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Inoculum Size and Age Studies on Single and Mixed Strain Fermentation of Grape Juice

Sandeep Kaur[1,2]*, Karamjot Kaur[2], Bharat Bhushan[2] Manveer Kaur[2] and Monika Hans[3]

[1]Department of Agriculture, Maharishi Markandeshwar University, Sadopur, Ambala - 134 007, India.
[2]Department of Biotechnology, Sri Guru Granth Sahib World University, Fatehgarh Sahib - 147 407, India
[3]Department of Food Science and Technology, Govt. P.G College for Women, Gandhi Nagar, Jammu - 180 004, India.

Abstract

Single and mixed strain fermentation were compared to check the effect on properties of wine. Two strains of Saccharomyces cerevisiae (MTCC 11815 & MTCC 170) were used to study the effect of inoculum age and inoculum size on fermentation of grape juice. The inoculum sizes used were 2%, 5%, 10% and 15%, while inoculum age effect was studied using 24 h, 48 h and 60 h old inoculum. Fermentation efficiency of 77.2% was achieved in mixed strain culture using 15% inoculum, 17% initial sugars giving ethanol concentration of 6.70% (w/v) after 48 hrs. Fermentation efficiency of 84.65% was achieved with MTCC170 using 15% inoculum and 17% initial sugars giving ethanol concentration of 7.34% (w/v) in 48 hrs. Strain MTCC11815 produced 8.5% (w/v) ethanol from 17% initial sugars giving 98% efficiency using 2 and 5% inoculum. Concentration of phenolics increased with inoculum concentration while nitrogen and phosphates did not show any regular trend. The nitrogen and phosphate concentration was affected by type of strain rather than other factors.

Keywords: Mixed strain fermentation, phenolics, inoculum age, inoculum size, Saccharomyces cerevisiae

***Correspondence:** ksandeep579@gmail.com; +91 9872193709

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INTRODUCTION

Wine making is one of the oldest techniques known to the civilization and even today it is one of the most commercially prosperous biotechnological processes (Moreno-Arribas and Polo, 2005). Microorganisms have a prominent role in determining the chemical composition of wine as they metabolize fruit sugars and other components into ethanol, carbon dioxide and hundreds of secondary end-products that collectively contribute to the wine character (Lambrechts and Pretorius*;* 2000).

The commercial alcoholic fermentation is normally batch type and requires preparation of fresh inoculum for every new batch as a result of which an initial lag phase is observed during fermentation . This lag may be reduced by recycling yeast from previous fermentation lots (Puri et al., 2012). Recycling of yeast inoculum has been considered as an important parameter in lowering wine production costs with improved fermentation performance (Krasucki, 2011). Inoculum size and age have always been critical in deciding fermentation yields. Low inoculum concentration adversely effects the mechanism of fermentation process, thus causing much lower yield and rate of product formation, while high cell concentration causes increased activities of organisms which end up utilizing the fermentation end results for their growth and in the process limit the yield and rate of formation of fermentation products as well (Lee et al, 2008).

Length of lag phase is affected by size of inoculum and its physiological conditions and its always preferred to transfer inoculum in log phase of growth, when the cells are still metabolically active (Lincoln, 1960). Age of inoculum is considered important in case of sporulating bacteria, as sporulation occurs at the end of log phase (Lincoln, 1960; Ray et al., 2007).

Phenolic compounds are one of the major quality factors in wine grapes and in the resulting wines. In addition, phenolic compounds have a direct effect on some important organoleptic characteristics of wines, such as color, flavor, bitterness, and astringency (Garrido & Borges, 2011). These compounds are present in grape skin, flesh and seed and are known to posses natural antioxidant and health protective properties.

According to Styburski et al. (2018) phosphorous content of the wort has strong influence on the quality, colour and taste of the beer. Nitrogenous compounds are known to effect fermentation process, final chemical composition of the wine and its aroma (tools.thermofischer.com).

Mixed strain fermentation have been explored since some time as to know about the synergistic effect of the varied cultures on the volatile and sensory properties of the wine.It has been confirmed that the volatile profiles created by mixed fermentation cannot be created by individual fermentations (Howell et al, 2006). But the interest is mainly on studying the effect of *Saccharomyces* and non- *Saccharomyces* species combination, in which many a times *Saccharomyces* is known to have antagonistic effect on the non- *Saccharomyces* species as toxic compounds produced by *Saccharomyces* starter culture are known to kill the non- *Saccharomyces* species added late in fermenting wort (Perez-Nevado et al., 2006).

During our literature search we did not come across mixed strain fermentation involving *Saccharomyces* species only. The current study was under taken with objective of observing effect of mixed *Saccharomyces* species fermentation and inoculum age and size on pure and mixed strain fermentation.

MATERIALS AND METHODS

Chemicals

All the chemicals and reagents used in the present study were of analytical grade and procured from Bio-Red, Himedia.

Procurement and maintainance of micro-organism strains

Yeast strain of *Saccharomyces cerevisiae* MTCC 11815 procured from P.A.U, Ludhiana and MTCC 170 procured from IMTECH, Chandigarh were used for the present investigations. and were maintained on Glucose yeast extract (GYE) agar slants.

Procurement of Raw Material

The fully ripened grapes was collected in the month of February from Khanna, Punjab and sorted by shape and ripeness. Then collected grapes was washed with water and mixed with a blender. The juice along with skins was stored at 4^{o}C for further use. The fruit juice thus obtained

was analyzed for pH, total soluble solids (°B), total sugars and reducing sugars after filtration through double muslin cloth.

Preparation of Must

The must was pasteurized by heating at 60°C for 30 min to inactivate wild microorganisms. The initial total soluble solids (TSS) of the wort was adjusted to 17°B. Diammonium hydrogen phosphate (0.1%, w/v) as nitrogen source for yeast was added. To inhibit the growth of undesirable microbes, potassium metabisulphite (100 ppm) was also added.

Production of wine

The must was inoculated with 10% (v/v) of 24 h old culture (unless and otherwise specified) of *Saccharomyces cerevisiae* MTCC 170 and 11815. Fermentation was carried out at 30°C till the readings were constant under stationary conditions.

Optimization of inoculum size

To investigate the effect of inoculum size on fermentation of wine, the must was inoculated with 2%, 5%, 10% and 15% inoculum containing 1 × 10^5 cells/ml.

To study the effect of age of inoculum

To investigate the effect of age of inoculum on wine, the fermentation was carried out with 24 hr,48 hr and 60 hr old inoculum.

Conditions for fermentation

- Sugar conc: as per brix of juice
- pH : 5.5
- Temperature: 30°C

All experiments were performed in triplicates and readings given are the mean of triplicate readings.

Analytical Techniques

Total soluble solids (TSS) in wine were determined by using Erma hand refractometer of 0-32°Brix.. The pH of the samples was determined by using digital pH meter. Reducing sugars of fruit juice/wine were estimated by 3, 5-dinitrosalicylic acid (DNSA) method (Miller *et al;* 1959) and ethanol content was estimated after distillation by method described by Caputi *et al;* (1968). Folin & Ciocalteu's phenol assay was used for determination of phenolic content in grape wine. Nitrogenous compounds analysis was done using Formol titration technique. Ascorbic acid method was used for determination of phosphate content in wine.

Cell count was determined by hemocytometer. Statistical analysis was performed by using the software origin 6.0

Calculations

The ethanol concentration was determined by the ethanol standard curve.

The other calculations were made as follows:-

1. Sugar utilized = Initial sugar conc. (%) – residual sugar conc. (%).
2. Ethanol fermentation efficiency (%) = actual ethanol produced % (w/v)×100/ theoretical ethanol produced
3. Theoretical ethanol production %(w/v) = sugar utilized × 0.51

RESULTS AND DISCUSSION

Effect of inoculum size

Four inoculum sizes viz. 2%,5%,10% and 15% were used to study the effect of inoculum size on ethanol content and other components of grape wine.

From 2% inoculum 8.5, 3.71 and 4.34 % (w/v) ethanol concentration was obtained from *S.cerevisiae* MTCC 11815 (Table 1), *S.cerevisiae* MTCC 170 (Table 2) and mixed strain (Table 3) respectively. While the same ethanol concentration was achieved with 5% inoculum using *S.cerevisiae* MTCC 11815 strain, 4.35 and 5.42% (w/v) and 8.07, 6.53 and 5.72 % (w/v) ethanol concentrations were obtained with 5% and 10% inoculum respectively. Use of 15% inoculum gave final ethanol concentration of 7.01, 7.34 and 6.7% (w/v) for *S.cerevisiae* MTCC 11815, *S.cerevisiae* MTCC 170 and mixed strain fermentation respectively. It was observed that fermentation efficiency was maximum (98%) for *S.cerevisiae* MTCC 11815 with 2 and 5% inoculum size and it decreased to 93% and 80% as the inoculum size increased to 10 and 15% respectively (Table 1). For *S.cerevisiae* MTCC 170, fermentation efficiency increased from 42.8% to 84.6% (Table 2) as the inoculum size increased from 2 to 15%. Similarly, fermentation efficiency increased from 50 to 77.2% for 2 and 15% inoculum size respectively in mixed strain fermentation. Although it still remained less than single strain fermentation. Duhan et al. (2013) observed that fermentation efficiency of *S.cerevisiae* MTCC 170 increased as inoculum size increased from 5 to 15% but maximum efficiency (91.39%) was achieved with 10% inoculum. According to Breisha (2010)

Table 1. Effect of inoculum size on ethanol production by *S.cerevisiae* MTCC 11815

Inoculum size	Initial sugars (°B)	Residual Reducing sugars (%)	Ethanol %(w/v)	Fermen-tation Efficiency (%)	Cell count (cells/ml)		pH	Productivity (ethanol produced-mg/ml/h)	Phenolics (mg/ml)		Nitrogen content (mg/ml)		Phosphate content (mg/L)	
					Initial	Final	Final		Initial	Final	Initial	Final	Initial	Final
2%	17±0.93	2.49±0.97	8.5±0.07	98	8×10^5	8.2×10^7	3.9 ±0.20	1.88	0.283 ±0.18	4.71 ±0.92	192.5± 2.48	385± 1.8	0.601 ±0.44	3.03 ±0.12
5%	17±0.90	2.23±0.71	8.5±0.076	98	8×10^5	4.9×10^6	3.5 ±0.28	1.88	0.283 ±0.18	4.61 0.36↓	175 ±1.5	380 ±.95	0.559 ±0.44	1.87 ±0.14↓
10%	17±0.90	2.3 ±0.80	8.07 ±0.077	93↓	8×10^5	4.3×10^6	3.5 ±0.35	1.68	0.280 ±0.35	4.57 ±0.87↓	192.5 ± 1.2	380± 2.01	0.559 ±0.44	2.56 ±0.26↑
15%	17 ±0.90	3.34 ±0.81	7.01 ±0.076	80↓	8×10^5	3.7×10^8	3.9 ±0.281	1.46	0.283 ±0.18	3.39 ±0.80↓	175± 1.5	367±1.5	0.559 ±0.44	4.09 ±0.31↑

Temperature: 30°C pH(initial): 5.5
*The readings are mean of triplicates.

Table 2: Effect of inoculum size on ethanol production by S. cerevisiae MTCC 170

Inoculum size	Initial sugars (°B)	Residual Reducing sugars (%)	Ethanol %(w/v)	Fermentation Efficiency (%)	Cell count (cells/ml)		pH	Productivity (ethanol produced-mg/ml/h)	Phenolics (mg/ml)		Nitrogen content (mg/ml)		Phosphate content (mg/L)	
					Initial	Final	Final		Initial	Final	Initial	Final	Initial	Final
2%	17.0± 0.97	2.77 ± 3.23	3.71± 0.74	42.8	5.8× 105	4.8x106	3.74± 0.04	0.77	0.293± 0.02	0.355± 0.12	210 ±2.0	414± 1.5	0.498	2.73
5%	17.0± 0.97	2.01± 2.8	4.35± 0.87	50.17↑	5.8× 105	1.7x106	3.78± 0.103	0.91	0.296± 0.02	0.395 ± 0.07↑	210± 3.48	367 ± 3.48↓	0.501	1.22↓
10%	17.0± 0.97	1.19± 2.17	6.53± 1.78	75.31↑	5.8× 105	2.1x106	3.76± 0.14	1.32	0.296± 0.02	0.508 ± 0.04↑	210± 2.19	352± 3.57↓	0.498	.378↓
15%	17.0± 0.97	1.31± 1.82	7.34± 3.03	84.65↑	5.8× 105	4.6x106	3.43± 0.17	1.73	0.296± 0.02	0.569± 0.063↑	210± 3.03	352± 3.3↓	0.501	1.11↑

Temperature: 30°C, pH(initial): 5.5
*The readings are mean of triplicates

Table 3. Effect of inoculum size on ethanol production by *S.cerevisiae* MTCC 11815 & *S. cerevisiae* MTCC 170

Inoculum size	Initial sugars (°B)	Residual Reducing sugars (%)	Ethanol %(w/v)	Fermentation Efficiency (%)	Cell count (cells/ml)		pH	Productivity (ethanol produced-mg/ml/h)	Phenolics (mg/ml)		Nitrogen content (mg/ml)		Phosphate content (mg/L)	
					Initial	Final	Final		Initial	Final	Initial	Final	Initial	Final
2%	17.0 ±1.1	2.9±0.7	4.34 ±0.4	50	1 x 10^3+ 1 x 10^3	5.4 x 10^6	3.6 ±0.04	0.90	0.265 ±1.0	0.901 ±0.3	178± 0.5	335 ±3.8	0.576	4.58
5%	17.0 ±1.1	2.7 ±0.7	5.42 ±0 .38	62↑	1 x 10^3+ 1 x 10^3	8.8 x 10^5	3.35 ±0.08	1.13	0.265 ±0.99	1.98 ±0.39↑	178 ± 0.5	396 ±2.5 ↑	0.576	3.98↓
10%	17.0 ±1.13	1.51 ±0.9	5.72 ±1.9	65↑	1 x 10^3+ 1 x 10^3	1.7 × 10^6	3.86 ±0.02	1.18	0.269 ±1.0	2.00 ±0.35↑	178 ± 0.5	385 ±1.9↓	0.573	3.81↓
15%	17.0 ±1.11	1.2±1.0	6.70 ±0.6	77.2↑	1 x 10^3+ 1 x 10^3	2.1×10^6	3.62 ±0.05	1.39	0.265 ±1.0	6.55 ±0.76↑	178 ± 0.5	305 ± 2.5↓	0.576	3.27↓

Temperature: 30°C, pH(initial): 5.5
*The readings are mean of triplicates.

Table 4. Effect of inoculum age on ethanol production by *S. cerevisiae* MTCC 11815

Inoculum size	Initial sugars (°B)	Residual Reducing sugars (%)	Ethanol %(w/v)	Fermentation Efficiency (%)	Cell count (cells/ml)		pH	Productivity (ethanol produced-mg/ml/h)	Phenolics (mg/ml)		Nitrogen content (mg/ml)		Phosphate content (mg/L)	
					Initial	Final	Final		Initial	Final	Initial	Final	Initial	Final
24	17±1.43	1.6 ±0.26	8.35 ±0.004	96.3	1.5×105	4.7×107	3.7 ±0.15	1.73	0.397±0.53	4.17 ±.87	175 ± 2.0	385± 4.2	0.345 0.08	5.75 ±0.006
48	17 ±1.43	1.19 ±0.13	7.19 ±0.068	83↓	1.3×106	2.8×106	3.78 ±0.22	1.49	0.397 ±0.53	3.43 ±0.33 ↓	173± 1.5	367± 1.5↓	0.345 0.08	1.40 ±0.014↓
60	17 ±1.43	2.98 ±0.48	5.79 ±1.24	66↓	1.4×105	3.6×106	3.5 ±0.09	1.2	0.397 ±0.53	3.12 ±0.25↓	175 ± 2.0	302 ± 5.0↓	0.345 0.08	1.28 ±0.018 ↓

Temperature: 30°C, pH(initial): 5.5, Inoculum : 10%
*The readings are mean of triplicates.

Table 5. Effect of inoculum age on ethanol production by *S.cerevisiae* MTCC 170

Inoculum size	Initial sugars (°B)	Residual Reducing sugars (%)	Ethanol %(w/v)	Fermentation Efficiency (%)	Cell count (cells/ml)		pH	Productivity (ethanol produced-mg/ml/h)	Phenolics (mg/ml)		Nitrogen content (mg/ml)		Phosphate content (mg/L)	
					Initial	Final	Final		Initial	Final	Initial	Final	Initial	Final
24	17.3 ± 0.82	2.62 ± 3.17	4.4 ± 1.83	50.74	4.3x 105	1.3*106	3.84± 0.073	0.92	0.354 ± 0.057	1.20 ± 0.48	210 ± 2.5	402 ± 3.0	0.351 ± 0.11	0.439 ± 0.03
48	17.0 ±0.81	1.73 ± 3.7	3.56 ±1.83	41↓	6.6x 105	3.2*106	3.71± 0.11	0.74	0.354 ± 0.057	0.651± 0.034↓	210 ± 2.5	398 ± 6.4↓	0.351± 0.11	2.29 ± 0.11↑
60	17.0 ±0.81	1.55 ± 3.60	2.7± 1.6	31↓	7.5x 103	1.4*104	3.52± 0.09	0.56	0.354 ± 0.057	0.576 ± 7.08↓	210 ± 2.5	382 ± 5.5↓	0.351± 0.11	0.779 0.49↓

Temperature: 30°C, pH(initial): 5.5, Inoculum : 10%
*The readings are mean of triplicates.

Table 6. Effect of inoculum age on ethanol production by S.cerevisiae MTCC 11815 & S. cerevisiae MTCC 170

Inoculum size	Initial sugars (°B)	Residual Reducing sugars (%)	Ethanol % (w/v)	Fermentation Efficiency (%)	Cell count (cells/ml)		pH	Productivity (ethanol produced-mg/ml/h)	Phenolics (mg/ml)		Nitrogen content (mg/ml)		Phosphate content (mg/L)	
					Initial	Final	Final		Initial	Final	Initial	Final	Initial	Final
24	17.8 ±1.47	3.61 ±1.0	5.33 ±0.6	61	1.1 ×105+ 1.1 ×105	9.6 ×105	3.53±0.1	1.11	0.378 ± 0.21	0.855 ±0.7	162 ± 0.9	359 ±1.5	2.29 ±1.18	1.44 ±0.45
48	18 ±1.12	3.9 ±0.9	5.02 ±0.5	57↓	1.2×105+ 1.2×105	3.0× 105	3.62 ±0.34	1.04	0.380 ± 0.21	0.968 ±0.22↑	162 ± 0.9	354 ± 2.5↓	4.29 ±1.18	1.54 ±0.28↑
60	18.0 ±1.12	4.76 ±0.9	4.69 ±0.9	54↓	1.1 x 105+ 1.1 x 105	2.7 x 105	3.12 ±0.09	0.97	0.378 ± 0.21	1.12 ±0.3↑	162 ± 1.0	347 ± 1.0↓	4.18 ±1.18	1.48 ±0.3↓

Temperature: 30°C, pH(initial): 5.5, Inoculum : 10%
*The readings are mean of triplicates.

increase in inoculum concentration from 3 to 6% reduced the fermentation time by 6%. Kaur et al. (2007) obtained 7% ethanol concentration from 15% initial sugars (substrate- malt) using 6% inoculum of *S.cerevisiae* MTCC 11815. Kaur et al (2019) obtained 8.84% ethanol (substrate rice) using 10% inoculum *S.cerevisiae* MTCC 11815 giving fermentation efficiency of 86.6%.

In fermentation with *S.cerevisiae* MTCC 11815 (Table 1), a drop (4.71 to 3.39 mg/ml) in the concentration of phenolics was observed while the phosphate content increased (3.03 to 4.09 mg/l) as the inoculum size increased from 2 to 15%. No constant trend was observed in nitrogen content. For *S.cerevisiae* MTCC 170 (Table 2), the phenolics concentration increased from 0.355 to 0.569 mg/ml with incoculum size. Nitrogen content decreased from 414 mg/ml to 352 mg/ml with increase in inoculum concentration and the phosphate content decreased from 2.73 to 1.11 mg/l.

Phenolics content continued to increase with increase in inoculum size in mixed strain fermentation (Table 3) also as it increased from 0.901 mg/ml (2% inoculum) to 6.55 mg/ml (15% inoculum). Both nitrogen (335 to 305 mg/ml) and phosphate (4.58 to 3.27 mg/l) content decreased with increase in inoculum concentration with mixed strain fermentation.

Effect of inoculum age

To study the effect of inoculum age on alcohol concentration three age parameters viz. 24 h, 48 h and 60h were chosen and 10% inoculum size was used.

As the age of inoculum increased from 24 to 60 h, the concentration of ethanol produced decreased and so did the concentration of different chemicals produced when *S.cerevisiae* MTCC 11815 (Table 4) was used for fermentation. From initial sugar content of 17°Brix, ehanol concentration of 8.35% (w/v) was obtained with 96.3% fermentation efficiency using 24 h old inoculum, which decreased to 7.19% (w/v) ethanol concentration giving 83% fermentation efficiency with 48 h old inoculum and 5.79% (w/v) ethanol was produced with 66% fermentation efficiency with 60 h old inoculum.

The concentration of phenolics and nitrogen and phosphates decreased from 4.17 to 3.12 mg/ml, 385 to 302 mg/ml and 5.75 to 1.28 mg/l respectively as the age of inoculum increased from 24 h to 60 h.

Similar results were obtained when *S.cerevisiae* MTCC 170 (Table 5) was used as fermenting strain. Ethanol concentration decreased from 4.4% (w/v) to 2.7% (w/v) as the age of inoculum increased from 24 to 60h. The corresponding fermentation efficiency for 24, 48 and 60 h old inoculum were 50.74, 41 and 31% respectively. The phenolics content was 1.2 mg/ml after using 24 h inoculum which decreased to 0.576 mg/ml with 60 h old inoculum. Similarly nitrogen content decreased from 402 mg/ml to 382 mg/ml. Phosphate concentration increased as age of inoculum increased from 24 to 48 h but decreased with further increase in inoculum age.

In mixed strain fermentation (Table 6), final ethanol concentration of 5.33% (w/v) giving 61% fermentation efficiency was obtained using 24 h old inoculum, which decreased to 4.69 % (w/v) with 60h old inoculum. The ethanol concentration obtained with mixed strain was better than results obtained from *S.cerevisiae* MTCC 170 single strain fermentation. Against the trend of decreasing compound concentration with increase in inoculum age, the phenolics concentration increased from 0.855 to 1.12 mg/ml with increase in inoculum age from 24 to 60 h respectively. The nitrogen content continued to decrease with increase in inoculum age, as it fell from 359 to 347 mg/ml for 24 and 60 h inoculum respectively. The concentration of phosphates increased with use of 48 h old inoculum but decreased with further increase in age. It was also observed that amount of reducing sugars were left unused during mixed strain fermentation as residual sugar content was comparatively higher the amount left in single strain fermentations. Nogueira et al. (2008) reported that nitrogen content in wine varied with cider variety and mainly depended upon the initial concentration of nitrogen present in the wort. Nogueira et al. (2008) also observed that concentration of different polyphenols varied with apple variety and increase or decrease in concentration of polyphenols depended upon factors like method of juice extraction, oxidation during extraction and interaction of the yeast cell wall with different varieties of polyphenols during fermentation. Zou et al (2017) observed that concentration of phenolic compounds increased

with alcoholic fermentation and decreased with acetic acid fermentation. Samoticha et al. (2019) reported decrease in concentration of phenolic compounds with increase in ethanol content.

Yeast inoculum size has a significant effect for ethanol production (Turhan *et al.,* 2010). Gibbons and Weastby (1986) reported that a 5% inoculum resulted in rapid yeast and ethanol production and higher inoculum showed no advantages. Tahira *et al.* (2010) using a different inoculum at 1-5% observed that the amount of ethanol produced gradually increased with the increase in the inoculum. Inoculum size for microbial growth which prevent growth vary with inoculum size.

The age of inoculum certainly had a detrimental effect on the final ethanol yield as ethanol produced decreased with increase in inoculum age. High inoculum size can also be the reason for decrease in fermentation efficiency as more substrate is utilized for maintaining high population of fermenting microorganism. Pramanik et al, (2003) observed that the maximum (9.1%) ethanol was produced by using 15 days old inoculum . Further increase and decrease in inoculum age resulted in decreased ethanol yield. At particular cell density, growth phase of yeast cells is slow and life cycle deviates from the growth path. Manikandan et al (2010) found that 24 h old slant of *S.cereviasae* gave higher yield of ethanol compared to 48 h old slant.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR'S CONTRIBUTION

The findings presented in this paper are Master's Research work of KK, BB and MK under guidance of Dr. SK. KK worked with *Sachharomyces cerevisiae* MTCC 1185 strain and MK worked with *S. cerevisiae* MTCC 170 strain. BB worked on mixed strain fermentation. The findings of all three have been combined in this research article. Dr. MH was minor guide to the scholars and helped in experiment performance.

The research work was performed at Sri Guru Granth Sahib World University, Fatehgarh Sahib, Punjab, India. Corresponding author and Major Guide of the scholars is currently employed at Maharishi Markandeshwar University, Sadopur, Ambala, India. Two of the scholars KK and BB are employed with Paraxel, Mohali,India while MK is preparing for competitive exams. Dr. MH is presently working at Jammu, India.

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ETHICS STATEMENT

Not applicable.

DATA AVAILABILITY

This report is compilation of thesis of three students and results submitted are summary of results obtained. The detailed data can be provided if required.

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