Isolation and Physiochemical characterization of yeast species from coffee wastes, from Majang zone of Gambella, a regional state of Ethiopia

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ABSTRACT

Microbial production of ethanol was a very popular concept in respect of alleviating energy demand nowadays. In this regard, two fermenting strains of yeast were isolated from coffee treatment plant container and coffee dump and grown in YEPD medium. They were characterized for carbohydrates fermentation using glucose, galactose, maltose, fructose sucrose, lactose, Trehalose and xyloses. Two isolate strain from coffee fermenter container (GM32) and coffee dump (GM73) further was tested against three fermentation parameters namely temperature, pH and ethanol to determine optimization parameters. Results were revealed that a temperature of 30℃ was optimal for isolate yeast strain, Which showed significantly high counts (8.62±0.31 CFU x 10^6/ml) and 8.12 ±0.38 CFU x 10^6/ml respectively at 30^0c temperature. Maximum yeast cell growth was (7.20 ±1.3010^6/ml) recorded and minimum was (1.42±0.22 CFU x 10^6/ml) recorded at pH 2.0. and pH.5.0 of coffee fermenter container (GM32) and coffee dump (GM73) respectively. highest yeast cell growth was recorded (7.80±.17 CFU x 10^6/ml) at (5%) ethanol concentration and lowest yeast cell growth was taken (1.21±0.20 CFU x 10^6/m) at (25 %) of isolate yeast strain of (GM73) and coffee dump (GM32) respectively.

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The optimum temperature, ethanol and pH of the isolate yeast strain were used as optimum parameter for ethanol production.

Key words: ***coffee, fermentation, ethanol, isolate yeast strain, , optimum***

INTRODUCTION

The continuously increasing population of Earth is dawning a new challenge for biotechnology: supplementation of mankind with commodity products from renewable resources, instead of fossil based ones. Biotechnology producing commodity products should aim to be responsive to societal needs for sustainable resource utilization and improved environmental quality. As raw materials are often dominant factors in determining the price of commodity products, renewable materials available at large scale are required as feedstock. In addition, these renewable sources are geographically more evenly distributed than fossil fuels; therefore, the products will be domestic and provide security of supply. At the beginning of the 20th century, many industrial materials such as dyes, solvents, and synthetic fibers were made from trees and agricultural crops. By the late 1960s, however, many of these bio-based chemical products were replaced by petroleum derivatives which could be produced at lower cost (Ragauskas *etal*., 2006). Over the last few decades, the negative impacts of fossil fuel on the environment and consequent global warming, progressive demand for energy, inevitable depletion of the world's energy supply, and the unstable oil market (such as the energy crisis of the 1970s) have renewed the interest of society in searching for alternative fuels (Solomon *etal*., 2007).

Lignocellulosic biomass is more preferred than starch or sugar-based crops for production of ethanol, since it does not compete with food and takes care of agricultural and plant residues in an

environmentally sustainable process. Therefore, the objective of this study to isolate and characterize wild yeast strain can convert an inexpensive and highly efficient integrated anaerobic fermentation process used to produce ethanol as an energy source directly from insoluble lignocelluloses coffee wastes

- **Materials and Methods**

Study area

The study would be conduct in Godare and Mangeshi district of Majang zone of Gambella people's regional state. Majang zone is located approximately 750 km far from Addis Ababa. Meti is capital town of the zone, laying latitude, 07^0 14' 35^0 19' E and longitude of 7.233^0 N 35.31'E. Elevation ranges from 550 - 1266 m above sea level. Majang is the only zone producing coffee from the region. *Coffee arabica L.* is mostly grown in those two Woreda. About 4000 tons of coffee annually produced (CSA, 2005) and the wet and dry processing method is uses for coffee bean extraction.

Sample collection and design

Fermenting juice of coffee cherries will be collect from dumping sites and fermented container of all 10 wet coffee processing plants found in the zone. All of coffee waste dumping site from each district will be select based on the waste disposal time. Samples will collect during mid-October end of December of high pick yield of coffee production and extraction time. Samples carefully collect and preserve in ice boxes then transported to the laboratory

Media preparation

A solid medium containing (g/l): (0.2% yeast extract, 1% peptone, 2% glucose, 1.5% agar) prepare and adjusted to pH 5.0 for yeast growth. The medium is autoclave at 121 °C for 15min. For preparation of inoculums broth (liquid) media (g/l) (0.3% yeast extract, 1% peptone, and 2%

glucose) is uses for yeast maintenance and growth. The medium is autoclave at 121 °C for 15 min and pour on conical flask and cool, and then inoculate with 48 hours old selected yeast strain from Petridish and incubate at 30 °C for 24 hours in vigorous shaking condition (180 rpm). Each treatment in this experiment is replicate six times.

Yeast isolation and Inoculum development

From each samples 1 g of sample was taken and dilute serially with a sterile saline solution (0.89% w/v NaCl) to a point where a reasonable number of cells can be count up to 10^6 ml. About 0.1 ml of serially diluted sample was transferred through the streaking technique on yeast peptone dextrose agar media (YPDA) and sub culturing until pure colony will appear. The yeast cells (10^3 10^6 ml) streak on Petri dish and, then incubate for 48 hours at 30ºC for yeast Inoculum preparation. Yeast cell in each conical flask count by hematocytometer. The culture of yeast will maintain by sub-culturing on slants using YMM (Yeast maintenance Media) media and incubating for 48 hrs. at 30ºC and thereafter storing in a refrigerator at 4ºC for future use.

Identification of the yeast

Yeast isolates will identify based on the morphological characters and physiological characteristics (Barnett *etal.,* 2000 and 1984; Mesa *etal*., 1999). The following features of the appearance of cultures are record for morphology on solid media; texture, color and surface of colonies using hand lens as well as its percentage and frequency will be record. The culture is examining visually on the surface of YEPD (yeast extract peptone dextrose) and for liquid media the shape of cells observed by microscope.

Physiochemical characterization

All yeast isolates are first screen for their ability to ferment carbohydrates. Yeast fermentation broth media will be used for identification of yeasts based on fermentation of specific

carbohydrates of fermentation pattern. The carbohydrates used are: glucose (dextrose), galactose, maltose, sucrose, Trehalose, fructose and xylose. The medium is prepared from Peptone 10 g, NaCl 5 g, Phenol red (Indicator), 5gm Carbohydrate and make the volume up to 1000 ml with distilled water. A 15 ml media dispensing in different McCartney tubes will autoclave after cooling the media then inoculate with 48 hrs. old selecting yeast strain and fermenting for 72 hrs.' to assess formation of co_2 in Durham tube and color change for each carbohydrate.

Detection of thermo tolerance

YEPD medium is uses for detecting thermo-tolerance growth in liquid media for selecting yeast strain. 10 ml portion of the medium is distributing into McCartney tubes, and then inoculate with 48 hours old selected yeast strain. The initial optical density of each tube is record on spectrophotometer at 600 nm against the medium as blank. All cultures will incubate at 25°C, 30°C, 37°C, 40°C and 44°C for 2 days for observing thermo tolerance of yeast strain. The increase in optical density in a tube will record as evidence of growth.

Detection of ethanol tolerance

YEPD liquid medium is uses for detecting yeasts for ethanol tolerance. 40ml portion of the medium is distributing into 125 ml flask containing varying percentages of ethanol concentration (0%, 5%, 10%, 15%, 20% and 25%) and then inoculate with selecting ethanol tolerant yeasts. The initial optical density of each flask is read off on spectrophotometer at 600 nm against the medium as blank. All cultures will incubate at 40°C for 5 days. After 5 days the increase in optical density in a flask is record as evidence of growth. The concentration of alcohol at which the growth of yeasts was just inhibit is assesses as the ethanol tolerance of yeasts

Growth in different pH

YEPD (yeast extract peptone dextrose) liquid medium will be used for detecting the ability to grow in different pH of selecting yeast strain. YEPD broth is prepare at different (2, 3, 4, 5, 6, 7) pH. Each test-tube contained 13 ml of YEPD media with different pH and blank media will uses as a control. Then each medium inoculates by half loop full of yeast cell and measured the initial optical density at 600 nm and incubated at 30°C for 48 hrs. After 48 hrs cell density was further recorded at 600 nm for growth

Data analysis

A completely randomized design (CRD) will be uses for all experiments. Produced data will subject to analysis of variance (ANOVA) and least significant difference (LSD) test using statistical data analysis software SPSS and at 0.05 probability level.

• Results

Isolation and identification of yeast

Pure cultures of total seventeen (17) morphologically distinct yeast isolate from eight (8) samples of coffee dump site and coffee treatment fermenter. Morphology of isolate yeast strain forms smooth surfaces with oval, spherical, ellipsoid, circular cell and white creamy colonies Colour on YEPD medium. While, smooth, circular and creamy white colonies were found to be more dominant.

Carbohydrate fermentation

The physiological study of each yeasts strain were carried out by using 7 tests for assimilation of carbon. Almost all the isolates utilized glucose, galactose and fructose. The result of this study indicated that most of the isolated yeasts from samples showed good fermentation attributes., Meanwhile isolates strain GM32 and GM73 could utilizes all types of carbohydrates. The most conversion fermenters of isolate strain (GM32 and GM73) shows remarkable properties of yeast by fermenting all carbohydrates were taken for further physiological characterization of stress tolerances.

Table 1: Carbohydrates fermentation by yeast isolates

NO	Isolate strain	Glucose	Fructose	Galactose	Sucrose	Maltose	Trehalose	Xylose	Total carbohydrate
1	GM25	++	++	++	++	++	++	-	6
2	GM30	+	+	+	+	+	+	+	7
3	GM31	++	++	++	-	++	++	-	5
4	GM32	++	+++	+	+++	+	++	++	7
5	GM45	++	++	++	-	++	++	-	5
6	GM46	++	++	++	++	++	++	++	7
7	GM47	+	+	+	+	+	+	+	7
8	GM48	++	++	++	-	++	++	-	5
9	GM55	+	+	+	+	+	-	+	7
10	GM61	++	++	++	-	++	++	-	5
11	GM62	++	++	++	++	++	++	++	7
12	GM71	+	+	+	+	+	+	+	7
13	GM72	++	++	++	++	++	++	++	7
14	GM73	+++	+++	+++	+++	++	++	++	7
15	GM85	++	-	++	+	++	++	-	5
16	GM91	++	+	++	++	++	++	-	6
17	GM92	+	+	+	+	+	+	-	6

+ = Fermentative, ++ = moderately fermentative, +++ = Highly fermentative (Durham tube empty), - = No carbohydrate utilization

Table 2: Colony characteristics of isolates yeast strain

NO	Isolate strain	Surface	Margin	Colour	Shape of cells
1	GM25	Smooth	Irregular	Creamy	Round/Oval
2	GM30	Smooth	Circular	Creamy white	Round/Oval
3	GM31	Rough	Irregular	Creamy white	Ellipsoidal
4	GM32	Smooth	Circular	Creamy white	Spherical/Oval
5	GM45	Smooth	Circular	Creamy white	Ellipsoidal
6	GM46	Smooth	Irregular	Creamy white	Spherical/Oval
7	GM47	Smooth	Irregular	Creamy white	Round/Oval
8	GM48	Smooth	Circular	Creamy white	Round
9	GM55	Smooth	Circular	white	Oval

10	GM61	rough	irregular	white	Round/oval
11	GM62	Smooth	Irregular	Creamy white	Round/Oval
12	GM71	Smooth	Circular	Creamy white	Round/Oval
13	GM72	Rough	Irregular	Creamy white	Ellipsoidal
14	GM73	Smooth	Circular	Creamy white	Spherical/Oval
15	GM85	Smooth	Circular	Creamy white	Ellipsoidal
16	GM91	Smooth	Irregular	Creamy white	Spherical/Oval
17	GM92	Smooth	Irregular	Creamy white	Round/Oval

Temperature

YEPD containing plates were streaked by Yeast cell and incubated for 48 hours at 25°C, 30°C, 37°C, 40°C and 44°C. both strain of yeast was able to grow within the range of (25-44°C). The isolates from coffee treatment plant (GM32) and dump site (GM73) gave maximum cell growth of yeast isolates counts 8.62±0.31 and 8.12 ± 0.38 CFU/ml were recorded at 30° C respectively, but as temperature increases low cell growth of dump site (GM73) of yeast isolate (2.38 ±0.25 CFU/ml) were recorded on 44°C.

Table 3: Temperature tolerance by the yeast isolates

Mean count (CFU×10^6/ml) of yeast isolates and optical density measure								
Temp	GM73				GM32			
	(CFU×1 0^6/mL)	O.DA at inoculation	ODA after 48	Change of O..D after	(CFU×1 0^6/mL)	O.DA at inoculati	ODA after 48	Change of O..D after
25	6.08 ±0.28^{b}	0.519	2.063	1.544	5.62±0.34^{c}	0.559	2.290	1.731
30	8.12 ±0.38^{a}	0.441	2.162	1.721	8.62±0.31^{a}	0.515	2.311	1.796
35	5.06 ±0.33^{bc}	0.523	1.901	1.378	6.56±0.28^{b}	0.671	2.058	1.387
37	4.34 ±0.64^{c}	0.413	1.623	1.210	4.82±0.28^{c}	0.568	1.988	1.420
40	2.64 ±0.17^{d}	0.350	1.261	0.911	4.40±0.21^{d}	0.465	1.918	1.453
44	2.38 ±0.25^{d}	0.687	0.471	-0.216	3.48±0.29^{e}	0.511	0.802	0.291

Means ± SE from five replications and mean values followed by different letter(s) in the same column indicate significant differences ($p < 0.05$)

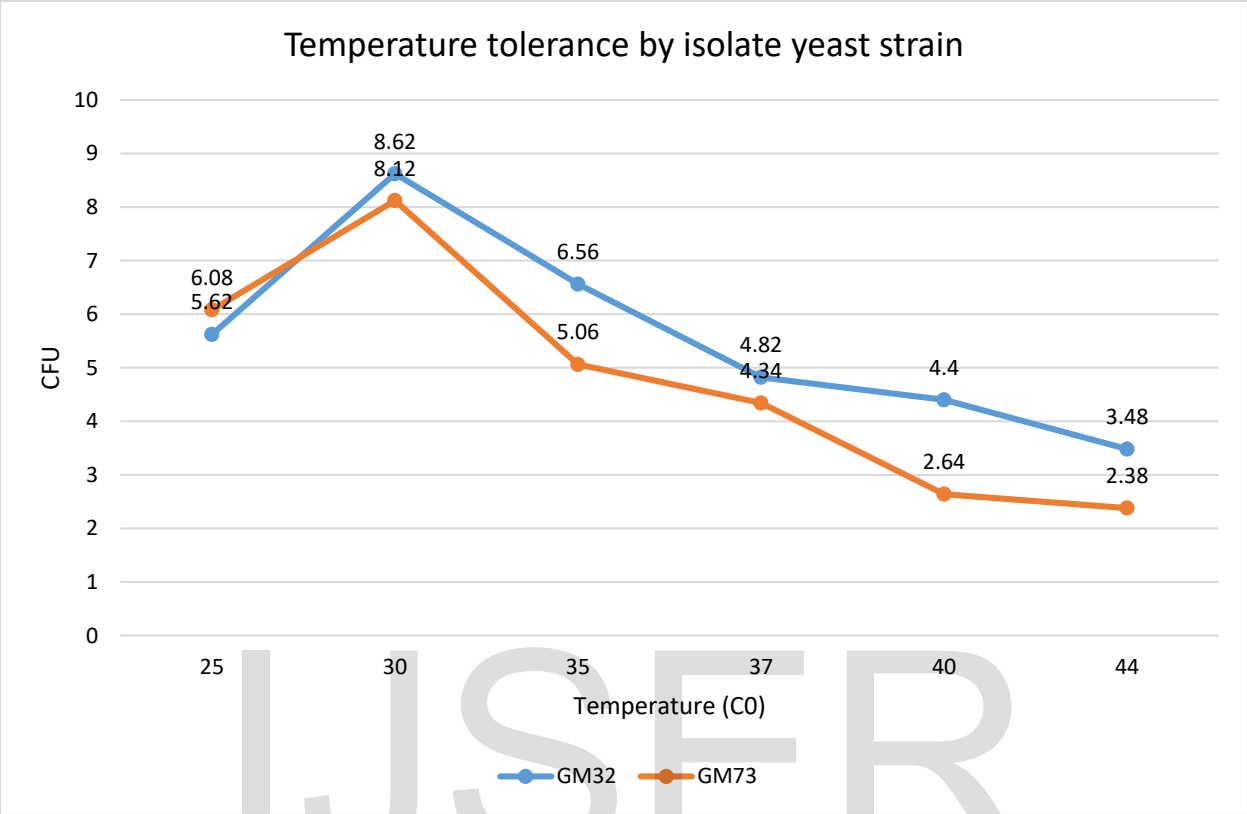

Figure 1. Yeast cell growth (GM32) in varying temperature within initial pH. 5

PH tolerance

YEPD containing plates were streaked by Yeast cell and incubated for 48 hours at 30^0c of different PH scale. The isolates from coffee fermenting container (GM73) gave maximum yeast growth (7.20 ±1.30 CFU×107/ml) were recorded on PH 5.0 and followed by dump site yeast isolate (GM32) with (7.13 ± 0.10 CFU x107/ml) on the same PH scale.

Ethanol tolerance

The isolates were selected for screening of ethanol tolerant yeast (Table 5.4). The isolates can grow in all ethanol containing liquid YEPD media. While the Maximum cell biomass for the GM 73 yeast isolate (7.80±.17 CFU×107/mL) was recorded on 5% ethanol containing media and followed by the GM 32 yeast isolate of cell biomass (7.60±0.30 CFU×107/mL) at the same ethanol concentration

Table 4: PH tolerance by yeast isolates at different pH. scale

Mean count (CFU×10^6/ml) of yeast isolates and O.D

PH	GM73				GM32			
	(CFU× 10^6/mL)	O.D at inoculation	ODA after 48 hrs.	Change of O.D after 48 hrs.	(CFU× 10^6/mL)	ODA at inoculation	O. D after 48 hrs.	Change of O.D after 48hrs
2	2.38 ±0.41^{d}	0.363	0.496	0.133	1.42±0.22^{c}	0.335	0.773	0.438
3	2.50 ±0.62^{cd}	0.404	1.466	1.062	1.46±0.09^{c}	0.361	1.407	1.046
4	3.40 ±1.14^{bc}	0.369	1.468	1.099	5.84±0.43^{b}	0.390	1.859	1.469
5	7.20 ±1.30^{a}	0.355	1.572	1.217	7.13±0.10^{a}	0.407	1.887	1.48
6	4.40 ±1.34^{b}	0.376	1.571	1.195	5.94±0.60^{a}	0.459	1.847	1.388
7	3.20 ±0.83^{bc}	0.439	1.319	0.88	4.06±0.3^{b}	0.473	1.822	1.349

Means ± SE from five replications and mean values followed by different letter(s) in the same column indicate significant differences ($p < 0.05$

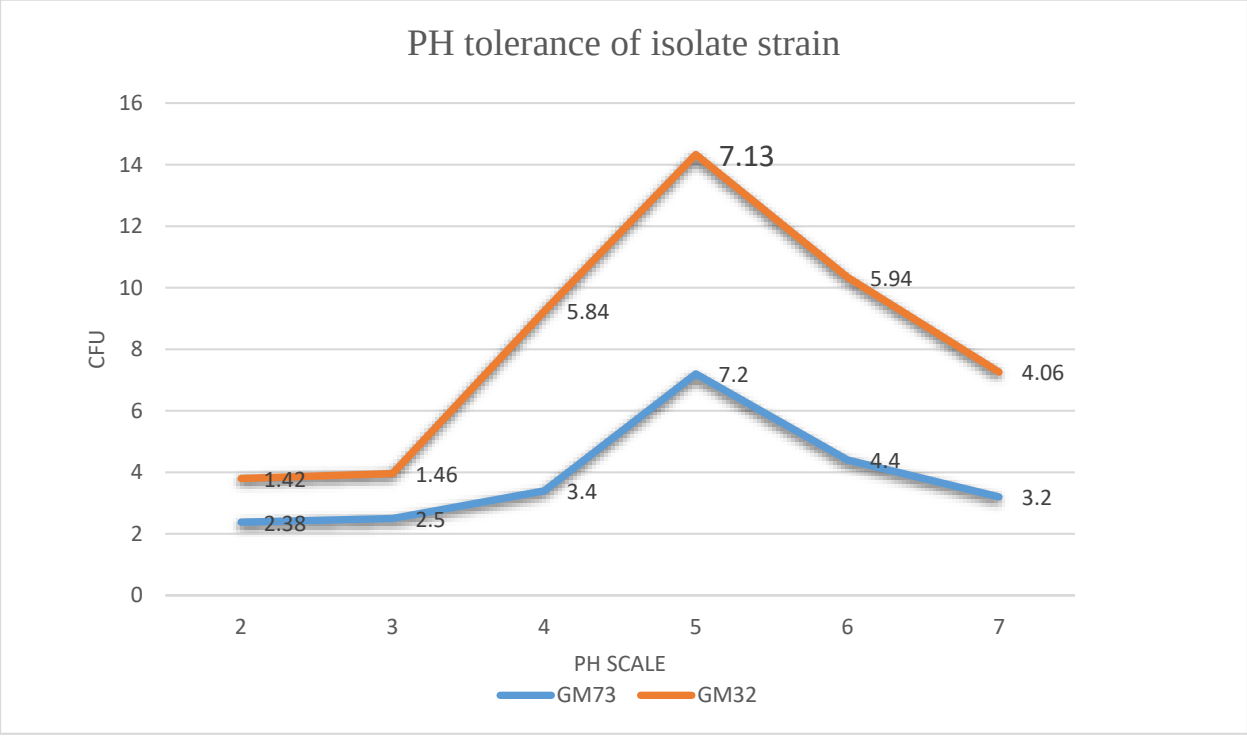

Figure 2. Yeast cell growth in varying pH within constant temperature

Table 5: Tolerance of the yeast isolates to different ethanol concentrations

Mean count (CFU×10^6/mL) of yeast isolates and O.D change								
Ethanol tolerance (%)	GM73				GM32			
	(CFU×10^6/mL)	O.D at inoculation	O.D after 48 hrs.	Change of O.D after 48hrs	(CFU×10^6/mL)	O. D at inoculation	O.D after 48 hrs	Change of O.D after 48hrs
5	7.80±.17 [a]	0.433	1.403	0.970	7.60±0.30[a]	0.364	1.920	1.556
10	5.80±.22 [b]	0.445	1.452	1.007	6.38±0.20[b]	0.365	1.825	1.460
15	5.30±.53 [b]	0.246	0.305	0.063	4.76±0.39[c]	0.352	0.833	0.481
20	3.84±.26 [c]	0.192	0.503	0.311	2.62±0.41[d]	0.290	0.384	0.094
25	2.22±.11 [d]	0.259	0.109	-0.150	1.21±0.20[e]	0.220	0.166	-0.054

Means ± SE from five replications and mean values followed by different letter(s) in the same row indicate significant differences ($p < 0.05$)

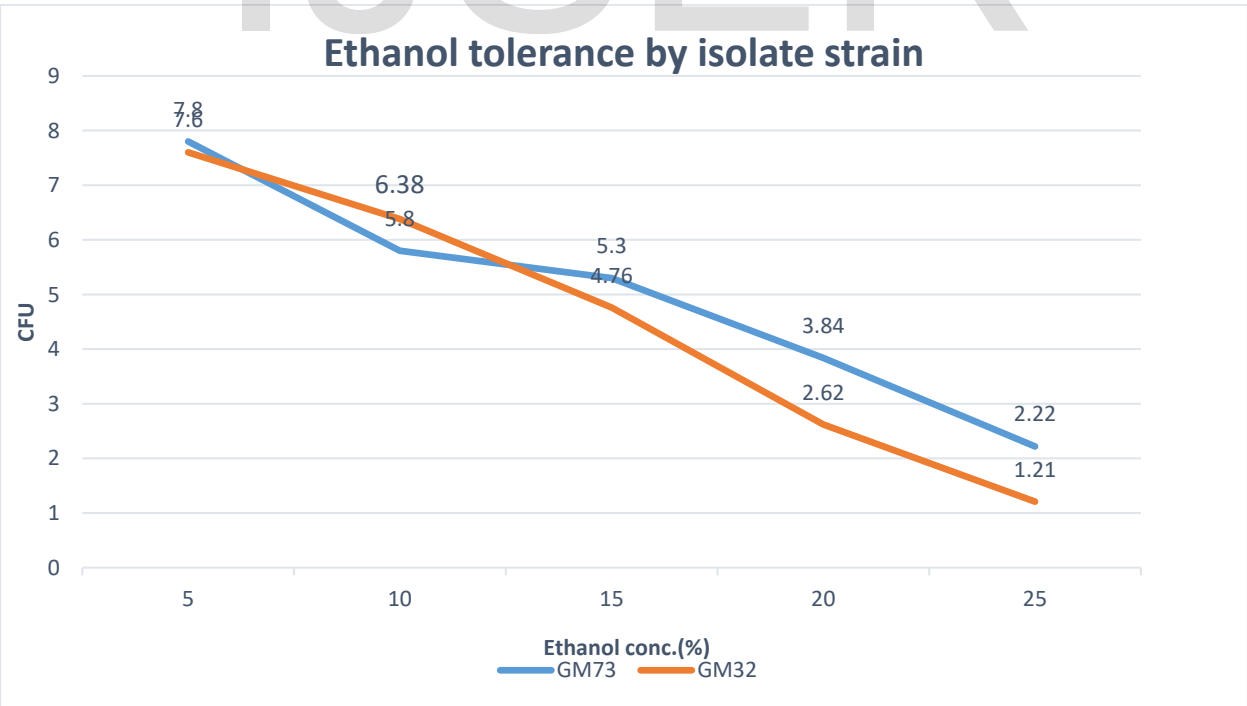

Figure 3. Yeast cell growth in varying ethanol concentration within initial pH.

- **Discussions**

Based on the colony characteristics (white and creamy texture) ovoid microscope shape, the presence of ascospore and budding pattern the selected isolate (GM32 and GM73) were found to belong *Saccharomyces* type unicellular organism

Morphological observations and other studied parameters of the yeast isolates shared similarities with the descriptions given by Lodder (1971) and Boekhout and Kurtzman (1996).

Accordingly, the coffee waste isolates (GM 73 and GM32) are tentatively assigned to a genus *Saccharomyces* type unicellular ascomycete. Furthermore, the features depicted by the isolates are consistent with the previous findings of (Buzayehu Desisa and Diriba Muleta, 2017) and (Tamene Milkessa, 2009) reported for yeast isolates recovered from Arabica coffee wet processing wastes and cereals based products respectively.

The all isolates were tested for assimilations of carbohydrates and coffee treatment plant strain (GM32) was capable to ferment six sugars out of the seven sugars tested (Table1). glucose, Sucrose, Lactose, Maltose and Trehalose were successfully fermented by this strain but it can't ferment xylose. The coffee dump strain failed to ferment Maltose and xylose, but utilized five other carbohydrates (Table), which proved the identity both of the microorganisms are Saccharomyces cerevisiae

Optimum temperature for yeast growth was crucial to ethanol production The results on the impact of temperature on isolate strain are reported on (table 4.2). The highest cell growth was counted after 48hr incubation at 30^0c of temperature. Similar study was done by Buzayehu Desisa and Diriba Muleta *etal*., (2017) described that, yeast isolated from Arabica coffee (Coffee Arabica L.) wet processing wastes counts higher population (7.1×10^7 CFU/ml) and (5.6×10^7 CFU/ml) at 30 °C, followed by the yeast isolate from batter (7.7×10^6 CFU/ml) and growth was decreased as the temperature increased. But, was also within the range of temperature optima for alcoholic fermentation by conventional yeasts.

this optimum temperature was optimal for alcoholic fermentation. The trends in lined with the findings of current experiment. Neelakandan and Usharani (2009) was reported maximum ethanol yield by *Saccharomyces cerevisiae* at 32^0C to be 8.53% after 24 hours of fermentation time which

was relative to this current experiment. However, a slightly higher temperature (33.2^0C) was reported by Chin *etal*. (2010) to be minimize growth rate. Temperature tolerance for growth and fermentation is said to be strongly strain dependent (Rousseau *etal*., 1992). As shown in (Figure 1) temperatures above 40^0C were a significant decrease in with lower growth counts which might be due to reduced yeast viability at high temperatures that causes retarding in yeast cell growth (Casey *etal*., 1984) narrated by Muruke *etal*., (2018). extremely high temperature greatly affects the enzymatic activity and membrane turgidity of yeast cells. These problems might be brought by higher temperature. **(**Hu. *etal.*, 2012) reports about effects of extreme temperature on two strain which representing potentials for bioethanol production from jerusalem artichoke, while extreme temperature poses low yeast growth. The current experiment was showed the same trends with his finding. Yeast growth was reduced considerably at 44℃ of extreme temperature of current study. This might be due to change in transport system which might increase accumulation of toxin including ethanol in the cell of yeast.

In this study, the isolate strain from coffee dump (GM73) recorded more tolerance (up to 15%) ethanol than isolate from coffee fermentation container (Table 4). The data also showed as the concentration of ethanol increased from 5% to 25% the growth of isolates slightly, but not significantly decreased. This was similar to the report of Subashini *etal*. (2011) that showed S. cerevisiae was tolerant to ethanol high (15%) concentration with cell count of (6.2×10^7CFU/ml) but slightly lower than the level of ethanol tolerance of 16%-16.5% by some yeast strains reported by (Buzayehu Desisa., *etal* 2017). *Saccharomyces* yeasts were the most ethanol tolerant of the eukaryotic organisms and able to tolerate over 20% ethanol. In a previous study by Muruke *etal*., (2018) yeast strain M4 could tolerate up to 16% ethanol concentration, which was similar with the current study. Almost Similar ethanol tolerance of 16.5% has been observed for

Saccharomyces cerevisiae by (Teramoto, *etal*., 2005). In current experiment both strains were screened for ethanol tolerance and showed up to 15% ethanol tolerance in YEPD growth media. While A slow growth rate was observed at 10-25% ethanol containing media (table 4.4)

The results from this study showed that the maximum growth was count after 48 hours of fermentation differed significantly at ($P<0.05$) with the variation in initial pH values. The lowest colony growth was counts ($2.38 \pm 0.41 10^6$ CFU/ml) in pH 2.0, but increased with increase in pH to a maximum growth of ($7.20 \pm 1.30 10^6$ CFU/ml) at pH 5.0, beyond which, it started to show a slight decreasing trend. According to these results, pH 5.0 provides optimal condition for isolate strain growth. Similar studies by Muruke *etal*., (2018) and Geetha *etal*., (2013) reported significant increase in ethanol yield from pH of 4.5 to 5.5, beyond this the levels did not increase much. Izmirlioglu and Demirci 2012 reported optimum pH for ethanol fermentation from waste potato mash with a maximum production of 30.99 g/l at pH 5.5. However, at lower pH (2-4) the production of ethanol was slightly lower compared to pH 5. This could be attributed to the fact that at lower pH, the yeast was unable to activate the enzymes due to its metabolic sensitivity. The optimal pH range for growth of yeast can vary from pH 4.0 to 6.0, depending on temperature, the presence of oxygen, and the strain of yeast. This likely was might due to the optimum pH value for the activity of plasma membrane bound proteins, including enzymes and transport proteins (Narendranath & Power, 2005). In our study the isolate strain from coffee fermenter container can grow in a wide pH range from 2 to 7, but pH. 5.0 showed to be the optimal for isolate yeast strain growth. This was in lined with the Muruke *etal*., (2018) report.

pH for ***S.*** *cerevisiae* BY4742 was in the range of 4.0–5.0 was reported by (Y. Lin, W etal.,2012); when the pH was lower than 4.0, the incubation period was prolonged though the ethanol concentration was not reduced significantly and when the pH was above 5.0, the concentration of

ethanol diminished substantially. Formation of acetic acid was enhanced when the pH was below 4.0 and pH above 5.0 favored butyric acid productions (Y. Lin, 2012)

Unlikely a wide range of optimum pH (4.0–8.0) was reported for ***S.*** *cerevisiae* JZ1C strain isolated from rhizosphere of Jerusalem artichoke using inulin and Jerusalem artichoke tuber as substrate at 35°C. however, optimum pH for isolate yeast strain for ethanol production was in the range of 4.0–5.0 reported by different scholars [B. Ortiz-Muniz *etal*., (2010), Lin.Y *etal*., (2012), Chin *etal*., (2010) and Muruke *etal*., (2018)**.** Then, the current study also shared similar phenomenon. which implied pH optimization strongly dependent on temperature

- ## Conclusion and Recommendation

The cell morphology of the yeast cells under microscope were observed as ovoidal to elongate, single or in pairs and budding cells were also recognized. All the physiological and biochemical characters observed, suggested that the strain demonstrated characteristics of *Saccharomyces cerevisiae*. This study demonstrates that the coffee processing wastes have a potential and are a promising alternative feedstock for bioethanol production in Ethiopia, which in turn contributes much to the proper management of environmental pollution. Yeast isolated from coffee wastes demonstrated remarkable tolerance to different levels of ethanol concentration, temperature and pH with highest cell growth of yeast. While isolated strain from coffee dump was found to be more tolerate than coffee fermenter container, to different pH scale and ethanol concentration.

The comparison of two wild-type isolates showed that the coffee dump strain was more potent microorganism, however this was not a remarkable significance. Therefore, these strains with optimized conditions need to be worked-up to make it an industrially suitable strain for Lignocellulosic biomass conversion of coffee waste for ethanol production. Productivity can also

be improved by further biotechnological methods using radiation or genetic manipulation or metabolic pathway engineering for direct ethanol production, perhaps this may be another way for improvement by studying the effect of inoculum size on ethanol yield thereby to improve cost effectivity.

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Appendix2: sample site of coffee wastes