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Hansunela polymorpha: A novel yeast strain for low-alcoholic beer production

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Abstract
The use of non-conventional yeast strains in brewing can produce beer with improved flavour and other organoleptic properties. These particular yeast strains are also capable of producing beer with reduced alcohol content. This study provides a detailed examination of using *Hansunela polymorpha* in the fermentation of producing low-alcoholic beer. The obtained beer contains an alcoholic level below 1%, which indicates that beer is low alcoholic. The real extract and apparent degree of fermentation of produced low-alcoholic beer are 10.73% w/w and 11.89% and comparable to the commercial low-alcoholic beer produced by *Saccharomycodes ludwigii* (10.77% w/w and 12.47%). The colour and FAN content of the HP beer is also equal to the SL beer. The HP beer's total phenolic and flavonoid contents are 171.34 GAE/L and 17.63 GAE/L of beer. The H. polymorpha yeast strain can be used to produce low-alcoholic beer with enhanced quality attributes that increase the consumer acceptability of low-alcoholic beer.

Keywords: *Hansunela polymorpha*, low alcoholic beer, phenolic content, flavanoids content, free amino nitrogen (FAN)

1. Introduction
Although non-alcohol beer (NAB) is not a common output of the brewing industry, there is an increase in demand due to an increase in concern for health and safety. Non-alcohol policies of the government especially for children and pregnant women are also reasons for the rise in demand
(Babor *et al*., 2017) [3]. NAB is referred to by a variety of names such as "low alcohol beer," "dealcoholized beer," "near beer," "alcohol-free beer" and "small beer," which commonly refer to an alcohol content between 0.00 and 0.50 percent by volume (ABV). Due to process conditions that are practising leave the taste to be compromised, so NAB faces modest consumer acceptance due to organoleptic issues.
De-alcoholization mainly involves removing ethanol from the beer, whereas biological methods are based on limiting ethanol production. Biologically, there is a special focus on non-Saccharomyces yeast species (Bellut *et al.,* 2019) [4].
The production of the NAB having the content of the volatile compounds as compared to that of the regular beer is a bit challenging task. However, these compounds are lost during the physical removal of alcohol. Hence, altering or interrupting the fermentation process would result in the production of a product that lacks the typical beer flavour (Catarino and Mendes, 2011) [7]. To overcome this problem, an alternative method of use of biological methods such as non-conventional yeast fermentation can be used (De Francesco *et al.,* 2015) [8]. Apart from dealcoholization, research works are in progress on biological methods to restrict ethanol production by using unconventional yeasts.
Commonly *Saccharomycodes ludwigii* yeast is used for the fermentation of the low-alcoholic beer production. This yeast fermentation leads to production of beer with low alcohol as well as low concentration of higher alcohols and esters (Adamenko *et al.,* 2020) [1]. Non-conventional yeast which can be able to ferment the glucose but not maltose can be employed in fermentation of the low alcoholic beer production. *Hansenula polymorpha* (*Pichia angusta*) is one such type of yeast strain that can ferment the glucose, xylose and mannose in limited amount and produce ethanol (Ryabova *et al.,* 2003) [13]. During fermentation *H. polymorpha* also produces esters and higher alcohols in more concentration compared to the traditional beer yeast. This compounds give typical beer flavour and aroma.
In this study physicochemical and sensory properties of the low alcoholic beer produced by *H. polymorpha* is compared with both standard alcoholic beer produced by *S. cerevisiae* and

commercial low alcoholic beer produced by *S. ludwigii.*

2. Materials and Methods

2.1 Materials

The raw materials such as barley malt and hops were purchased from brewof (Pune, India). The yeast strains *Saccharomyces cerevisiae* (3052), *Saccharomycodes ludwigii* (3261) and *Hansenula polymorpha* (3377) were procured for (CSIR-NCL) Council for Scientific and Industrial Research – National Chemical Laboratory, National Collection of Industrial Microorganisms (NCIM) (Pune, India). These yeast strains were stored at 4 ^{0}C on YPD slants (Yeast extract-Peptone-Dextrose) and are sub-cultured when required.

2.2. Brewing

The beer was produced based on infusion mashing technique, briefly, the barley malt were ground using a hammer mill with 0.2 mm mesh. Mashes were subjected to heating by following four steps: i) 45 ^{0}C for 30 min, ii) 60 ^{0}C for 60 min, iii) 72 ^{0}C for 30 min and iv) 78 ^{0}C for 10 min (mashing-off). Once the temperature reaches to 72 ^{0}C, saccharification time was measured. Hot mashes are filtered with Whatman No - 595 filter paper, and sediments are rinsed with the same volume of hot water. Wort extract is analyzed after cooling to 20 °C. The filtered worts were boiled with addition of hop pellets at concentration of 1.5 gL^{-1} for 60 min at 90 ^{0}C. The worts are filtered to remove hot break. Yeast propagation is done by inocluating yeast into 100 ml Erlenmeyer flasks containing 20 ml of YPD (Yeast Extract-Peptone-Dextrose). They were cultivated on a rotary shaker for 24 h, at 25 ^{0}C and 180 rpm. Primary fermentation is carried out in cork capped flask placed in a shaking incubator at 16 ^{0}C for 4 days. Beer maturation is done at 4 ^{0}C for 7 days and filtered with bentonite powder using Whatman filter paper No – 595 for further analysis.

2.3 Physicochemical analysis

Basic physico-chemical analysis like wort extract (^{0}P), ethanol concentration (% v/v) and degree of fermentation of beer and wort samples were done in Anton Paar DMA 4500 M density meter (Graz, Austria). Beer samples are degassed and filtered using Whatman filter paper no. 597 before analysis.

2.4 Colour and Bitterness

Colour: Using Shimadzu UV-1800 UV/Visible Scanning Spectrophotometer (Kyoto, Japan) the colour of wort and beer samples was determined by following EBC-9.6 and 8.5 (Analytica-EBC, 2008) [2]. For colour, the beer and wort samples were filtered using 0.45 µm syringine filter. Expressed as EBC, we measured absorbance at 430 nm of filtered wort and beer samples.

Bitterness: Take 10 ml chilled beer and add one drop of 1-octanol, 1 ml 3 N HCl and 20 ml isooctane in a 50 ml centrifuge tube. Close the cap tightly and shake vigorously using a mechanical shaker for 15 min. Separate the phases by centrifuging the tubes for 3 minutes at 3000 rpm. Using isooctane as the blank, the absorbance of the supernatant at 275 nm was measured and the results were expressed in IBU (Analytica-EBC, 2008) [2]. The pH of the samples were analysed by using pH meter.

2.5 Free Amino Nitrogen

FAN content in wort and beer samples were determined by using an official method EBC -9.10.1 (Analytica –EBC, 2008) [2]. In detail, 4 ml diluted beer with distilled water is added to 3 ml ninhydrin colour reagent in a screw capped test tube and close it loosely to prevent the loss of evaporation. Boil the test tubes for 15 minutes in hot water and cool to 20 ^{0}C rapidly. 10 ml of 2% KIO_3 in 60% ethanol solution is added and incubated at room temperature for 3 minutes. The absorbance was determined spectrophotometrically at 570 nm against blank. Results were given in mg/L of beer.

2.6 Total Phenolic compounds

The phenolic content of beer samples was determined using Folin-Ciocalteu spectrophotometric technique described by Kawa-Rygielska *et al.* (2019) [10]. In a 10 ml test tube, dilute 0.5 ml of beer sample with 2 ml of distilled water. Add 2.5 ml of F-C reagent and 20% aqueous sodium carbonate solution to the diluted beer. Incubate for 1 hr in a dark place at room temperature and spectrophotometric measurements were taken at 765 nm against distilled water as a blank. We expressed the data in terms of GAE (gallic acid equivalents) per litre of beer.

2.7 Total Flavonoid content

Total flavonoid content of obtained beers were determined by using EBC-9.12 method
(Analytica-EBC, 2008) [2] spectrophotometrically. In brief, 0.1 ml of beer is added to 5 ml of 0.1% 4-(Dimethylamino)-cinnamaldehyde in methanol and 37% HCl solution in a 3:1 ratio. Incubate in room temperature for 10 min and absorbance was taken spectrophotometrically at 475 nm using distilled water as blank. Obtained data was expressed as QE (quercetin equivalents) per litre of beer.

2.8. Statistical analysis

Graphpad Prism 9 developed by GraphPad Software, Inc. (San Diego, USA) was used to analyze the data. The brewing trails and analysis were performed in triplicate and data is presented as mean ± standard deviation.

3. Results and Discussion

3.1 Physicochemical properties

The basic physicochemical properties like alcohol (% v/v), degree of fermentation (%), wort extract (^{0}P) and specific gravity of the wort and beers are given in Table 1. The obtained results were compared with both a commercial strain of *S. ludwigii*, which produces low alcohol beer since maltose cannot be converted into alcohol, and a commercial brewer's yeast strain of S. cerevisiae. The original extract of all three beer samples were close to 12 ^{0}P, which is commonly present in all commercial beers. The present study shows that use of *H. polymorpha* yeast in brewing results in beer with less than 1% (0.93% v/v). This indicates the beer obtained is low-alcoholic beer.

H. polymorpha fermented beer also contains the same amount of ethanol as compared to the S. ludwigii fermented beer. The alcohol concentration in obtained low alcoholic beers (SL and HP) is -% lower than the standard alcoholic beer obtained by fermentation of *S. cerevisiae*. The previous study on the *Pichia angusta* fermented beer also showed the lower RDF nearly 12% and RE approximately 10% w/w (Larroque *et al*., 2021) [11]. The degree of fermentation is also very low in SL and HP beer (11.89-12.47%) compared to the alcoholic beer (46.73%).

However, the RDF and ADF are almost similar in SL and HP

beers. The RE of the *H. polymorpha* beer is almost double that of the standard alcoholic beer. But, there is no significant difference in RE of SL and HP.

pH of the beer is a key parameter in the aspect of quality (Guyot-Declerck *et al.*, 2005) [9]. The present study results shows that the pH of the obtained beers is less than the wort. It shows that during fermentation there is production of acid in the beer. The pH of the SC beer is lower than the other beer, due to less fermentability of *S. ludwigii* and *H. polymorpha*. There is no significant difference ($p<0.05$) in the pH of the low alcoholic beers. However, the pH of all three beers are in range of standard beer.

3.2 Colour and Bitterness

The colour and bitterness of the beers and wort are given in the Table 1. The results shows that the colour of all three beer samples reduced during fermentation. This reduction in colour is due to production of acid results in the precipitation of the colour pigments. The HP beer shows highest colour than other beer samples. However, differences are not significant ($p<0.05$) in colour of SL and HP beer samples. The higher value of colour in SL and HP beers indicate that there is limited amount of acid is produced during fermentation. This is attributed to the low fermentability of *S. ludwigii* and *H. polymorpha* yeast strains.

The present study shows that the bitterness of the beer is reduced during fermentation. The bitterness of the all three beer samples are almost similar and there is no influence of yeast on bitterness. The bitterness of the obtained beers are lower than the results found in Caballero *et al.* (2012) [5] due to the avoid of dry hopping and the variety of hops used.

Table 1: Physicochemical properties of beer and wort

	Alcohol		Extract			Degree of fermentation		Specific gravity	Density
	%v/v	%w/w	Apparent (% w/w)	Real (% w/w)	Original (% plato)	Apparent (%)	Real (%)		g/cm^3
SC	3.73 ± 0.05^a	2.89 ± 0.05^a	5.14 ± 0.07^b	6.5 ± 0.06^b	12.09 ± 0.07^a	57.53 ± 0.64^a	46.73 ± 0.57^a	1.0253 ± 0.00^b	1.0488 ± 0.00^a
SL	1.02 ± 0.02^b	0.76 ± 0.01^b	10.44 ± 0.24^a	10.77 ± 0.22^a	12.27 ± 0.26^a	14.91 ± 0.19^b	12.47 ± 0.35^b	1.043 ± 0.00^a	1.0495 ± 0.00^a
HP	0.93 ± 0.02^b	0.7 ± 0.02^b	10.4 ± 0.08^a	10.73 ± 0.07^a	12.09 ± 0.07^a	14.03 ± 0.34^b	11.89 ± 0.29^b	1.043 ± 0.00^a	1.0488 ± 0.00^a

Values are expressed as the mean ± standard deviation. Values in the same column followed by different letter are statistically different ($p<0.05$).

3.3 Free Amino Nitrogen content

The present study results shows that free amino nitrogen of the beer is reduced compared to the wort. The reduction in FAN is due to utilization of the FAN by yeast for meeting it's metabolic activities during fermentation. The results shows that the HP beer contains higher amount of FAN due to low fermentability of the *H. polymorpha.* The lowest FAN content was seen in SC beer as (66.51 mg/L) followed by SL beer (78.41 mg/L). This shows the fermentability of the S. cerevisiae and S. ludwigii is higher than the *H. polymorpha*. FAN content is an important attribute of beer as higher amount of FAN deteriorates the beer flavour and lower amount of FAN results in reduction yeast fermentability (Lekkas *et al.*, 2005) [12].

3.4 Total phenolic and flavonoids

Total phenolic and flavonoids in the beer and wort samples were given in Table 3. The present study results shows that the phenolic and flavonoid contents increased during fermentation. This is due to the release of bound form of polyphenols into the beer as a result of fermentation by yeast. The highest total phenolic compounds was found in SC (117.78 GAE/L) beer and lowest in HP beer (171.34 GAE/L). HP beer contains less amount of polyphenols due to the lower fermentability of the yeast *H. polymorpha* compared to the *S. cerevisiae*. The results shows that there is no significant difference ($p<0.05$) in the total phenolic content of SL and HP beer samples.

The total flavonoid content of the HP beer is 17.63 QE/L of beer which is lower than the SC and SL beer samples (22.05 and 18.02 QE/L). This is also due to the lower fermentability of the

H. polymorpha results in the reduction in the liberation of flavonoids in the final beer.

The polyphenolic components such as total phenolic content and flavonoids in the beer are mainly derived from the barley malt and their concentration depends on the variety of malt used in brewing. The phenolic content in the beer plays a major role in the stability and flavour of the beer. Besides the phenolic compounds in the beer contribute to the health benefits of the beer (Callemien and Collin, 2009) [6].

Table 2: Colour, bitterness, FAN and pH of wort and beer samples

	Colour EBC	Bitterness IBU	FAN g/L	pH
Wort	8.37 ± 0.21^a	24.08 ± 0.09^a	123.6 ± 0.92^a	5.71 ± 0.02^a
SC	4.33 ± 0.06^c	18.26 ± 0.06^b	66.51 ± 0.22^b	4.67 ± 0.05^d
SL	4.6 ± 0.10^c	18.3 ± 0.06^b	78.41 ± 0.36^c	5.04 ± 0.07^c
HP	5.23 ± 0.15^b	18.13 ± 0.08^b	80.89 ± 0.48^d	5.3 ± 0.02^b

Values are expressed as the mean ± standard deviation. Values in the same column followed by different letter are statistically different ($p < 0.05$).

Table 3: Total phenolic content and total flavonoid content

	Phenolics mg GAE/L	Flavonoids mg RE/L
Wort	117.78 ± 0.65^c	9.21 ± 0.04^c
SC	212.67 ± 2.22^a	22.05 ± 1.05^a
SL	174.31 ± 1.56^b	18.2 ± 0.54^b
HP	171.34 ± 2.61^b	17.63 ± 0.04^b

Values are expressed as the mean ± standard deviation. Values in the same column followed by different letter are statistically different ($p<0.05$).

4. Conclusion

The present study results confirm that the use of *H. polymorpha* in beer fermentation produces a beer with low alcoholic content i.e., below 1%. The real extract and attenuation degree of fermentation are almost equal to the beer produced by *S. ludwigii* fermentation. The biochemical properties such as total phenolic content and total flavonoid content of HP beer is lower than the SC beer. However, there is no significant difference in these biochemical compounds between HP and SL beers. Use of *H. polymorpha* in brewing industry may results in the production of beer with improved

quality and sensory attributes. The present study may form a basis for the further investigation into non-alcoholic and low-alcoholic beer produced by non-conventional yeast fermentation.

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