Optimization of bioprocess parameters for wine from household vegetable waste production by employing response surface methodology

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Abstract

Wine prepared from vegetable peel which is a household waste, is a novel beverage rich in anti-oxidant. The quantitative effects of temperature, pH and time on fermentation of ethanol from the hydrolysed product using wine yeast *Saccharomyces cerevisiae* (NCIM 3206) were investigated. The conditions for the fermentation were optimized using Response Surface Methodology (RSM). The optimum conditions for the yield of 6.69% ethanol were found to be: temperature 32°C, pH 5.5 and time of fermentation 48 h. The wine had phenol 52.28 mg/l, flavonoid 41.5 mg/l. The wine had a DPPH (2, 2-diphenyl-1-picrylhydrazyl) scavenging activity of 55.18%. In this study heavy metals content like Cu (0.357mg/L), Pb (0.123mg/L), Zn (0.385mg/L) and Fe (0.285mg/L) content were determined. The structural degradation of vegetable waste starch and penetration of yeast cell into the inner tissue were observed by scanning electron microscope.

Introduction

Processing of fruits and vegetables result in high amounts of waste materials which include peels. Disposal of these materials usually represents a problem that is further aggravated by legal restrictions (Mohadaly *et al.*, 2010). Edible vegetable peels normally discarded as a traditional culinary practice contains large amounts of substances important for human metabolism and could be used as alternative nutritional sources. There is another reason to choose peel as a substrate for making wine. Peels of several fruits such as apple mango have also been found to contain higher amount of phenolics than edible flesh parts. Flavonol glycosides are located mainly in leaves, flowers, and outer parts of the plants such as skin and peel and decrease in concentration toward the central core (Aherne and O'Brien, 2002). Concentration of flavonoids is maximal in external and /or aerial tissues because sunlight stimulates biosynthesis. Producing wine from wastes offers a unique way for waste utilization. Vegetable wastes from potato, pumpkin, carrot and parwal are available throughout the year and are mainly used. These wastes contain low amount of starch material and are used as raw material in wine making. To make a safe globally acceptable wine it becomes essential to check the heavy metal. These wastes containing low amount of starch material mainly act as substrates of wine making. Banana peel was added as a supporting material for enhancing starch content suitable for yeast growth. The aim of the current work, was therefore to optimize fermentation process conditions using response surface methodology (RSM) and analysing structural feasibility for fermentation by scanning Electron Microscope (SEM), which has not been reported till date. In addition antioxidant activity, phenolic content, flavonoid content were analysed. Given the lack of information in published articles about the heavy metals in wine, a pertaining problem in India due to excessive pesticide use and improper sewage disposal, its study was necessitated before promoting it as marketable wine as excess heavy metal content leads to potential toxicity.

Materials and Methods

Chemicals

Dextrose, KH_2PO_4, K_2HPO_4, $MgSO_4.7H_2O$, $FeSO_4.7H_2O$, Urea, (Merck, India) were used. Yeast extract, Peptone (Himedia, India), 2,2- Diphenyl - 1 picryl – hydrazyl (DPPH) (Himedia, India), (+)-Catechin hydrate (Sigma-Aldrich, USA), Gallic acid (SD fine Chem Ltd India), Falin-Ciocalteu's phenol reagent (Merck India) were used.

Sample preparation

Parwal *(Trichosanthes dioica)*, potato *(Solanum tuberosum)*, pumpkin *(Cucurbita pepo)* and carrot *(Daucus carota)* were purchased from market in

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Table 1. Central composite design matrix

Run no.	Temperature(°C) (X1)	Time (X2)	pH (X3)	Ethanol %(Actual) (Y1)	Ethanol% (Predicted) (Y1)
1.	28	24	3.5	3.67	3.57
2.	32	24	3.5	3.84	3.93
3.	28	72	3.5	3.46	3.68
4.	36	72	3.5	4.25	4.08
5.	28	24	7.5	3.67	3.78
6.	36	24	7.5	4.67	4.39
7.	28	72	7.5	4.02	3.86
8.	36	72	7.5	4.48	4.52
9.	28	32	7.5	5.87	5.80
10.	36	32	7.5	5.98	6.31
11.	32	24	7.5	5.76	5.94
12.	32	72	5.5	5.98	6.06
13.	32	32	3.5	5.18	5.15
14.	32	32	7.5	5.18	5.47
15.	32	32	5.5	6.90	6.69
16.	32	32	5.5	6.89	6.69
17.	32	32	5.5	6.73	6.69
18.	32	32	5.5	6.55	6.69
19.	32	32	5.5	6.89	6.69
20.	32	32	5.5	6.71	6.69

Kolkata. These were preserved at -50^0C in an ultra-low temperature Freezer (Model C340, New Brunswick Scientific, England).The peel were extracted and added with fermentation media. The fermentation medium consisted glucose 10, urea 3, KH_2PO_4 0.5, K_2HPO_4 0.5, $MgSO_4.7H_2O$ 0.5, $FeSO_4.7H_2O$ 0.01 (g/l). The fermentation process was carried out in a 250 ml flask; 100 ml of fermentation media were inoculated with yeast culture.

Yeast culture preparation

Stock culture of *Saccharomyces cerivisiae* (NCIM 3206) was procured from National Chemical Laboratory (NCL) Pune, India. The culture media consisted of 0.3 malt extract, 1.0 glucose, 0.3 yeast extract and 0.5 peptone all in g/100 mL. The organisms were grown at a temperature of 30^0C, pH 6.5 with a incubation period of 45 hours.

Alcohol estimation

Five ml of fermented sample was centrifuged (Remi C-24, Mumbai, India) at 3500 rpm for 10 minutes. Gas Chromatography of supernatant was done to determine the ethanol concentration. Gas chromatography (GC) analysis was carried out using Agilent Technologies: GC system – 7890A gas chromatography, column- Agilent JK WDB-624 with column ID- 250 μm, length - 60 m and film length- 1.4 μm. The chromatograph was fitted with column (28 x 31 x 16 cm) with column gas flow rate 1.4615 ml/min. Helium was used as a carrier gas and the solvent used is dimethyl sulfoxide (DMSO). The ethanol content was calculated by the GC peak areas.

Experimental design

Statistical analysis and experimental design were performed according to the response surface analysis method using Design Expert 7.1.5 (Stat-Ease Inc., Minneapolis, MN, USA) software. Central composite experimental design (CCD) (Box and Wilson, 1951) with quadratic model of three independent variables namely temperature (X1, ^{0}C), pH (X2) and incubation time (X3, hrs) was employed to study the combined effect. Ethanol (Y1, %) of the wine were measured with the dependent variables (Y). These dependent variables were expressed individually as a function of the independent variables known as response function. In CCD, the range and the levels of the variables investigated in this study are given in Table 1. The second degree polynomial equation (1) was used with the statistical software to estimate the response of the dependent variable.

$$Y = b_0+b_1X_1+b_2X_2+b_3X_3+b_{11}X_1^2+b_{22}X_2^2+b_{33}X_3^2+b_{12}X_1X_2+b_{23}X_2X_3+b_{13}X_1X_3 \quad \quad (1)$$

where Y is the predicted response, X1, X2, X3 are independent variables, b0 is the offset term, b1, b2 and b3 are linear effects, b11, b22 and b33 are squared effects and b12, b23 and b13 are interaction terms. With F-value at a probability (P) of 0.001, 0.01 or 0.05 the significance of all terms in the polynomial functions were assessed statistically. The regression coefficients were then used to generate contour maps from the regression models. The three-dimensional (3D) plots were generated by keeping one variable constant at the centre point and varying the other variables within the experimental range. For optimisation in wine production, the three factors of temperature, pH and incubation time, which highly influence the wine fermentation were considered. Using CCD, a total number of twenty experiments with different combinations of temperature, pH and incubation time were performed in triplicate. The

encoded values of the initial temperature, pH and incubation time, as well as the mean values of the triplicate results obtained in the twenty performed assays executed in terms of ethanol production have been recorded.

Determination of total phenolics content

The total phenolic content (TPC) by the Folin-Ciocalteu (FC) reagent was measured according to Di Stefano (1989) and Singleton (1999) method. An aliquot of 20 μL samples was taken along with 150 μL of Folin- Ciocatlteu reagent in a spectrophotometer cuvette, 600 μL of a 15 % Na_2CO_3 solution and distilled water were added to make the final volume of 3000 μL. The increase in absorbance was measured at 784 nm and the concentrations of TPC after 2 hours and expressed as mg/L gallic acid equivalent (CE), were determined by a calibration curve graph.

Total flavonoid content

The total flavonoid concentration was measured. Firstly 5.0% 75 μL of $NaNO_2$ was added, shortly followed by 10% 150 μL of $AlCl_3 \cdot 6H_2O$ and left for 5 min. After adding 500 μL of NaOH, the absorbance was measured at 510 nm. The total flavonoid content of samples was measured using a mg catechin equivalent of 100 mL.

Determination of DPPH radical scavenging activity

The effect of the extracted sample on DPPH radical was estimated according to the procedure described by Brand – William *et al.* (1995). The sample extract (0.1 mL) was added to 3.9 mL of DPPH (100 μM) in ethanol that was prepared daily. After incubation time of 45 min the absorbance was determined at 515 nm. Ethanol was used as blank and 0.1 mL ethanol solution and 3.9 mL of DPPH solution were used as control. The inhibitory percentage of DPPH was calculated according to the following equation (1):

DPPH scavenging effect (%) = [1- (absorbance$_{sample}$ / absorbance$_{control}$)] X 100

Metal determination

Of several methods for metal determination, techniques of atomic spectroscopy are the most sensitive and rapid (Acetoy *et al.*, 2002). Metal content of samples such as copper, lead, ferrous and zinc were determined using Flame Atomic Absorption Spectroscopy (AAS) (Perkin Elmer, A Analyst-200) (Table 3). Precision of analysis usually very good, being on average >1%, for all elements considered at mg/L concentration levels.

Scanning electron microscopy

For the microscopic studies, samples were freeze dried using a Lyophilizer and then transferred to a desiccator. After the samples were dehydrated, they were mounted over the stubs with double-sided conductivity tape and were observed using a scanning electron microscope (model FEI Quanta -200 MK2) at a specific magnifications of 400x.

Statistical analysis

Data was presented as mean ± SD for three replications for each sample. The Fisher Least Significance Test was used to check equality of variances and one way ANOVA was used to estimate statistically significant difference (p≤0.05).

Result and Discussion

RSM

RSM is a sequential procedure with an initial objective of leading the experimenter rapidly and efficiently to the general vicinity of the optimum. The information about few variables identified during screening as having the greatest impact on performances can be judged through optimization of experimental condition (Ghosh *et al*., 2012). Effects of temperature, pH and time on ethanol production were investigated using RSM. The importance of individual factors, the appropriateness of this functional form and sensitivity of the response to each factor can be accessed through statistical influence techniques. The effect of each factor and their interaction were analysed using the analysis of variance (ANOVA). The calculated regression equation for the optimisation of fermentation condition showed that the ethanol (Y, %v/v) production is a function of temperature (X1, ^{o}C), pH (X2) and time (X3). By applying multiple regression analysis on the experimental data, the following second order polynomial equation was found to represent the ethanol production.

$$Y_i = 6.69 + 0.25X_1 + 0.05X_2 + 0.16X_3 - 0.64X_1^2 - 0.69X_2^2 - 1.38X_3^2 + 0.010X_1X_2 + 0.063X_2X_3 - 0.005X_1X_2 \dots \dots (2)$$

Statistical significance of wine from household waste fermentation model is explained by analysis of variance. The effectiveness of the model can be checked by different criteria. The R^2 values for all these response variables were higher than 0.90, indicating that the regression model explained the reaction well. The Model F-value of 54.21 implies the model is significant. There is only a 0.01% chance that a high "Model F-Value" could occur

due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A^2, B^2, C^2 are significant model terms. The "Lack of Fit F-value" of 4.96 implies there is a 5.18% chance that a "Lack of Fit F-value" this large could occur due to noise. The "Pred R-Squared" of 0.7949 is in reasonable agreement with the "Adj R-Squared" of 0.9618."Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Our ratio of 18.198 indicates an adequate signal. This model can be used to navigate the design space. Nature of fit of the regression model is determined by the adjusted co-efficient of determination (R^2_{adj}). The high value of R^2_{adj} 0.9618 indicates the goodness of fit of the regression equation. The predicted co-efficient of determination (R^2_{pred}) value was 0.7949. As per thumb rule, these values should be within 0.2 of each other. Here the difference was less than 0.2 and so the model is significant. The probability of p-value for models of less than 0.05 indicates that models are significant, p-value less than 0.0001 indicate the models are highly significant. So our model p value <0.001 is significant. The words lack of fit refers to the fact that the simple linear regression model may not adequately fit the data. There is evidence that the simple linear regression model is not appropriate because the treatment means do not appear to have a straight-line relationship with the amount of the treatment factor. Our p value for lack of fit of model was insignificant it indicted that our experimental model system was statistically significant.

Temperature tolerance for growth of yeast and fermentation are strongly strain dependent (Rousseau *et al*., 1992). In batch culture, waste ethanol production was highest (6.69%) at 32°C. Initially the rate of ethanol production at 34°C was higher than 32°C (data not shown). It has been reported that ethanol producing yeast could grow rapidly at temperature 25-33°C and again ethanol production was high at 30-37°C (Ozcelik *et al*., 1996). But in our experiment we observed initially the yeast growth and then ethanol production was at the same temperature. Actually wine yeast is very particular for their optimum condition (Torija *et al*., 2003). This optimum condition also varied with strains. 36°C was not the favourable temperature for our yeast strain and 28°C was also another stressful condition. At 30°C and 34°C, the yeast cell was moderately activated and ethanol concentration was not high (data not shown).It can be said that, at 32°C the cell viability, growth rate, exponential phase, enzyme activity and membrane function was well controlled for this strain. The response surface and the contour plot show the conditions for ethanol production. The interaction
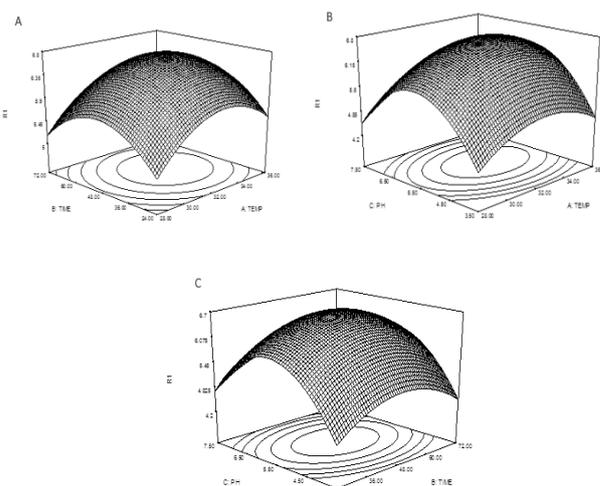

Figure 1. Response surface and contour plot of (A) time vs. Temperature (B) pH vs. Temperature (C) pH vs. time on ethanol production

between the two variables and their optimum level can be determined from Figure 1a which shows the interaction between the two variables i.e. temperature and time. The temperature 32°C and 48 h are the optimum conditions for highest ethanol yield. With higher temperature and longer incubation time, the production of ethanol decreased. Again with long time incubation at optimum temperature, the ethanol concentration also decreased because ethanol was utilized by the yeast. Figure 1b shows the interaction between temperature, pH and their effect on ethanol production. For highest ethanol yield, the temperature of 32°C and pH 5.5 are found to be the optimum conditions. It was observed that with increase in pH and temperature, ethanol production increased, upto a certain limit, beyond the temperature 32°C and pH 5.5 production of ethanol decreased.

The growth of biomass and ethanol production is highly affected by variations in pH. The yeast growth is affected by environmental pH which in turn was controlled by acetic acid or lactic acid produced by yeast (Narendranath *et al*., 2001). Figure 1c shows the interaction between pH and time and the optimum pH for the highest ethanol production was 5.5. Low pH and long time of incubation decreased the production level. The surrounding pH influenced the enzyme activity. During the phase of yeast growth, the key enzymes for metabolisms were influenced by pH (Kumar *et al*., 2004). According to the response surface graph, at pH 5.5 and 48 h incubation time ethanol produced was the highest. Table 3 shows the response of the variables, temperature, pH, time, temperature^2, pH^2, time^2 and temperature x pH, temperature x time which were checked with p-value of less than 0.05. But most of the values were significant and thus the whole model was significant.

Table 2. Antioxidant properties of wine from vegetable waste

	Phenol (mg/l)	Flavonoid (mg/l)	DPPH (%)
Wine from Vegetable waste	52.28	41.5	55.18

Temperature, pH and time were linear term of the model and had great influence on wine fermentation and therefore their p values were significant. The squared value of pH was most effective than the other parameters. The quadratic term of the model, the temperature^2, pH^2 and time^2 and their p value were highly significant. It means that the temperature, pH and time play a significant role in ethanol production in terms of quadratic relationship.

Total phenolics and flavonoids content

It is essential to know the chemical composition of wine in order to establish a potential relationship between its constituents and its role in beneficial biological activities enhancing human health benefits. The mode of action of phenolic compounds are mainly primary oxidation or through scavenging of free radical. The antioxidant properties of phenolic compounds stimulate the need to design strategies to enhance its content in plant tissues (Heredia and Cisneros-Zevallos, 2009). The Folin-Ciocalteu method measures the reduction of the reagent by phenolic compounds via the formation of a blue complex. The amount of phenol in wine is found to be 52.9 mg/l. But individually carrot peel, potato peel, pumpkin peel, and parwal peel show phenolic content 73.5 mg/l, 50.82 mg/l, 55.09 mg/l, 46.48 mg/l respectively. It was reported that the phenolic content of potato peel was 2.74± 0.03 mg GAE g^{-1} (Mohdaly *et al.*, 2010), total phenolic content in fresh carrot peels was approximately 1380 mg GAE/100 g dry weight (Chantaro *et al.*, 2008). Flavonoids, such as quarcetin and kaempferol are phenolic compounds that can be synthesis by plants as a response to the attack of pathogens and the level of phenolic compounds in plants depends on their maturity, stage, variety, and genetic factor among others. In this study, the amount of total flavonoid compounds in household waste wine was found to be 41.5 mg/l. But flavonoid content in carrot peel, potato peel, pumpkin peel and parwal peel were found to be 101 mg/l,89.75 mg/l,41.5 mg/l,38.75 mg/l respectively. Total flavonoids content in potato peel is 0.81+0.04 mgQEg^{-1} DW (Mohdaly *et al.*, 2010). Some fruit and vegetable contain lower amounts of phenolics and flavonoids which are influenced by a number of intrinsic and extrinsic factors (Jagtap *et al.*, 2011). The phenolic composition in different wines is varied

and determined by variety, environmental factors and winemaking processes including fermentation, maturation and aging conditions.

DPPH free radical scavenging assay

Free radicals involved in the process of lipid peroxidation are considered to play a major role in numerous chronic pathologies such as cancer and cardiovascular diseases. DPPH is considered to be a model of a stable lipophilic radical. A chain reaction of lipophilic radicals is initiated by lipid autoxidation. Antioxidants react with DPPH reducing the number of DPPH free radicals to the number of their available hydroxyl groups (Mohdaly *et al.*, 2010). The DPPH free radical scavenging assay has been widely used to evaluate antioxidant capacities. The DPPH free radical scavenging capacity of wine from vegetable waste is shown in Table 2.The extracts were capable of scavenging DPPH radicals in a concentration dependent manner. It showed 55.18 % DPPH scavenging capacity. These findings suggest that total phenolic content may be important contributors to the DPPH radical scavenging capacity of wine. We examined the antioxidant of raw vegetable peels extract before fermentation (Data not shown). Fermentation is responsible for a decrease of a drop in polyphenols content because of their adsorption onto yeast cell walls and the reaction with cell wall proteins (Czyzowska and Pogorzelski, 2002). The process of wine ageing and wine deacidification decreased the content of polyphenols as well. The use of pesticides and fertilizers may be responsible for decreasing of antioxidant molecule. The results of the DPPH free radical scavenging assay suggest that components within the wine, are capable of scavenging free radicals via electron- or hydrogen-donating mechanisms and thus should be able to prevent the initiation of deleterious free radical-mediated chain reactions (Mohdaly *et al.*, 2010). Antioxidant properties of compounds presents in wine from vegetable wastes are important as they delay, retard or prevent oxidative stress.

Heavy metal concentration

The determination of heavy metals in wine is routinely carried out in oenological laboratories. In order to make a safe globally marketable wine it becomes essential to keep in view of the legislations

Table 3. Elemental concentrations measured in wine from vegetable peel and law limits for the same elements

Element	Allowed concentrations of heavy metals in specific countries (mg/L)			Experimental vegetable peel wine value (mg/L)
	Italy	Germany	Australia	
Cu	10	5	5	0.357
Pb	0.3	0.3	0.2	0.123
Zn	5	5	5	0.285
Fe	-	-	-	3.385

Adapted from Acetoy *et al.*, 2002

permitting heavy metals globally. The mean elemental concentrations measured in the wine are shown in Table 3. The concentrations of all the measured elements are much lower than the allowed ones with the reported values to be: Cu- 0.357 mg/L, Pb- 0.123 mg/L, Zn- 0.585 mg/L and Fe- 0.285 mg/L. Elements like Zn had greater concentration while Pb is absorbed in small quantities, but both might be carried over into wine from the use of copper and zinc-based pesticides. However freshly fermented wine generally has low concentration of Cu due to the ability of dead yeast cells to absorb copper (Scollary, 1997). Lead (Pb) content was lower than the limit allowed and also in good agreement with those reported by Roses *et al.* (1997) for wines. However in this case the values are in compliance with the prescribed law limits. Iron (Fe) is found in substantial quantities in all wine varieties and its evaluation is of major importance due to the changes in stability caused by its effects on the oxidation and wine aging (Lara *et al.*, 2005). In the present study, iron concentrations are in concurrence with those of allowance values. Acetoy *et al.*, 2002 specified that the lead content should be less than 0.3mg/L, according to Italian legislation. In order to popularize and gain export rights in global markets, rules restricting metal content in wines must be fulfilled by producers. Thus this wine is suitable for consumption and export according to its heavy metals content.

Scanning electron microscope (SEM) studies on changes of structure during fermentation process

Due to the utilization of the starch converted to sugar by saccharification, the volume of the waste starch was reduced. Electron microscopic examination shows that due to the proliferation of yeast cells in the free medium inside surface of vegetable wastes, it has high populations of yeast cells on the external surface (Reddy *et al.* 2008) as observed during fermentation (Figure 2). This may be due to the greater availability of nutrients (sugars and nitrogenous compounds) near the surface of the support (Reddy *et al.*, 2011). In addition, the greater
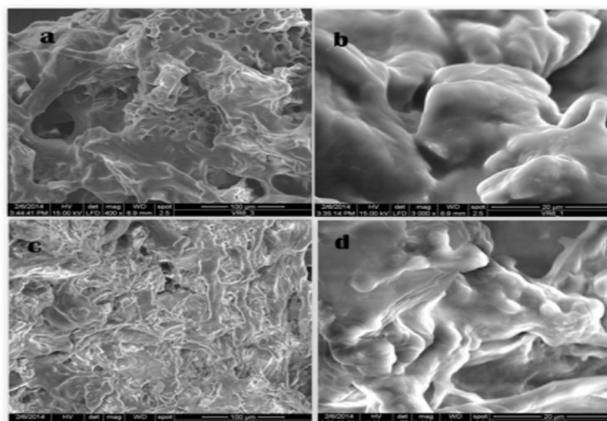

Figure 2a. Sacnning electron microscopic image of raw vegetable waste. b. Scanning electron microscopic image of raw vegetable waste (closer view). c. Scanning electron microscopic image of yeast inside the parenchyma the fermented vegetable waste. d. Scanning electron microscopic image of yeast inside the parenchyma of the fermented waste (closer view).

nutrient availability in the cavities of parenchyma cells is also an attraction to yeast cells, which encouraged yeast cells to migrate into the inner parenchyma cells during the process. Adhesion of *S. cerevisiae* is essentially dependent upon electrostatic interactions between the support and the normally negatively charged cell surface and the attachment of the yeast cells on the support may have occurred as a result of hydrogen bonding, entrapment of the cells in vegetable wastes, and the van der waals forces (Reddy *et al.*, 2011). Because of this, wastes are believed to have natural entrapment of yeast cells into the porous structure of the materials and due to physical adsorption by electrostatic forces between the cell membrane and the yeast cells. Similar results have been observed in yeast cells when used in apple pieces for wine preparations (Kourkoutas *et al.* 2001).

Conclusion

The results found in this work show that the optimum conditions for production of wine from household wastes of vegetable peels having maximum

yield 6.69% ethanol are temperature 32°C, pH 5.5 with incubation time of 2 days. The antioxidant potential of the wine was found to be significant and the concentration of heavy metal concentration is in concurrence with the allowed values. Electron microscopic studies revealed noteworthy structural degradation of starch penetration of the yeast cells into the inner parenchyma during fermentation.

Acknowledgement

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