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# Production and investigations of antioxidant rich beverage: utilizing *Monascus purpureus* IHEM LY2014-0696 and various malts

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**Abstract.** Antioxidant evokes numerous health benefits to the consumer as well as stabilisation of the beverages flavours. Therefore, this paper provides detailed information on the application of *Monascus purpureus* IHEM LY2014-0696 in combination with various malts in brewing antioxidant rich beverage (ARB). Starter culture Angkak was prepared by solid state bioprocessing (SSB). Single infusion method of mashing was used. Physicochemical parameters, volatile compounds, DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging activity and fermentability of the wort were evaluated. Distillation procedure based on pycnometry technique was use to quantify the alcohol content (%ABV). Fermentability of the wort was found to be 97.6 ± 0.46% whilst %ABV was equal to 5.42 ± 0.03. It was observed that ARB showed a strong DPPH radical scavenging activity of $1.00 \times 10^{-4}$ mol × equ ($R^2 = 0.91$) whereas $3.43 \times 10^{-5}$ mol × equ ($R^2 = 0.81$) for wort. The strong antioxidant activity (AOA) is thought to be caused by pigments produced by *M. purpureus* IHEM LY2014-0696 and other compounds originated from the malts and hops utilised in brewing ARB. A total of 4 volatile compounds were identified in the present study. Incidence of microbial load ranged from 2.14 ± 0.04 × 107 and $0.8 \pm 0.1 \times 10^5$ for *M. purpureus* IHEM LY2014-0696 and bacterial respectively was observed in the ARB. This study contradicts some previous ones, as the ARB brewed did not take the red pigment produced by the *M. purpureus*. Panellists generally expressed their acceptance for the ARB as they assessed it as a new product, moreover, taking account its health benefits.

**Key words:** Volatile compounds, Fermentability, Angkak, Solid state bioprocessing, Pigments

## INTRODUCTION

The genus *Monascus*, subdivided into four species: *M. pilosus*, *M. purpureus*, *M. ruber* and *M. floridanus*, belongs to the family *Monascaceae*, the order *Eurotiales*, the class Ascomycetes, the phylum Ascomycota, and the kingdom Fungi (Hawksworth & Pitt, 1983; Barnard & Cannon, 1987; Lin et al., 2005), can be cultured on a substrate containing starch. These starches are broken into different metabolites. The pigments from which any product fermented with *Monascus* species derives its distinctive red

colour are among them. For centuries, product like rice (Red yeast rice (RYR)) cultivated on red mould was stapled dietary and food additives in Asia continent (Erdoğrul & Azirak, 2004). Moreover, it is used as preservatives in meat and fish, to add colour and flavour to food. RYR is not known by Asians alone but other continents as well. As such, different names are used in identifying the same product, i.e. the Japanese, Chinese, Europeans, and Americans called it Beni-Koji or Red-Koji, *angkak*, *Rotschimmelreis*, Red Mould respectively, (Bakosova et al., 2001). According to Palo et al. (1960), RYR originated from China, however was kept in secrecy probably due to the fear of revenue loss from this product, if other Asian countries get to know this technology. For a decade, the Chinese and the Philippines utilize RYR as starters in brewing *Anchu* and *somsu* alcoholic beverages respectively.

In brewing, the starter culture transform wort into beverage, moreover, that helps to develop volatiles compounds (esters) which give any beverage its distinctive flavour with contributions from other ingredients (hops, species, etc). Alcoholic beverages brewed with *M. purpureus* has higher antioxidant activities than beer brewed with conventional yeasts (Takeshita et al., 2016) and these beverages (food) tend to play a substantial role which evokes health benefits i.e. Cholesterol Lowering, Anti-Diabetic Activity, Effects on Osteoporosis, Anti-Inflammatory inhibitions (Arunachalam & Narmadhapriya, 2011). Moreover, *M. purpureus* can withstand stresses (higher temperature, low pH) than other microorganisms, which makes it more suitable in brewing (Huang, 2000).

Antioxidant plays a role in flavour stability, one of the important characteristics in beverages (Zhao et al., 2010). Malt specifications are vital to brewers all over the globe. Different types of beverage are produced from the variety of malts since malts have different properties and each contribution is crucial in developing sorts of products. However, the geographical location, variety of barley, malting technology could not be ignored as crucial factors that contribute to these properties variation.

To the best of our knowledge, no research has been conducted to investigate the kind of beverage produced from the combined malts (Pilsner, Vienna, Biscuit) and *M. purpureus* (*angkak*: see Fig. 2, b). Therefore, the first objective of this study was to brew antioxidant rich beverage (ARB) from different malts using *angkak* as starter. The second objective was to analyse the antioxidant activity, the microbial load and sensorial properties of the beverage.

## MATERIALS AND METHODS

Malts (Pilsner, 181 g, Vienna, 230 g, Biscuit, 200 g), hops (Perle hops, 3 g) and rice (Miracle seeds, Mistral trading, Moscow, Russia, 100 g) used in this study were purchased from Beerfan brewery company and supermarket respectively, whereas, Monascus purpureus IHEM LY2014-0696 was taken from the Belgium Co-ordinated collection of microorganisms (BCCM-IHEM) of the Scientific Institute of Public Health-Section Mycology and Aerobiology, Belgium and maintained on Potato Sucrose Agar (PSA) (100 g of potato, 10 g of sucrose, 10 g of agar, 350 mL of distilled water, pH 5.6) plates.

Starter culture was prepared according to the method described by Takeshita et al., (2016) with some modifications. Solid state bioprocessing (SSB) was performed in preparing the starter called Angkak (Fig. 2, b): 50 g of rice was weighed and soaked in

200 mL distilled water in a 300 mL Erlenmeyer flask for 1 hour. It was then, drained, and autoclaved at 121 °C for 15 min. Autoclaved rice was cooled to ambient temperature and transferred into a plastic Petri dish (Fig. 2, a). M. purpureus IHEM LY2014-0696 (0.2 g) colonies on the PSA plate was cut off with the aid of using a sterile knife and inoculate onto the autoclave rice, and incubated at 30 °C for 6 days to prepare the angkak seed. Again, 50 g of rice was weighed and soaked in 200 mL distilled water for 1 hour then drained, and autoclaved at 121 °C for 15 min. Autoclave rice was once again cooled to an ambient temperature and transferred into plastic Petri dish. 10 g of angkak seed was uniformly mixed with the steam rice with the aid of sterile spatula under lamina flow hood. The mixture was then incubated at 32 °C with 90% humidity for 5 days. The resulting red rice (angkak) was then soaked in 100 mL distilled water for 3 days at room temperature. Water was then drained and angkak ready for pitching. Flowchart of the entire production process is shown in Fig. 1.

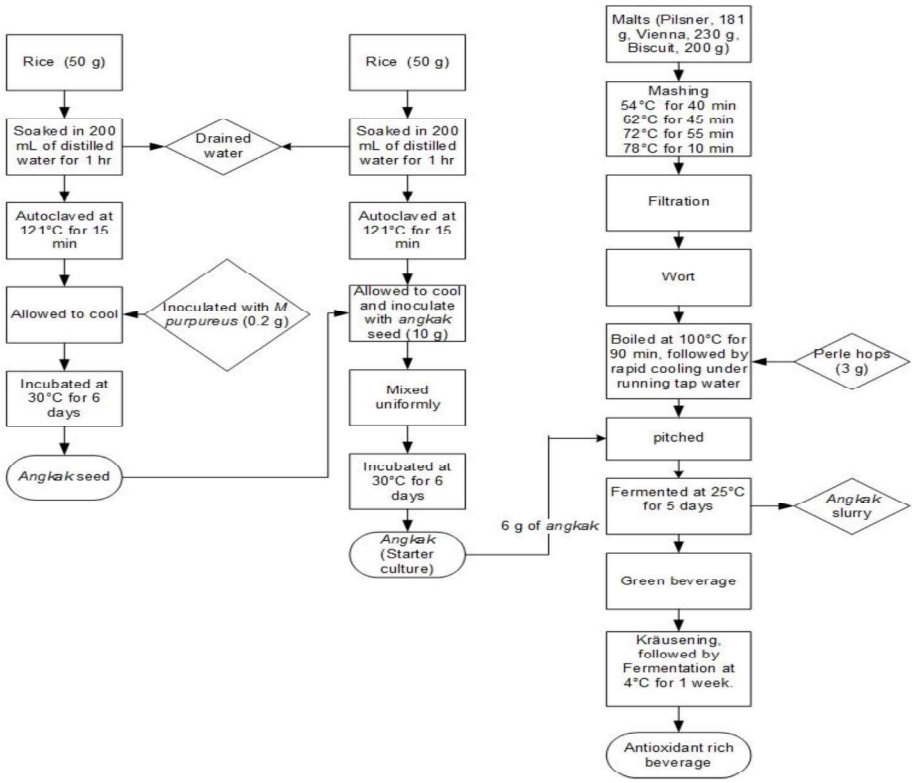


**Figure 1.** Flowchart of *angkak* and antioxidant rich beverage production.

Single infusion method of mashing was used, malts was mixed with 1.5 L distilled water in mash turn and heated up to 45 °C for 30 minutes. The temperature was increased upto 54 °C and 62 °C with a step times of 40 and 45 min respectively. After these step times, the temperature was once again increased upto 72 °C for 55 mins. Finally, the temperature was increased to 78 °C for 10 minutes. Filtration was carried out whilst 1 L distilled water was used for the sparging process. Wort was then boiled at 100 °C for 90 min, Perle hops (3 g) were added 80 min before the end of the boiling time. Wort was allowed to cool to a desired temperature by placing the boiled wort under

running cold water. Wort was then transferred into 5 L sterilized improvised fermenter (Fig. 3) equipped with airlock bubbler. The entire processes of ARB production is shown in Fig. 1 as well.

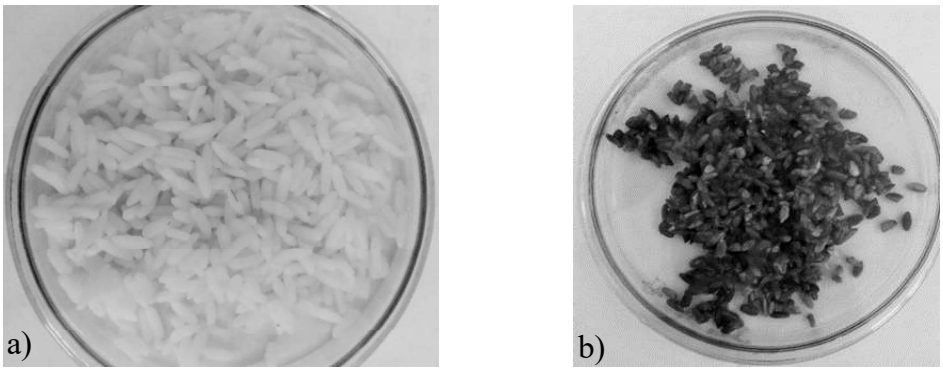


**Figure 2.** Autoclaved rice (a) and rice cultured with M. purpureus IHEM LY2014-0696 (angkak) (b).

Angkak (6 g) was measured under lamina flow hood, pitched and mixed thoroughly with the wort. Fermentation was carried out at a temperature of 25 °C for 5 days. Filtration was performed to separate green beverage from the fermented angkak. Kräusening was practice to carbonate the green beverage, where 2 mL of sterile wort was added to green beverage and ferment at 4 °C for 1 week. The matured beverage was termed antioxidant rich beverage (ARB).

**Figure 3.** Hand-made fermenter for fermentation of wort pitched with *angkak*.

Physicochemical parameters (oBrix, Titratable acidity (TA), colour), volatile compounds composition and DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging activity were evaluated according to the method described by Adadi et al. (2017b). Alcohol content (%ABV) was quantified by distillation technique using pycnometry method (GOST, 2011). Fermentability of the wort was determined according to formula stated by (Briggs et al., 2004).

Serial dilution was performed mixing 1 mL of ARB with 9 mL of sterile peptone physiological saline solution (5 g peptone, 8.5 g NaCl, and 1,000 mL distilled water, pH = 7.0) and homogenized with the help of pipette. Decimal dilutions were plated. Aerobic mesophiles were enumerated by pour plate on nutrient agar (15 g peptone, 3 g yeast extract, 6 g NaCl, 1 g dextrose and 12 g agar, pH = 7.5) and brain heart infusion agar (HiMedia, Vadhani, India) incubated (Memmert GmbH, model 30-1060, Germany) at 30 °C for 3 days. *M. purpureus* IHEM LY2014-0696 was enumerated by spread plate on PSA and incubated at 25 °C for 5 days. Plates with countable colonies (30–300 colony

forming units (cfu)) were removed and counted according to the method describe by Adadi & Obeng (2017). Sensorial analysis was performed according to previous method use in Adadi et al. (2017a) and Adadi et al. (2017b).

The data were statistically analyzed by Origin statistical software (version 8.1). Values were presented as mean ± standard deviation (S.D.). One-way ANOVA and Fisher test were conducted to test the means results for sensory analysis at 5% significance.

## RESULTS AND DISCUSSION

Changes in physiochemical parameters (pH, titratable acidity, °Brix, colour, fermentability and ABV) of the wort and final beverage are shown in Table 1. There was a gradual decrease in average pH value from 5.53 ± 0.06 to 4.57 ± 0.06 for wort and final beverage respectively. TA increased from 0.83 ± 0.01 to 1.88 ± 0.02% for wort and beverage. Substance in wort and beer can affect their buffering abilities, notable the residue of proteins like aspartate and glutamate (Bamforth, 2001). According to Adadi et al. (2017b), utilisation of substrate in wort and accumulation of metabolites is responsible for fluctuations in TA.

**Table 1.** Physicochemical characteristics of wort and antioxidant rich beverage

| Parameters | Wort | beverage |
|---|---|---|
| pH | 5.53 ± 0.06 | 4.57 ± 0.06 |
| Titratable acidity (%) | 0.83 ± 0.01 | 1.88 ± 0.02 |
| °Brix | 13.8 ± 0.17 | 0.33 ± 0.58 |
| Colour (EBC units) | 47.7 ± 0.13 | 33.5 ± 0.24 |
| Alcohol (% ABV) | N.A | 5.42 ± 0.03 |
| Fermentability (%) | 97.6 ± 0.46 | N.A |

N.A-not applicable, (n = 3).

The enzyme GOX in *M. purpureus* IHEM LY2014-0696 could produce gluconic acid, which might affect the pH and subsequently TA. Dimerumic and γ-aminobutyric acids found in *M. purpureus* fermented products could contribute to the fluctuation of both the pH and TA (Aniya et al., 2000; Su et al., 2003).

According to Briggs et al. (2004) starter culture (*angkak*) transforms simple sugars in wort to alcohol. °Brix measures amount of sugar in wort and since starters utilize these sugars a decrease was observed from 13.8 ± 0.17 to 0.33 ± 0.58 for wort and final beer respectively.

It was observed during the fermentation that; the fermenting wort was hazy due to the activity of *M. purpureus* IHEM LY2014-0696. Furthermore, a thick-light layer was seen covering the surface of the fermenting wort after 24 hours of fermentation time, which probably might be the mycelium of the *M. purpureus* IHEM LY2014-0696. In our previous works (Adadi et al., 2017a; Adadi et al., 2017b), we did not observed any layer, when we were working with conventional starters (*S. cerevisiae* and Kölsch Yeast). The mycelium could alter the colour of the fermenting wort. There was a change in colour from 47.7 ± 0.13 to 33.5 ± 0.24 EBC units for wort and beverage respectively which differs averagely ($P < 0.05$). After the end of primary fermentation, it was observed that, *angkak* settled at the bottom of the fermenter.

The alcohol content (% ABV) 5.42 ± 0.03 was measured in the present study. Takeshita and his colleagues, also measured alcohol (% v $v^{-1}$) 8, 7.9 and 8.6 when they brewed their alcoholic beverages using polished rice, wild rice and black rice respectively (Takeshita et al., 2016). It was noted that, the kind of starting raw material, affected the ethanol content of the final beer. Variety of starters (yeast, bacteria or fungi) utilizes nutrients in wort to support growth, generate energy (Boulton & Quain, 2007) in other to transform wort to beverage.

Fermentability of the wort was found to be 97.6 ± 0.46% in this study (Table 1). This percentage indicates that not all the sugar in the wort was consumed by the *M. purpureus* IHEM LY2014-0696. Inability of conventional yeasts to ferment wort 100% was report by MacWilliam (1968) when various wort from different countries were used in his work. The procedure and material used to produce wort make it inexorable that is complex was not well characterised. Sugar spectra in these wort differs (Hoekstra, 1974), likewise their fermentability. Fraction of unfermentable sugars (dextrins) accounts roughly 25% in wort. However, it involves both mono (arabinose, xylose and ribose) and trisaccharides (panose and isopanose and β-glucans). Unfermented substances are inevitable in brewing process as majority of α-(1 -6) linkages of malt amylopectin survive wort production intact (Enevoldsen & Schmidt, 1974).

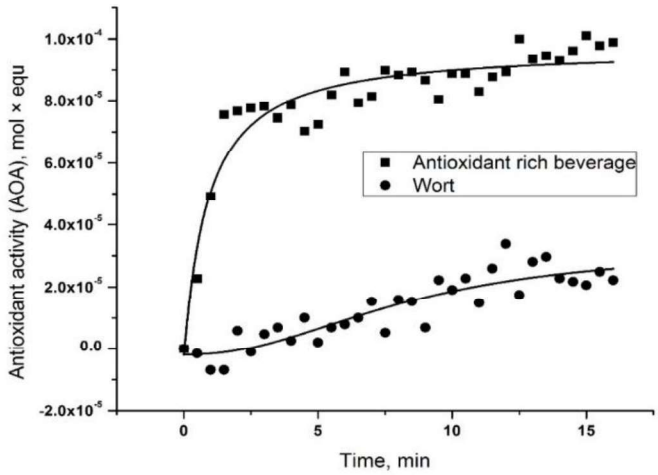


**Figure 4.** DPPH radical scavenging activity of samples vs. time.

The antioxidant activity (AOA) of the wort and beverage brewed with *angkak* was determined. The DPPH radical scavenging activity of the beverage and wort is shown in Fig. 4. It was observed that beverage showed strong DPPH radical scavenging activity of $1.01 \times 10^{-4}$ mol × equ ($R^2 = 0.91$) whereas $3.43 \times 10^{-5}$ mol × equ ($R^2 = 0.81$) for wort. Alcoholic beverage brewed with *beni koji* (*angkak*) showed stronger AOA (3,400 µM Trolox equ.) than the beverage brewed with *ki koji* (1,700 µM Trolox equ.) (Takeshita et al., 2016). The beverage brewed in the current study exhibited stronger AOA than Baltica beer we examined in our previous work (Adadi et al., 2017a). The AOA is thought to be caused by the pigments produced by *M. purpureus* IHEM LY2014-0696 and other compounds originated from the malts and hops utilised in brewing the beer. According to (Sato et al., 1992; Juzlova et al., 1996; Watanabe et al., 1997), the genus

*Monascus* produced six pigments (rubropunctatin, monascorubrin, rubropunctamine, monascorubramine, monascin and ankaflavin) of polyketide origin which are grouped into 3 major categories.

Monacolin K, γ-aminobutyric acid (GABA), and dimerumic acid (antioxidant) were found in food fermented by *M. purpureus* (Aniya et al., 2000; Su et al., 2003) and are thought to have AOA. Antioxidants are related with the prevention of cardiovascular, neurological diseases, cancer and oxidative stress dysfunction (Bolck, 1992; Diplock, 1995). AOA of beverage decreases as the storage time increases and this phenomenon was observed by Ditrych et al. (2015).

A total of 4 volatile compounds were identified in the presented study (Table 2). Among the volatiles identified 3 were alcohols and 1 ester. These compounds are thought to contribute to flavour of beverage and their availability depends on raw material, mashing style, and fermentation conditions under which the beverage is produced (Adadi et al., 2017a; Adadi et al., 2017b).

**Table 2.** Volatile compounds identified from antioxidant rich beverage

| No | Volatile compounds | Retention time (min) | Formular |
|---|---|---|---|
| 1 | Ethanol | 1.51 | $C_2H_6O$ |
| 2 | 2-Methyl-1-propanol | 2.27 | $C_4H_{10}O$ |
| 3 | Ethyl Acetate | 2.31 | $C_4H_8O_2$ |
| 4 | 3-methyl-1-Butanol | 3.27 | $C_5H_{12}O$ |

According to Briggs et al. (2004) yeast metabolites, which contribute to beverage flavour, are diversed chemically and consisted of organic acids, medium chain-length aliphatic alcohols ('fusel alcohols'), aromatic alcohols, esters, carbonyls and various sulphur-containing compounds. It could be obvious that the metabolites produced by *M. purpureus* IHEM LY2014-0696 during fermentation turned to have an inhibitory effect on the formation of these volatiles. Moreover, we practiced Kräusening during the carbonation, this can be another factors why we identified 4 volatiles in this study. Bottles lacks the ability to hold all the gases and volatiles formed during the secondary fermentation as keg, hence the volatiles formed ended up escaping from the bottle (due to pressure built up by the gases formed). In our previous works, 32 volatile were identified in Kölch fruit beer (KFB), (Adadi et al., 2017b) whereas 11 in low alcoholic beer and, 22 sorghum beer (Adadi et al., 2017a) which were all carbonated using kegs.

The incidence of microbial load ranged from $2.14 \pm 0.04 \times 10^7$ and $0.8 \pm 0.1 \times 10^5$ CFU $mL^{-1}$ for *M. purpureus* IHEM LY2014-0696 and bacterial respectively (Table 3) in the ARB. The incidence of *M. purpureus* IHEM LY2014-0696 was shown to be higher than the bacterial load but theren't significantly difference ($P > 0.05$). *M. purpureus* IHEM LY2014-0696 was the sole starter culture use in this study, and after the secondary fermentation there were still viable and capable of fermenting another sterilized wort hence the higher load on the ARB. However, conventional yeast are usually weakened by the alcohol content after secondary fermentation unlike *M. purpureus* which are able to withstand these higher ethanol content and other stressful factors (Huang, 2000). According to Hill (2009) and Suzuki (2011) beer has been recognized as a microbiologically stable beverage due to it range of antimicrobial hindrance that, under most circumstances, prevents the growth of pathogenic microorganisms. Other factors like presence of alcohol, bitter compounds in hops, low pH, played a vital role in keeping

beer safe from microbial contamination. Nevertheless, microbiological contamination sporadically occurs in beverages. Pasteurization was not performed in this study and this might be the reason for the incidence of growth we observed on the plated Petri dishes. We noticed, in our previous work that, the observation strict hygienic protocol could curb the incidence of microbial contaminations (Adadi et al., 2017b).

**Table 3.** Frequency of microbial load on ARB (Mean ± S.D × CFU $mL^{-1}$)

| Microorganisms | Mean | SD | CFU $mL^{-1}$ |
|---|---|---|---|
| Bacteria | 0.8 | 0.1 | $10^5$ |
| *M. purpureus* | 2.14 | 0.044 | $10^7$ |

Incidences of bacterial contamination were also observed by Elio (2013), in all beer brewed (young beer; centrifuged beer; beer with priming; bottle beer; bottle conditioned beer) excepting the wort. The presence of bacteria in our study assumed that, the contamination could have happened during the carbonation stage after the primary fermentation as we practiced Kräusening. Hygienic management in brewery and disinfection using hydrogen peroxide, peroxacetic acid and quaternary ammonium compounds (Praeckel, 2009) could help in dealing with the menace of contaminations.

Food acceptance by consumers goes in hand with the impressions of the five human senses. Beverage acceptance by consumers has a role to play by the impression of the human senses i.e sight, hearing etc. Raw materials, technological procedures and storage could positively or negatively influenced the sensorial of beverage. The variables accessed were different statistically ($P < 0.05$) and are tabulated in Table 4. Transparency was not good as the score 1.57 ± 0.53 tells it all. Poor filtration of wort and green beverage might be the reason for the poor feedback from the assessors. Colour was somehow satisfactory with mean scores of 2.14 ± 1.07. Colour and transparency go together as transparent beverage could definitely receive positive responses from panellists. The colour was like conventional beer (brewed from *S. cerevisiae*), did not take the colour (red) of the pigments produced by the *M. purpureus*. Flavour of the ARB was good (2.57 ± 0.98) and this could be attributed to contribution from the hops, malt, and some metabolites produced by *M. purpureus*.

**Table 4.** Scores of sensory properties of antioxidant rich beverage

| Parameters | Mean ± S.D |
|---|---|
| Transparency | 1.57 ± 0.53 |
| Colour | 2.14 ± 1.07 |
| Flavour | 2.57 ± 0.98 |
| Taste | 3.14 ± 1.35 |
| Hops bitterness | 4.29 ± 0.49 |
| Foaminess | 4.45 ± 0.98 |
| Overall acceptance | 3.86 ± 0.89 |


**Figure 5.** Sample of antioxidant rich beverage indicating foam formation.

Bitterness from hops balanced the sweet taste of wort when it was fermented with the starter and consumers could reject the sweet beer as they perceived it as juice.

Bittering compounds from hops is thought to play a vital role in stability of the beverage from microbial staling. Kräusening method of carbonation was excellent in this study, judging from the average scored of the beverage foaminess (4.45 ± 0.98). The image of the glass after pouring ARB with massive foam formation is shown in Fig. 5. Foaminess plays a vital role in acceptability of beverage and consumers might reject beverage with poor foaminess. Panellists generally expressed their acceptance for the beverage as they considered it a new product taking into account the health benefit (AOA potential). Flavour of the product also played a role in the acceptability of the beverage studied.

## CONCLUSION

Antioxidant rich beverage was brewed from various malts and traditional Asian fungus *M. purpureus* IHEM LY2014-0696. However, the beverage did not take the colour (red) of the *angkak* (*Monascus* pigments) and this contradicts previous work (Takeshita et al., 2016). A thick-light layer was observed covering the surface of the fermenting wort during the 24 hours of fermentation time. It was experimentally established that beer brewed showed a strong DPPH radical scavenging activity when compared with the wort. Incidence of microbial load was recorded in this study and this could be attributed to many factors. The beverage was accepted taking into account the health benefits and flavour.

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