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Encapsulation of *Saccharomyces cerevisiae* in Alginate Beads and its Application for Wine Making

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A study was carried out on encapsulation of wine yeast (Saccharomyces cerevisiae) and its use in wine making compared to free yeast. Rehydrated active dry wine yeast was encapsulated in a 2% sodium alginate solution, cross linked with different molar concentration of $CaCl_2$ solution (0.1, 0.2, 0.3,0.4 and 0.5 M) for 30 minutes. The molar concentration with minimum cell leakage (0.2 M) was used for yeast encapsulation. Colony count (CFU/ml) was analyzed for both free yeast (FY) and encapsulated yeast (EY) so as to equilibrate the rate of yeast pitching in wine fermentation. Physicochemical properties; total soluble solids (TSS), acidity and pH of red and white grapes were analyzed and were found to be 16.4±0.10°Bx, 0.38±0.02% and 3.90±0.02 for white grapes and 19±0.15°Bx, 0.64±0.01% and 3.1±0.10 for red grapes. During the fermentation process in both wines, a gradual reduction in TSS was noted while an alternate of increase and decrease trend in acidity was noted which finally stabilized after 12 days. The final TSS of wines was not significantly different for yeast types (FY or EY) but higher values were noted for red wine (FY, 7.11±0.26 & EY, 7.33±0.19) than for white wine (FY, 6.1±0.10 & EY, 6.2±0.10). Similar trend was noted for final acidity of red wine (FY, 0.83±0.01 & EY, 0.84±0.02%). Though, no significant effect of yeast type on alcohol production was noted, the average alcohol content of red (FY, 13.22±0.26% & EY, 13.72±0.44%) and white (FY, 9.21±0.21% & EY, 9.64±0.38%) wine were found to be significantly different. However, wine prepared from EY was less turbid (Red wine, 95 NTU & White wine, 140 NTU) and had higher clarity (L) than wine from FY. So, from this study it was concluded that encapsulating wine yeast does not affect its fermenting capability but will aid in production of less turbid wine which will definitely simplify the filtration process.*

Key words: Encapsulation, Alginate beads, Wine

Introduction

Wine is an alcoholic beverage, an outcome of grape juice fermentation. Intrinsic factor such as enzymes and extrinsic factors such as yeasts and bacteria play an important role in converting grape juice into wine. Winemaking starts with adjusting the composition of grape must followed by microbial fermentation, especially by manipulating the biochemical activities of yeasts and lactic acid bacteria (Diviès *et al*., 1994). The biotechnology involved in the fermentation of must in wine making is considered as one of the oldest method. At present, considerable developments in wine making techniques have led to better understanding of the fermentation kinetics, which leads to improvements in quality factors of wine. Recent developments such as immobilizing wine yeast for the production of alcoholic beverages shows some potential. Enhanced fermentation productivity, feasibility of continuous processing, cell stability and lower costs of recovery and recycling and downstream processing have been the key advantages of yeast immobilization (Kourkatous *et al*., 2004). Most frequently, calcium alginate beads has been used as a matrix to immobilize living cells, such as *S. cerevisiae* (Colagrande *et al*., 1994). The use of calcium alginate is favored by it being non-toxic food additive. Furthermore, it involves a very simple gel entrapment process (Blandino *et.al*., 1999). As compared to free cells, the use of immobilized cells in wine making is a rapidly expanding research area with the objective to increase fermentation productivity, to improve quality through low temperature fermentation or to produce sparkling wines (Tsakiris *et al*., 2004). Yeast performance in alcoholic fermentation depends directly on yeast activity which can be seen as a function of cell viability as well as the physiological state of viable cells.

Clarity of wine in terms of its ability to either absorb or reflect light, is an important factor in wine quality. Though important, it is difficult to judge clarity in wines with dark colors. There are several methods: natural settling, filtration, centrifugation, tangential microfiltration and carbondioxide or nitrogen flotation, use of filtration aids (Ribéreau-Gayon *et al*., 2006) to render clarity to wine, but at the cost of quality and time, not to mention the extra cost. Thus, using encapsulated yeast can be an option to reduce the amount of lees especially the sediment of yeast after the completion of fermentation and their removal thereafter.

Wine making is a growing sector in Nepal. Though, many small and cottage wineries use different clarifying agents, the clarity of wine produced has remain an upmost technological challenge to achieve clear wine. In such context, better options in wine clarification to achieve quality improvement in wine needs to be explored. This study was conducted with the objective to immobilize wine yeast using a simple gel entrapment method using calcium alginate in a convenient and economical way in order to apply it for wine making. Furthermore, the efficacy of

encapsulated yeast on clarity enhancement and other quality parameters of wine as compared to clarifying agent and free yeast systems were evaluated.

Materials and Methods

Raw material collection

Active dry wine yeast (ADWY: Saccharomyces cerevisiae, Springer oenologie, Belgium), sodium alginate, pectinase enzyme (Lesaffre, Safizym pres, France), Calcium chloride dehydrate (Himedia, Japan), Potassium metabisulphite (Fischer scientific, India), Buffered peptone water and Potato dextrose agar (Himedia, India) and all other required raw materials and ingredients were bought from local market of Kathmandu.

Rehydration of Active dry yeast

1 g of ADWY was rehydrated into 10 ml final volume at 37 ºC for 30 min in accordance with the manufacturer's specifications.

Encapsulation

Calcium alginate capsules was prepared by using a simple one-step process similar to that described by Nigam *et al*. (1988). Sodium alginate was dissolved in warm water (40±5 ºC) to prepare sodium alginate solution of concentration 3.0%. Sodium alginate solution was then mixed with activated yeast suspension using magnetic stirrer to obtain uniform yeast alginate suspension having 2% sodium alginate. Droplets of alginate yeast suspension (5 mL) were then dropped through 22G 1″ sterile hypodermic syringe (Lifeline, Nepal) into 30 ml of (0.1, 0.2, 0.3, 0.4, 0.5 M) $CaCl_2$ solution. The $CaCl_2$ solution was maintained under constant stirring (330 rev/min) using a magnetic stirrer (MH-2L; Vitco laboratory equipment, India). A dropping height of 10 cm was used to ensure the formation of spherical droplets. The gelation time was kept for 30 min and cell leakage efficiency was evaluated for varied concentration of $CaCl_2$ solution. A cross-linking time of 30 min was adopted according to the finding of Bokkhim *et al*. (2016) as longer time led to higher leaching of active components into the cross-linking solution. Finally, the formed beads were rinsed with distilled water to remove excess calcium chloride. All of the above procedures were carried out at room temperature.

Viability of yeast in alginate beads

1 g of calcium alginate beads loaded with microbial cells was mechanically crushed and homogenized with 9 mL of distilled water in a Stomacher Blender (Stomacher 400 circulator; Seward, UK) to obtain complete and homogeneous dispersion of cells. The *Saccharomyces cerevisiae* cell density and viability was calculated by spreading cell dilutions on Yeast Extract-Peptone-Dextrose (YPD) agar medium. The plates were incubated at 28 ºC for 48 h and the colony forming unit (CFU) was counted using a colony counter (Electronic, India).

Cell leakage determination

The cell leakage determination was determined by measuring the cell density in different $CaCl_2$ solution after recovering the beads after 30 min of cross linking (Callone et. al, 2008).

Physicochemical analysis of grape juices and wines

The total titrable acidity was assessed by titration with standardized sodium hydroxide. The pH value was measured using a digital pH meter (HI 2216; Hanna, Romania). Total soluble solid (TSS) was measured as ºBrix (ºBx) using hand refractometer (DR 201-95; Kruss, Germany).

Wine making

Red wine was prepared by following standard procedure as mentioned by Sacchi *et al*. (2005). Cleaned red grapes were de-stemmed, crushed and sulfited at the rate of 75 ppm.

TSS of must was adjusted to 25 ºBx by adding sugar. Also pectolytic enzyme was added at the rate of 0.01g/kg. Must was then pitched with yeast at the rate of 0.3 g/L free cell and left for fermentation at room temperature till residual sugar decreased to a constant value. After completion of primary fermentation, the clear wine was siphoned away from lees and kept for a week to settle further which was afterward racked, bottled and aged.

Likewise, white wine was prepared as mentioned by Pacock *et al*. (2011). Grapes were cleaned and juiced by using juicer. The juice was sulphited at the rate of 75 ppm TSS maintained at 25 ºBx and then pasteurized at 72 ºC for 1 min. The settled juice was separated by drawing off and pectolytic enzyme was added at the rate of 0.01 g/kg. From here forth, pitching and fermentation until aging was done similar as in red wine making mentioned above.

TSS, residual sugar and alcohol content were determined each day and fermentation kinetics was studied for free yeast (FY) and encapsulated yeast (EY).

Analysis of wine

TSS and acidity was determined every 2 days and fermentation kinetics was studied for FY and EY. TSS was determined by a hand refractometer and acidity by titration method as per Ranganna (2003). Ethanol content was determined by specific gravity method and free SO_2 was determined as per AOAC Official Method 990.28. Color was measured using hand held Chroma Meter CR-400 (Konica Minolta, Japan) using the color space parameters (L*, a*, b*, C & hº) values developed by Commission International de l'Eclairage (CIELab). Turbidity of the prepared wines were assessed with a turbidity meter (HI 88703; Hanna, Romania) and is expressed as Nephelometric turbidity units (NTU).

Statistical analysis

For experiments conducted in triplicates, values are presented as mean ± SD and the significance of differences between the values was assessed by analysis of variance (ANOVA single factor) at 95% confidence level using Excel 2013. For other experiments, the number is indicated by 'n'.

Results and Discussion

Optimization of molar concentration of calcium chloride

Activated yeast suspension (1 g/10 mL) was mixed with 3% sodium alginate solution to achieve a final mixture of 2% sodium alginate and cross linked with different molar concentration of $CaCl_2$ solution for 30 minutes. The encapsulated yeast was recovered and the left over $CaCl_2$ solution was incubated at 28 ºC for 48 h and the CFU was counted. The result obtained is shown in figure 1. The result shows that highest leaked cell density was observed in 0.5 M concentration of $CaCl_2$ solution which was found to be 78 CFU/mL $CaCl_2$ solutions. Minimal leakage was found at 0.2

M concentrations of $CaCl_2$ solutions which was only 16 CFU/mL $CaCl_2$ solution. Thus, 0.2 M of $CaCl_2$ was used for cross linking the sodium alginate beads.

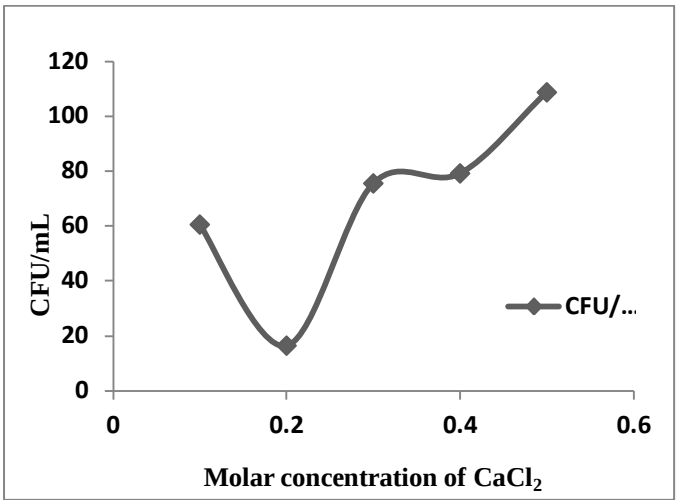

Fig 1: Number of cell leakage in different molar concentration solution

Lotfipour *et al*., (2012) had also observed that when concentrations of $CaCl_2$ were varied in the range 1.3–5.5% w/v and the concentration of the sodium alginate solution was fixed at 1.0% w/v, the use of concentrated $CaCl_2$ solutions significantly reduced the percentage of *Lactobacillus acidophilus* that diffused out of the capsules. Similarly, the result of this study is also in accordance with Hariyadi *et al*., (2014) findings who reported that $CaCl_2$ concentration of 0.1M did not form microsphere but form irregular shaped gelling sheets when forming ovalbumin loaded alginate microspheres using aerolisation techniques.

Cell count of encapsulated yeast and free yeast.

Table 1: Total colony count of free and encapsulated yeast

S.N	Yeast type	CFU/g
1	Free yeast	3.3×10^9
2	Encapsulated yeast	4.9×10^8

Viable cell count of active dry yeast and encapsulated yeast was done by YPD agar medium and result obtained is shown in table 1. Live yeast cells were more than 10 fold less in per gram beads as compared to free yeast. Also, according to Shi *et al*., (2013) the cell loading on encapsulated yeast can be affected by various factors such as nozzle size, polymer concentration, hardening time in calcium chloride, initial cell concentration. So, CFU/g beads was found and equilibrated with free yeast cell count so that similar yeast concentration could be used for wine preparation with FY and EY. In our study, 1 g. active dry yeast was equal to 6.65 g. beads of EY in terms of live yeast cell count.

Physiochemical properties of white and red grapes

Table 2: TSS, acidity and pH of white and red grapes

Parameters	White grapes	Red Grapes
TSS	16.5 °Bx	19.1 °Bx
Acidity (% Tartaric acid)	0.40%	0.64%
pH	3.9	3.2

TSS, acidity and pH of red and white grapes used for wine making in this study was analyzed and results obtained are tabulated in table 2. Red grapes used were of high TSS and acidity as compared to white grapes used in this study. According to Joshi *et al* (2013) physicochemical properties of wine vary according to the variety and environmental conditions of the region in which the grapes are grown. Higher TSS of grape juice is associated with higher alcohol content of wine and acidity of wine juice influences the taste and flavor of thus formed wine. The wine with a pH of 3.2 will have bright fruit flavors, but it will also be thin, acidic and aggressive on the palate. On the other hand, the wine at 4.0 will be softer and rounder than the wine at 3.2, but also less vibrant. Also, for red wine fermentation ideal acidity is 0.6 - 0.7% and ideal pH is 3.4 to 3.7 and TSS is 22 to 25 °Bx. Likewise, he mentioned for white wine fermentation, optimum acidity of must is 0.6 to 0.9, pH is 3.2 to 3.5 and TSS is 17-24%. In our study, we maintained the TSS of must to 25 °Bx for both red wine and white wine by adding sugar. However, acidity was not maintained though red wine must was in optimum level as mentioned but white wine must wasn't adjusted which is limiting in this study.

TSS profile of red wine and white wine prepared with FY and EY

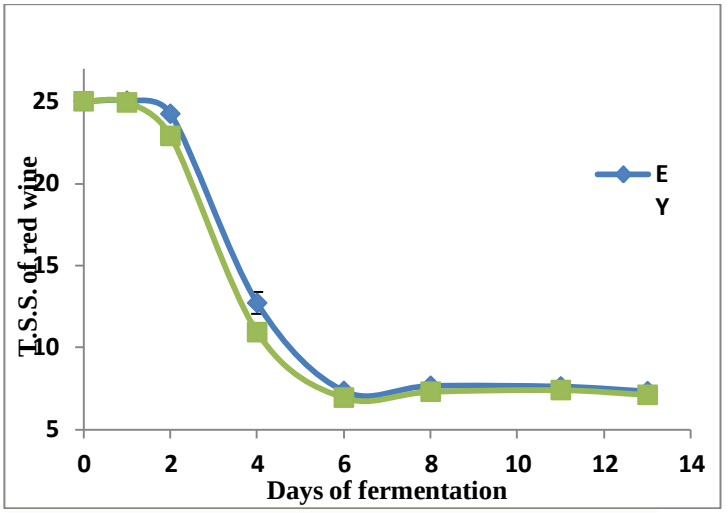

Figure 2: TSS profile of red wine for free (FY) and encapsulated yeast (EY)

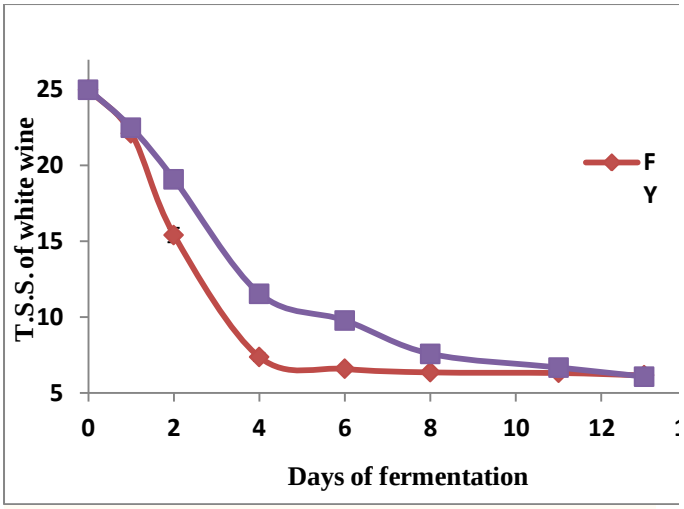

Figure 3: TSS profile of white wine for free (FY) and encapsulated yeast (EY)

TSS profile of red and white wine produced by using FY and EY was observed for 14 days of fermentation (Figure 2 & 3). It is evident from the figures that TSS profile of red and white wines produced by FY and EY were quiet similar. TSS decreased steeply for a week and then leveled off thereafter. A constant TSS of approx. 6 and 7 ^{o}Bx for white and red wine were achieved after 12 days of fermentation. The rate of decrease in TSS was slightly faster for FY compared to EY in the first week of white wine fermentation. But this did not affect the efficiency of fermentation process as both FY and EY achieved the same final TSS.

Acidity profile of red wine and prepared from FY and EY

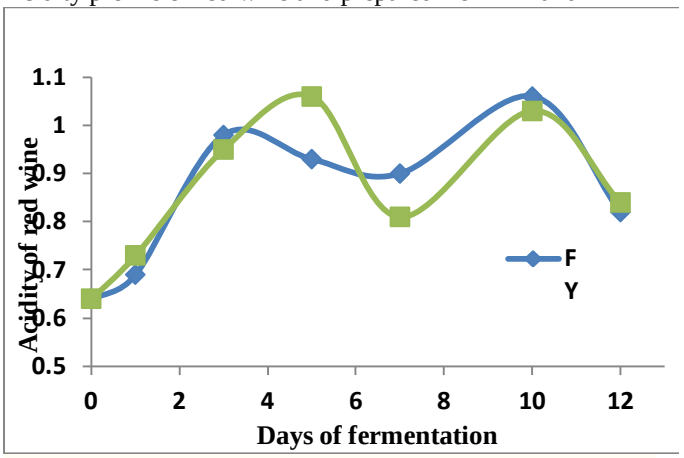

Figure 4: Acidity profile of red wine for free and encapsulated yeast

The total acidity profile of red wine prepared from free and encapsulated yeast was measured for 14 days and result obtained is shown in the figure 4. The pH affects flavor, aroma, color, tartrate precipitation, carbon dioxide absorption, malolactic fermentation, stability, ageablity, and fermentation rate. The acidity increased initially, decreased midway, increased thereafter before finally getting stabilized at final acidity of 0.8% as tartaric acid. The result is in accordance with the finding of Joshi *et al*. (2013) who reported that in wine making there is initial increase in acidity. However, when alcohol is produced the acidity start to decrease.

Alcohol content of red and white wine with FY and EY

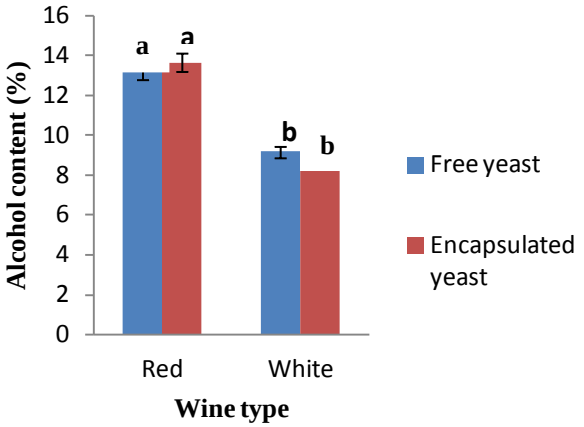

Figure 5: Alcohol content of red and white wine with FY and EY. The columns sharing the same alphabet are not significantly different (p>0.05).

Alcohol content of white wine and red wine produced by FY and EY was determined and the result obtained is shown in figure 5. It was observed that wine produced by FY and EY wasn't significantly different for both red wine and white wine. Fumi *et al.* (1988) also noted no difference in the alcohol content of sparkling wines prepared by free and immobilized yeast. However, yeast in both conditions shows higher fermentability in red wine compared to white wine for similar production conditions. This could be due to the difference in composition of juice used for wine making. According to Sacchi et.al (2005), the nitrogen content of juice greatly influences the fermentation characteristics of yeast.

Clarity and color of wine

The visual clarity can be measured by turbidity level, which is a measure of particulate levels in wine and usually used as an indication for its readiness for bottling. Significantly different levels of turbidity were found for white and red wines from FY and EY (Table 3). Further filtration of wines from EY through a membrane filter (0.45 µm) lead to reduction of turbidity level, especially of red wine. This showed EY has an advantage over FY on wine clarity, though filtration is still necessary to reduce the turbidity to acceptable level prior to bottling. An NTU ≤ 1 is preferred for wines (Bowyer *et al*., 2012).

Table 3. Comparative chart of turbidity levels (NTU) of different wines

Samples	Turbidity Level (NTU)		
	Free Yeast	Encapsulated Yeast	EY after filtration (0.45µm)
White wine	170	140	33.0 ± 2.65
Red wine	340	95	1.6 ± 0.10

Clarity of wines can also be expressed by the CIELab or chromaticity coordinates, where L* denotes clarity, which is directly related to the visual sensation of luminosity (Resolution Oeno 1/2006). Clarity of wine was strongly affected by the types of yeast forms used in both red and white wines as the values of L* were significantly higher (p < 0.05) for EY compared to FY. Furthermore, color in wine being the visual indicator for its acceptability, can also be

measured by other chromaticity coordinates a* (Redness), b* (Yellowness), C* (Chroma) and h° (Hue), which were also significantly higher for EY red wines. This indicated the deepening of the red color and shifting from yellow towards red color. Whereas in white wine, though a*, b* and C* significantly increased, h° decreased indicating the shifting of redness towards yellowness (Figure 7A & 7B).

Chromaticity coordinates for Red wines (L) & White wines (R) prepared from FY & EY

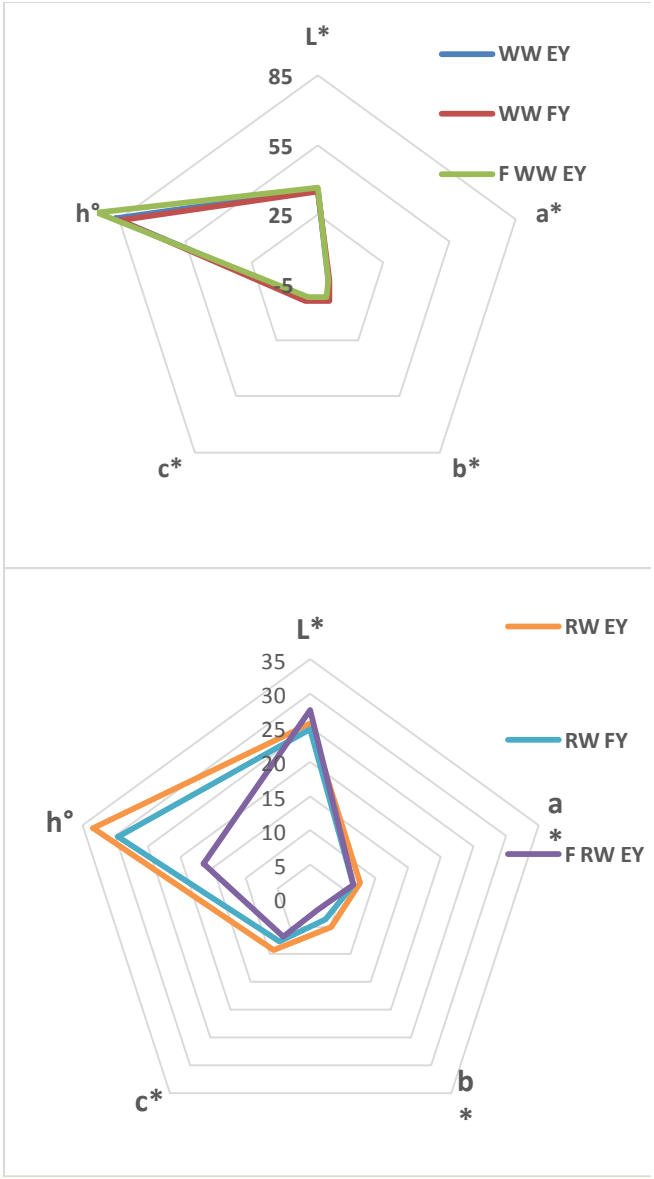

Figure 7. Chromaticity coordinates of red (7A. Left) and white (7B. White) wines from FY and EY

Conclusions

From this study it was found that wine yeast *Saccharomyces cerevisiae* could be encapsulated effectively in 2% sodium alginate solution and crosslinking with 0.2 M $CaCl_2$ solution for 30 minutes. There was no significant difference in TSS and acidity profile of wine with the use of EY as compared to FY within the same type of wine. Also, EY was equally efficient in alcoholic fermentation in wine. Furthermore, wines with higher clarity were obtained with the use of EY. In conclusion, encapsulating wine yeast in calcium alginate can offer better wine quality in terms of clarity without compromising other quality factors of wine.

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