et al, 1999; Lee, 1999; Subba, 2016). The isolation and study of brewing/fermentation potential of murcha yeasts have been carried out by several workers, including KC et al. (1999), Rai and Subba (2004), and Rai (2006) but work on improvement aspects of the isolates has largely been ignored. Literatures on protocols for the improvement of yeast strains abound (Bridges, 1976; Bacila et al, 1978; Chambers et al, 2009; Reed and Nagodawithana, 1991; Walker, 1998; Smith and Burke, 2014; Steensels et al, 2014) but these generally deal with only 'culture' or laboratory yeast species, polyploid/anueploid *Saccharomyces cerevisiae* in particular. Wild yeasts have not been used so far for the study. Of the several improvement methods described, UV-mutation is the simplest, most rapid, and requires minimal laboratory facilities (Bridges, 1976). This approach for producing respiratory deficient mutants (RDMs) has recently been reviewed by several workers and its possibility for improving brewing potential of yeasts has been conclusively proved (Bacilla et al, 1978; McCann and Barnett, 1984; Hammond, 2003). However, research on the improvement of strains of feral yeasts in general, and *murcha* yeasts in particular, is almost non-existent. Since UV-mutation is the simplest (yet very powerful) technique of strain improvement strategies (Bridges, 1976), this approach can be an attractive proposition for preliminary studies on improving the fermentation potential of *murcha* yeasts.

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Materials and Methods

Murcha cakes were collected from different parts of Eastern Nepal. Screening of the potential fermentative yeasts was carried out following the protocol developed by Rai and Subba (2016). Characterization of the yeast species was done by auxanography (sugar assimilation- and fermentation tests) (Harrigan and McCance, 1976; Payne et al, 1998; Kurtzman et al, 2003).

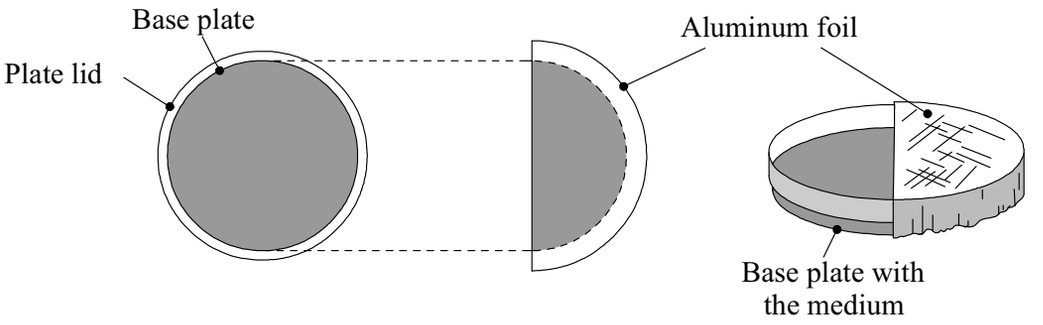

Fig 1. Wrapping of the spread-plated base plate with Al foil

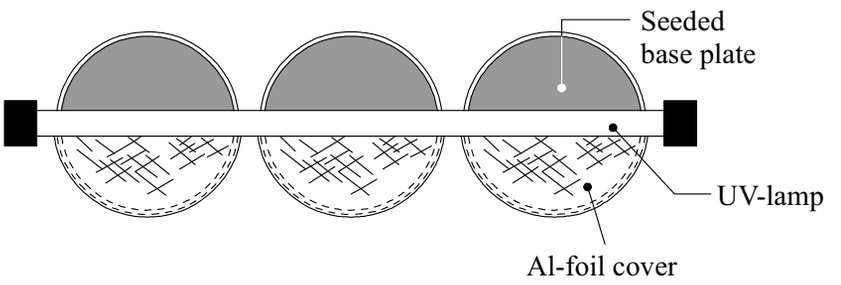

Fig 2. Arrangement of seeded plates for UV irradiation

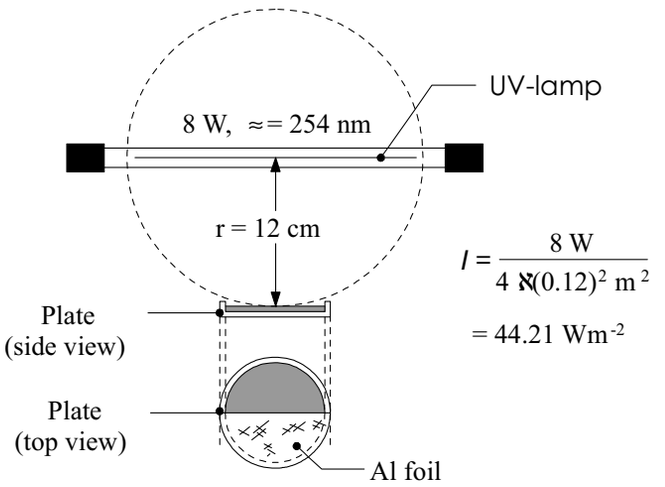

Fig 3. Exposure of seeded plate to UV irradiation

Briefly, the method involved enrichment of fermentative yeast cells (in *murcha*) by fermenting 15% high-test cane molasses solution (pH 4.0 with citric acid) for 15 days to ensure the profuse (and selective) growth of yeast cells, microscopic examination (negative staining), spread-plating on molasses agar, stock culture preparation (molasses agar slants), and auxanography. UV-mutation study was based on the protocols described by Markert (1953), Winston (2008), D'Costa and Santoro (2009), and Rai and Subba (2016). Briefly, the isolates were washed twice by suspending the cells in distilled water and centrifuging at 3000 rpm for 5 min, diluting in distilled

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water to give 20-30 cells/mL, and spread-plating (~ 0.1 mL) on molasses agar. Mutation was carried out in UV cabinet (Labtronics®, India). For this, the seeded plates were exposed to UV radiation (8 W lamp at λ = 254 nm and an intensity of 44.21 Wm^{-2} for 5-50 s, at 5 s interval) (Fig. 1 – Fig. 3), immediately incubated in dark at 30 ^{0}C for 24 h, and survival rates observed. Plates with survival rates of 10-15% (von Borstel and Mehta, 1976) were selected and RDM cells screened by replica-plating and TTC (Merck®)-overlay method (Boulton and Quain, 2001; White and Zainasheff, 2010). Finally, 8 randomly selected mutant colonies were compared with normal cells for fermentation kinetics (cell growth and substrate utilization) in high-test cane molasses medium of 30 Brix (pH 4.0 with citric acid). Fermentation was carried out in 1000-mL bottle with a working volume of 750 mL and the pitching rate of 10^6 cells/mL/Brix (Rai, 2012). Substrate concentration at 30 °Brix was selected to test the hardiness of the yeast as this sugar concentration is taken as the practical limit for fermentation by *Saccharomyces cerevisiae* (Reed, 1987). Cell growth was determined during the fermentation by modified direct microscopic count (DMC) (Rai and Subba, 2016).

Substrate utilization was determined by measuring drop in total soluble solids (TSS) (^{0}Brix) with a portable refractometer (Hanna® make, ± 0.01 unit) for a fermentation period of 12 days under ambient temperature (28-30 ^{0}C). Due consideration was also given to the findings of Atala et al. (2001), McKay et al, (2011), and Felix et al, (2014) on alcoholic fermentation. Alcohol contents of the final broths were also determined employing the routine pycnometric method (Rai and Subba, 2016).

Results and Discussion

Screening of fermentative yeasts

All of the *murcha* samples (a total of 8) were found to have different fermentation vigor, as inferred by the rate of drop in TSS. Only two *murcha* samples, viz., from Udayapur (UD) and Laxmimarga (LM) were considered worth further investigation. Both the *murcha* types were found to harbor strains of *Saccharomyces cerevisiae* as confirmed by auxanography (Harrigan and McCance, 1976; Kurtzman *et al*., 2003). The result agrees with the earlier report (Rai, 2006) that brewing potential of *murcha* varies a lot (due to variation in microflora composition.

Survival curve

The survival curves of UV-irradiated cells originating from UD and LM are shown in Fig. 4. The effect of UV-radiation on microbial cells can be expressed in two ways, viz., in terms of (i) % survival, and (ii) the D-value (time required for the surviving population to be reduced by 1 log cycle) but the former is more common (D'Costa and Santoro, 2009) because the objective of UV-mutation is to get the largest number of mutants with the least possible cell death, rather than to get the highest kill.

From Fig. 4, the slopes of survival curves for UD and LM are 0.0424 and 0.0414, respectively. The D-values ($D_{44.21}$ Wm^{-2}) for UD and LM are therefore 23.58 s and 24.15 s. von Borstel and Mehta (1976) recommend that the mutant search be carried out at a 10% survival rate. From Figure 4, this approximates to 25 s of UV-exposure at an intensity of 44.21 Wm^{-2}. In the present work, the survival rates for the said condition were found to be 12% for Udayapur yeast and 8% for Laxmimarga yeast.

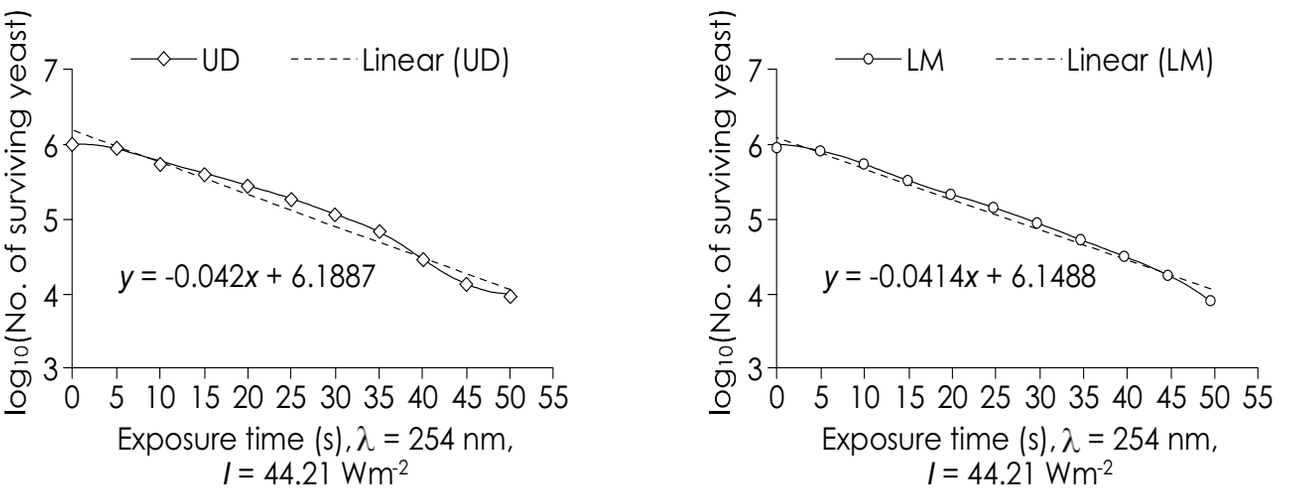

Fig 4. Survival curve of UD and LM yeasts after UV-treatment

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Mutation rate

Mutation rate, obtained by simple averaging of mutant cells in replica plates (TTC overlayed), showed 26% and 17% RDMs for Udayapur- and Laxmimarga yeast, respectively (Figure 5).

Fig 5. TTC overlay test for the presence of RDMs. (a) before TTC overlay and (b) after TTC overlay

The left-halves of the plates in Figure 5 are control colonies (not exposed to UV radiation) and the sparsely populated right-halves of the plates are the surviving colonies. In (b), pink colonies represent healthy cells (respiratory-sufficient) whereas white colonies represent RDMs.

Test fermentation of 8 randomly selected RDM isolates, 4 each from UD (coded as UDm1 to UDm4) and LM (coded as LMm1 to LMm4) in molasses broth (15^{0}Brix, pH 4.0) for 12 days at 28-30 ^{0}C showed UDm4 as having the most rapid fermentation rate and was thus selected for the comparative kinetic study. The rest of the RDMs isolates were either poor or very weak fermenters.

Substrate utilization

Comparison of substrate utilization pattern growth of the yeast isolates LM, UD and UDm4 is shown as a compound plot in Fig. 6. From the graph, the TSS curves appear to stabilize after about 6-8 days of fermentation, under the fermentation conditions employed (pitching rate, temperature, substrate concentration, initial pH), which is in agreement with earlier reports of alcoholic fermentations (Atala et al, 2001; McKay et al, 2011; Felix et al, 2014).

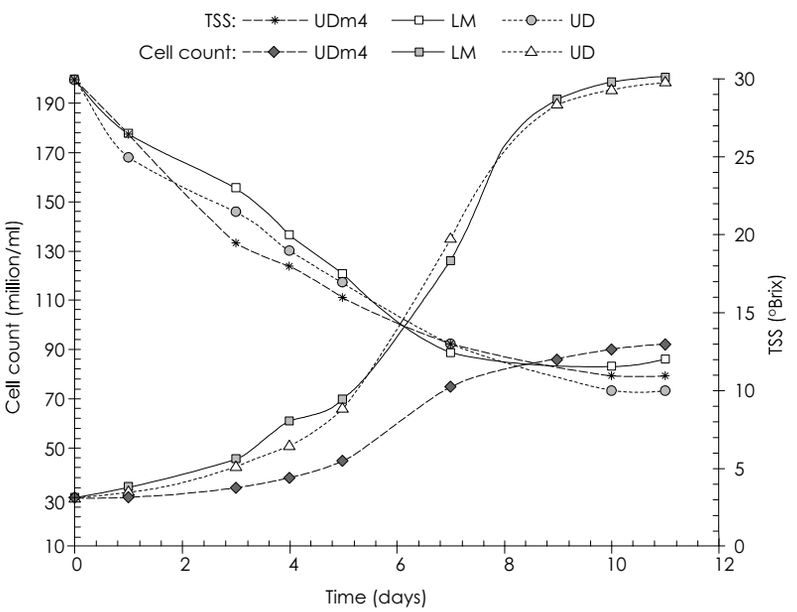

Fig 6. Growth- and substrate utilization kinetics of LM (Laxmimarga yeast), UD (Udayapur yeast) and UDm4 (UD-derived RDM isolate No. 4) in molasses broth (30^{0}Brix)

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Changes in cell number

The cell count increased by 6-7 times the original concentration in the case of normal yeasts (UD and LM) but only 3-4 times in the case of RDM yeast (UDm4). In all fermentations, the cell growth reached near-stationary phase after 6-8 days of fermentation (Figure 7). The graphs show considerable lag time for growth, which is in contrast to commercial yeast fermentation. This could be due to the inherent property of the feral yeast used in this study. Using this presumptive data as the guideline, a confirmatory study can be carried out for detailed kinetic studies.

The mutant yeast did not appear to respond well, vis-à-vis cell multiplication. This is explicable because the mutants have lost the ability to respire glucose, a property of the RDMs (Bacila et al, 1978; Hammond, 2003).

Alcohol yield

Udm4, UD and LM were found to produce 13.3, 12.2 and 11.65% alcohol by volume (abv) from molasses media of 30 °Bx. The values are significantly different ($p<0.05$), the sequence in the decreasing order being UDm4 > UD > LM. The significant enhancement in ethanol yield by UDm4 yeast (~ 9% compared to UD and ~ 16.5% compared to LM) can be explained on the basis of work done by Bacila et al (1978) and Hammond (2003). The authors mention that rho-negative (ρ^-) yeast cells (mutants that have undergone sub-lethal mutation in mitochondrial DNA thereby resulting in RDMs that lack many components of the electron transport chain) are committed to ethanol production because they cannot respire glucose: because of the failing respiratory mechanisms, the ρ^- RDMs resort to enhanced ethanol production as a means to derive energy. The enhanced production of ethanol in spite of low cell count (growth) follows the same logic. The metabolite production is only partially growth-associated.

Conslusions

Subjecting *murcha* to molasses enrichment medium for a week or two results in strains of *Saccharomyces cerevisiae* as the dominant yeast. At UV radiation intensity of 44.21 Wm^{-2} ($\lambda = 254$ nm) with an exposure time of 20-25 s (plates seeded with feral yeast from *murcha* cakes) can yield 8-12% cell survival. RDMs comprise less than $1/4^{th}$ (~ 22%) of the UV-survived colonies, with even small number acquiring the property for enhanced ethanol production. Feral yeasts from *murcha* can be UV-mutated to increase ethanol yield.

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