Nutritional and logarithmic fungal count of brewery spent grain in different conservation techniques and brewery factories'

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Abstract

The objectives of this study was to investigate the effect of conservation practices (soaking, sun drying and ensiling) on nutritional and fungal load dynamics of wet brewers' grain (WBG). About 40 kilogram of WBG was collected from four breweries (Meta Abo, Habesha, Dashen and Bedele) located in different districts of the country. Sample was labeled and kept in a separate sterile bag, stored under – 20 ^{O}C. For soaking, two kg sample was uniformly treated with salt at 3% on DM basis and placed in cold water submerged plastic container and covered with a lead for partial aerobic storage. Two kg fresh sample was exposed to sun drying for three days, eight hours per day and another 2 kg fresh sample was ensiled in a plastic bottle. Molasses was added at the rate of 3% on DM basis to enhance proper fermentation and compaction inside the silo. All samples except sun dried, were subjected to oven drying at 55^{0}C for 72 hrs. Chemical compositions, dry matter losses and, fungal count (yeast and mold colony) were determined by standard laboratory procedure. To determine insacco degradability, samples were incubated 6, 12, 24, 48, 72 and 96 hours in nylon bags (6.5 × 14 cm, 50 µm pore size) placed ventral sac of thee cannulated Boran-Friesian steers (550 ± 15 kg live weight). The sample received from Meta Abo brewery factory had y higher ($p < 0.05$) acid detergent fiber, lignin and digestible organic matter in the dry matter (DM) but comparable dry matter, ash, crude protein (CP) and neutral detergent fiber with the other breweries. The minimal loss on DM and other nutrients, lower fungal, yeast and mold colony counts and the higher CP digestion kinetics was observed in ensiling techniques. If supply is not a constraint under local conditions, ensiling can be recommended as a best WBG conservation practice.

Introduction

Fresh brewer's grain is a cheap, non-conventional protein source which is becoming increasingly available to urban and peri-urban dairy farms in Ethiopia. In the year 2016/17, the annual total wet brewery spent grain (WBG) production from the twelve factories in Ethiopia was estimated at 26, 723 tons DM (Getu et al., 2018). Nutritionally, WBG is basically a lignocellulosic material and the major constituents of the byproduct are fibre (hemicellulose and cellulose), protein and lignin (Mussatto et al., 2006; Xiros and Christakopoulos, 2012). It is a heterogeneous substance particularly with respect to inter-brewery variation. This is due to a number of factors, such as the cereal variety, type of hops added, the malting and mashing regime, and whether adjuncts were employed during brewing (Steiner et al., 2015). Fresh brewer's grain contains 70–80% water by weight (Kunze, 210). This high moisture level poses two major difficulties when using it as a feed for different classes of animals. Firstly, transport of wet WBG can be costly, this being a particular reason why supply to local farmers as cattle feed has primarily been the main outlet. Secondly, the rich polysaccharide and protein content and the high moisture content of WBG make it susceptible to microbial growth and spoilage, this being identified as a potential problem area which might restrict its successful exploitation as livestock feed. This being the case, improper storage of WBG results in a large loss of dry matter and nutrients, characterized by an unpleasant odor, and even stimulates mold to produce mycotoxins (Asurmendi et al., 2013; Amézqueta et al., 2009). To this end a number of methods have been examined for their suitability to preserve WBG. These methods includes:

acid solutions such as lactic, acetic, formic and benzoic acids have been used, however, use of such chemicals under local context can be at odds with the livestock producers who not only had access to the chemicals but also lack proper knowledge and skill to handle such chemicals. Several physical methods of preservation have also been examined elsewhere, including oven-drying, freeze-drying, freezing and use of superheated steam (Bartolomé et al., 2002). Under local farmers conditions these are not affordable associated to initial procurement costs and costs of energy maintainace. In Ethiopia, farmers traditionally depend on either fresh wet brewery grain (WBG) or treated and soaked with a brine solution (Getu et al., 2018). According to same source sun drying and ensiling are also seldom used. The efficiency of these WBG conservation practices, particularly from perspectives of nutritional and fungal load dynamics have not so far been well documented. This study was, aimed to investigating nutritional variations of WBG received from different brewery sources and to exploring the best WBG conservation practices based on nutritional merits and fungal load dynamics.

Materials And Methods

Experimental location, sampling procedures and measurements

Two laboratory based experiments were conducted at Holetta agricultural research center (HARC), animal nutrition research and dairy microbiology research laboratories. For the first experiment about a kilogram of pooled fresh samples were received in an ice box over two days' samplings from Meta Abo, Dashen, Habesha and Bedele breweries. For each day about 500 g of pooled fresh sample was taken from three batches in order to make the samples more representative. Each sample was labeled and kept in a separate sterile bag, stored under -20 ^{O}C temperatures. In the second laboratory trial, three local WBG conservation practices including soaking, sun drying and ensiling were compared to a control, freeze-dried sample. After sub-sampling of some 40 kg of fresh sample brought from same brewery above in an ice box, 2 kg fresh WBG sample in five replication was immediately subjected to frozen inside the freeze dry system (Lyph Lock 4.5 L (77510-00)) between temperatures −50 °C and −80 °C, pressure lowered at 1,33x 10^{-3} mbar. Samples were allowed to dry until the moisture in WBG reached 4% by removing the ice in the frozen WBG through the sublimation process (Freeze Dry Systems Catalog, 2004). The remaining 30 kg of fresh sample was divided in to three equal parts and subjected to the first local storage treatment option called soaking in which 2 kg fresh WBG sample was uniformly treated with salt at roughly 3% on DM basis and placed in cold water submerged plastic container of 2 kg capacity, finally covered with a lead for partial aerobic storage. To mimic local farmers' practice, cold water re-filling was maintained for over fifteen storage days. For sun drying technique, 2 kg fresh WBG sample was exposed to sun for about three days, eight hours a day. Sun dried brewery spent grin was prepared by using a clean sterile plastic sheet with the recommendation Boessinger et al. (2005). Another 2 kg fresh WBG sample was ensiled in a plastic bottle of 2 kg capacity. Molasses was added at the rate of 3% on DM basis to enhance proper fermentation and compaction inside the silo, sealed with caps and left to properly ferment under anaerobic conditions for about 42 days. Samples from ensiled WBG were

subjected to PH reading upon opening each silo to ensure whether or no proper fermentation has taken place. In each conservation technique, samples were replicated five times.

In-situ CP degradability characteristics of brewer's grain conserved under different conservation

The crude protein degradability was carried out through Ørskov and McDonald (1979) procedure. Three cannulated Boran-Friesian steers (550 ± 15 kg live weight) were used to measure the degradability of the feed. The steers were fed with maintenance diet i.e., *adlibitum* natural pasture hay (6.8% CP) and 2 kg concentrate (20% CP) head^{-1} day^{-1}on DM basis. All samples were ground to pass through 2 mm sieve size. Three-gram sample was placed into nylon bags (6.5 × 14 cm, 50 µm pore size) and incubated for 6, 12, 24, 48, 72 and 96 hrs. All bags were subjected to hand washing using tap water until clean water comes out of the washing. The bags with residues were dried at 55°C for 72 hours in an air forced oven, hot weighed and finally the residues recovered for further CP analysis. Ruminal CP degradability characteristics were calculated as the difference of the DM and CP in the residues and original samples.

Laboratory analysis

Brewers' spent grain samples from the different brewery factory and conservation practices were dried in a forced-ventilation oven (55 °C for 72 h) and ground to pass through 1 mm, Cyclotec sample mill screen (Tecator 1093, Tecator AB, Hoganas, Sweden). All samples and residues after degradability were analyzed for DM, total ash and crude protein (CP) by using AOAC (1990) procedure. Neutral detergent fiber (NDFom-NDF), acid detergent fiber (ADFom-ADF) and lignin (pm-Lignin determined by oxidation of lignin with permanganate) were determined by Van Soest and Robertson (1985) procedure. Tilley and Terry (1963) two-stage in-vitro digestibility technique was employed to analyze and calculate the digestible organic matter in the dry matter. Metabolizable energy (ME) was estimated as ME (MJ/kg) =0.16 x DOMD (dry matter digestibility) (McDonald et al., 2002). Dry matter losses were calculated as a difference of DM for the fresh WBG sample (control) and the same samples that were subjected to aerobic and anaerobic conservational practices. Yeasts and molds were counted by pour plating. The samples (25g) were dissolved in 225 ml of peptone water. Potato Dextrose agar medium was injected with 1ppm per each 100 ml of agar with chloramphenicol and streptomycin to restrict bacterial growth (FAO, 1997). Plates were incubated aerobically at 28±1°C for 3 day and growing molds and yeast colonies were directly counted (MoH, 2010).

Statistical analyses

The model used for lab trial was a completely randomized design with five replications: $Y_{ijk} = m + C_i + e_{ij,}$ Where; m = Overall mean; C_i= Effect of brewery source (experiment-1) and/or effect of local storage practices (experiment-2); e_{ij} = Random error.

In situ CP degradability trial was fitted into the exponential models of Ørskov and McDonald (1979) as: $P = a + b (1 - e^{-ct})$; where; P= CP disappearance in rumen at time t; a = the washing loss fraction; b = the

slowly but potentially degradable fraction; c = the rate at which the "b" fraction degraded (in % h^{-1}). Similarly, effective CP degradability was calculated applying the equation of Ørskov and McDonald (1979) as ED = a + b[c / (c+k)], Where; k = the rumen outflow rate (3 % per h). Data from all trials were subjected to analysis of variance using the general linear model (GLM) procedures of statistical analysis system, version 9.3 (SAS, 2014). Mean separations were made using least significant differences (LSD) analysis at $p \leq 0.05$.

Results

Nutritional variations of brewers' grain collected from different breweries

The nutritional composition of brewery spent grain is significantly affected ($p<0.05$) by brewery factory and presented in Table (1). The spent grain from Meta Abo and Dashen brewery factory had the greater dry matter (DM) but lower ($P <0.05$) neutral detergent fiber (NDF) content than the Habesha D/B and Bedele breweries. Ash content of the brewery spent grains was in the order of Meta Abo = Habesha D/B > Dashen > Bedele; while the CP content was in the order of Meta Abo = Habesha D/B > Dashen > Bedele ($p< 0.05$). The highest ($p < 0.05$) acid detergent fiber (ADF) content of BSG was observed in Habesha brewery factory than the other factory while the lignin content was highest in Habesha and Bedele brewery factories. The DOMD values differ ($p < 0.05$) among breweries being in the order of Meta Abo > Dashen > Bedele, while the value for Habesha differ only with Meta Abo.

Table 1:-Chemical composition and in-vitro digestibility (g/kg DM) of brewery's spent grain from different factory

Brewery factory	DM	Ash	CP	NDF	ADF	Lignin	DOMD
Meta Abo	242^{a}	46^{a}	265^{a}	630^{b}	252^{d}	66^{b}	699^{a}
Habesha D/B	208^{b}	44^{a}	263^{a}	673^{a}	315^{a}	76^{a}	655^{bc}
Dashen D/B	240^{a}	37^{b}	235^{c}	636^{b}	271^{c}	51^{c}	668^{b}
Bedele	219^{b}	33^{c}	236^{b}	677^{a}	293^{b}	72^{a}	630^{c}
SEM	9.6	4.5	6.3	8.5	11.9	6.7	15.3
P-value	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0006

^{a,b,c}Means within a column with different superscripts differ ($p < 0.05$);DM=Dry matter; CP=Crude protein; NDF=Neutral detergent fiber; ADF=Acid detergent fiber; DOMD=Digestible organic matter in the dry matter; D/B= Debre Birhan; SEM = Standard error of the mean

Chemical composition of brewer's grain stored under different conservation practices

The effects of conservation practices on the chemical composition and logarithmic fungal count are presented Table (2). The dry matter content of brewery spent grain (BSG) was in the order of control > ensiled > sundried > soaked, while crude protein content of BSG was in the order of control = ensiled > sundried > soaked ($p< 0.05$). The values of digestible organic matter were similar between the control and ensiling treatment ($p > 0.05$. Dry matter loss was the lowest in ensiling and the highest in soaking ($p< 0.05$). The current results indicated that ensiling preserves fresh WBG better than soaking and sun drying.

Table 2:- Chemical compositions and DOMD (g/kg DM) of spent grain under different conservation practices

Parameter	Control	Soaking	Ensiling	Sun drying	SEM	P-value
DM	247^{a}	217^{d}	240^{b}	226^{c}	7.5	<0.0001
Ash	45.3^{c}	55.6^{b}	63.0^{a}	38.8^{d}	1.20	<0.0001
CP	260^{a}	216^{c}	265^{a}	231^{b}	3.5	<0.0001
NDF	635^{b}	587^{c}	610^{bc}	719^{a}	13.8	<0.0001
ADF	246^{c}	267^{b}	245^{c}	297^{a}	5.4	<0.0001
Lignin	62.3^{b}	65.0^{ab}	62.0^{b}	66.9^{a}	1.02	<0.0180
DOMD	687^{a}	630^{b}	693^{a}	637^{b}	4.6	<0.0001
DML (%)	-	12.4^{a}	2.9^{c}	8.5^{b}	0.62	<0.0001

$^{a-d}$ Means with in a row with different superscripts differ ($p<0.05$); Control= freeze dried; DM= Dry matter; CP=Crude protein; NDF=Neutral detergent fiber; ADF= Acid detergent fiber; DOMD=Digestible organic matter in the dry matter; DML=Dry matter loss; SEM =standard error of the mean

Fungal load dynamics of brewery spent gain conserved under different practices

Fungal load dynamics of brewery spent grain was affected by the different conservation practices and presented in Table (3). Yeast count in brewery spent grain was in the order of soaking > sun drying > ensiling = control ($p< 0.05$). The minimum number of mold count ($p<0.05$) was observed in the control group and the maximum number of mold count was also recorded in soaking than the other conservation practices. The total fungal count in brewery spent grain was lowest in the control than the other conservation practices. This result also suggests that ensiling will be a better storage practice than sun drying and soaking.

Table 3:-Effect of different brewer's grain conservation practices on fungal load dynamics

Parameter	Control	Soaking	Ensiling	Sun drying	SEM	P-value
Yeast (log CFU/g DM)	2.7^{c}	4.9^{a}	3.0^{c}	3.8^{b}	0.22	<0.0001
Mold (log CFU/g DM)	2.1^{c}	3.7^{a}	2.8^{b}	3.1^{b}	0.16	<0.0001
TFC (log CFU/g DM)	4.8^{d}	8.6^{a}	5.8^{c}	6.9^{b}	0.29	<0.0001

$^{a-d}$ Means with in a row with different superscripts differ ($p<0.05$); Control= freeze dried; WBG = Brewers' grain; cfu= Colony forming unit; DM= Dry matter; TFC=Total fungal count; SEM =standard error of the mean

Crude protein degradability of brewer's grain conserved under different storage practices

In-situ* crude protein (C*P) degradability of brewery spent grain stored under different conservation techniques are presented in Table (4) and fig (1). Soaking and sun drying increased and that of ensiling decreased the "a" fraction of CP ($p < 0.05$). However, the "b" fraction for CP was lower for ensiling than soaking and sun drying but was comparable to the control. The rate at which "b" fraction degraded (i.e., c value) was greater ($p<0.05$) in soaking and was lower in ensiled and sun dried than the other preservation techniques. The brewery spent grain conserved by using the ensiling method had the least effective CP degradability (ED) followed by sun drying, control and soaking treatments in that order ($p<0.05$). Calculated ED in soaking were 10.1, 26.73 and 18.42% higher ($p<0.05$) than that recorded in the control, ensiling and sun-dried, respectively. Potential degradability (PD) fraction of wet brewery spent grain was significantly ($p<0.05$) affected by conservation methods. The PD of crude protein (CP) was higher ($p<0.05$) in the soaked brewery spent grain than other preservation methods. As indicted in fig (1), at early phase of ruminal incubation period (≤ 48 hrs.), CP disappearance of the grain in the all treatments were higher, and the rate slowed down afterward. The trend of ruminal CP disappearance of the brewery spent grain at any incubation period was in the order of soaking > control (fresh) > sun drying > ensiling.

Table 4:-Effect of conservation practices on the in-situ CP degradability characteristics

Parameters	Control	Soaking	Ensiling	Sun drying	SEM	P-value
a (%)	11.1^{c}	17.8^{a}	8.1^{d}	14.2^{b}	0.72	<0.0001
b (%)	55^{ab}	58.1^{a}	51.8^{b}	55.8^{a}	0.99	<0.0087
ED (%)	46.2^{b}	56.3^{a}	41.8^{d}	43.6^{c}	0.84	<0.0001
PD (%)	66.1^{b}	73.1^{a}	66.25^{b}	66^{b}	0.9	<0.0002
c (h^{-1})	0.05^{b}	0.07^{a}	0.04^{bc}	0.04^{c}	0.006	<0.0002

[a-d] Means within a row with different superscripts are significantly different (p<0.05); Control= fresh sample subjected to freeze-drying; CP=Crude protein; SEM =standard error of the mean Soluble fraction (a), Slowly degradable fraction (b), rate of degradation of b fraction (c), Effectively degradable fraction (ED) and Potentially degradable fraction (PD).

Discussion

Nutritional variations of brewers' grain collected from different breweries

All chemical compositions and in vitro digestibility with exception of some nutritional parameters of wet brewery grain from the present trial were found within ranges a recent finding (Heuzé et al., 2017). However, slight deviations were noted against NRC (2001) for all measured parameters. The acid detergent fiber and neutral detergent fiber components of wet brewery grain in this study were differed with the report of Westendorf and Wohlt (2002) and dry matter was also different with the report of Senthilkumar et al. (2010). The reasons for this variations maybe speculated to a variety of factors that include: grain and/or varietal difference among the malt grain used as foundation grain, harvesting time and the conditions under which it was cultivated; the conditions used for malting and mashing and the amount and type of the adjuncts added in mixture with the barley malt during the process of wort production. In addition, period of fermentation, processing techniques and analytical procedures followed may be partly responsible for the observed variations.

In agreement to the present finding, Mussatto et al. (2006) and Waters et al. (2012) found nutritional variations among wet brewery grain (WBG) derived from brewing processes without addition of adjuncts (i.e. using 100% barley malt), but the former used Brazilian barley malt while the latter used barley malt from Ireland. Differences can also be observed when comparing the results of these authors with those reported by Meneses et al. (2013) and Carvalheiro et al. (2006) who used WBG derived from a process using barley malt with adjuncts obtained from two different Portuguese breweries. The variations observed in the results of these authors suggest that the differences in the source of malt grain and the brewing process conditions affected the composition of the residual WBG material. In fact, the conditions used for the brewing process (i.e., heat applied during the malting and mashing process, and the type of procedure followed for starch extraction during the wort filtration process etc.) were not reported in any of the studies reported and this is probably another factor with significant influence on the results indicated in Table 2 above.

Chemical composition of brewer's grain stored under different conservation practices

Soaking is the most prominently used wet brewery grain (WBG) conservation practice under on-farm conditions despite extensive losses in feed and microbial quality compared to the other storage methods evaluated in the current study. Sodium chloride is reported to have good anti-microbial property and often considered as "fermentation inhibitor" in feed preservation process (McDonald et al., 1991). However, sufficient scientific literature was not found to substantiate this with the result obtained from the soaking

method in the present study. In line to this, the level of salt used in the soaking process and the storage duration that optimizes proper storage of fresh WBG need further research. Lower dry matter (DM) loss for the ensiled sample was a good sign of the absence of any significant degradation of nutrients during the ensiling process as compared to the other two local WBG conservation practices. Higher DM loss in the soaked and sun-dried brewery grain compared to the control (freeze-dried) and ensiled brewery grain could probably be associated to the relatively higher fungal colony count that arise from the slow sun drying process mainly attributed to the lower solar energy and to the frequent opening of the storage container for feed removal that often induces aerobic deterioration in the soaked sample. In general, during sun drying and soaking process, residual soluble carbohydrates in brewery grain possibly have been converted into ethanol, CO_2 and water in the presence of large colonies of yeast and mold (McDonald et al., 1991) thereby leading to excessive loss of dry matter (DM) and other nutrients from the WBG. Moreover, in agreement to same author, the lower CP observed for brewery grain conserved using these two preservation practices could probably be associated to the extensive proteolysis that might have occurred during the open air storage conditions. The greater values of neutral detergent fiber, acid detergent fiber and permanganate lignin across the preservation practices except ensiling indicated that there were undesirable microbial activities which can be witnessed from the larger fungal colony counts in the present study, as soluble nutrients have been degraded, the proportion of fiber components tended to have proportionally increased. Likewise, lower values of digestible organic matter in sun-dried and soaked sample, can be possibly linked to the loss in DM and other soluble nutrients and a sharp increase in cell wall fractions. In a related study Baskett et al. (2009) also reported losses in DM equivalent to 8.6% and 9.6% for wet distiller grains stored in aerobic and anaerobic storage conditions in bunker silos.

Fungal load dynamics of brewery spent gain conserved under different practices

The higher fungal count in the soaked brewery spent grain (WBG) compared to the other two conservation practices and the control can be speculated to the aerobic exposure of WBG during feed removal for routine feeding on the one hand and the proportion of salt to water that might not have optimized proper fermentation partly due to lack of research recommendations for the soaking technique. Longer storage durations may also hold responsible for the larger fungal contamination seen in the soaking process. Nutrient loss from the sun drying brewery grain in the present study was also relatively higher since drying was done under a low temperature that increased risks of mold growth and mycotoxin production (Chulze, 2010). According to Thus, The efficiency of ensiling in brewery spent grain conservation has also been noted by some other authors (Heuzé, et al. 2017; Geron et al., 2008). According to Woolford (1990) and Dairy one (2017) the presence of yeasts and mold in a stored feed greater than 5.00 CFU/g DM, is considered undesirable leading to higher losses of DM and other essential nutrients. Hence, based on these recommendations both yeast and mold colony counts from the present study were considerably lower implying such feeds can safely be fed to dairy cattle. The average yeast and mold forming colony units (log CFU/g DM) obtained from the current study were slightly higher than the values of 2.7 and 1.8 which was reported earlier in aerobically and anaerobically conserved WBG

using organic acids (lactic acid strains) (Marston et al., 2009), the variation being attributed to the strong inhibitory effect of the organic acid used in the latter case.

Crude protein degradability of brewer's grain conserved under different storage practices

The result of degradability constants excluding water-soluble crude protein fraction from the current study is in line with the previous report (NRC, 2001; Kazemi et al., 2014; Heuzé et al., 2017). The variation of the "a" fraction in crude protein degradability can related with differences in the type of WBG conservational practices employed and differences in the type of washing methods (hand Vs machine). In agreement to the present finding, Gao et al. (2015) noted low repeatability for rapidly soluble ruminal and post-ruminal nitrogen and amino acids fractions of three supplemental protein sources. The "c" and "ED" values of CP fraction varied greatly among the conservation practices and the control sample. This variation could be related in the differences modes of dry, fresh or ensilage (Nocek, 1985. In agreement to the present finding, Kamalak et al. (2005) noted that in situ DM disappearance after 48 and 96 h to be negatively correlated with neutral detergent fiber of the WBG stored under different preservation practices. The poorest 'ED' and rate constant 'c' values in CP degradability of the ensiled wet brewery grain than the control, soaked and dried grain in the present study provided a clear evidence of ensiling have relatively lower rumen fermentable protein. Similar observations have been reported earlier by Armentano et al. (1986) and NRC (2001). The estimated rumen undegradable protein (RUP) in this study was 53.8, 43.7, 58.2 and 56.4% in control, soaked, ensiled and sun-dried samples, respectively. This estimated RUP percentage for the fresh and the remaining local storage techniques from the present study was slightly higher than the mean values reported by NRC (1989) for cottonseed meal (41%) and sunflower meal (26%) but was lower than the content of RUP for dried distillers' grain (67.1%) (Kelzer et al., 2010). Lower potential CP degradability (a + b) values was found for the control, ensiled and sun-dried samples compared to WBG preserved using the soaking method. Similar results were noted from related research work by Promkot et al. (2007). But, in general, WBG reportedly have lower ruminal protein solubility and degradability (Armentano et al., 1986) while in-situ degradability results have been affected by different factors such as feed particle and sample size, bag material pore size; test animal diet and washing procedures (Nocek, 1985; Gao et al., 2015).

Conclusions

Brewery spent grain from Meta Abo brewery factory had the higher nutritional values than the remaining beer factories. The minimal loss of dry matter and other nutrients, lower fungal, yeast and mold colony counts and the higher crude protein digestion kinetics was observed in ensiling techniques. If supply is not a constraint under local conditions, ensiling can be recommended as a best wet brewery spent grain conservation practice.

Declarations

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Figures

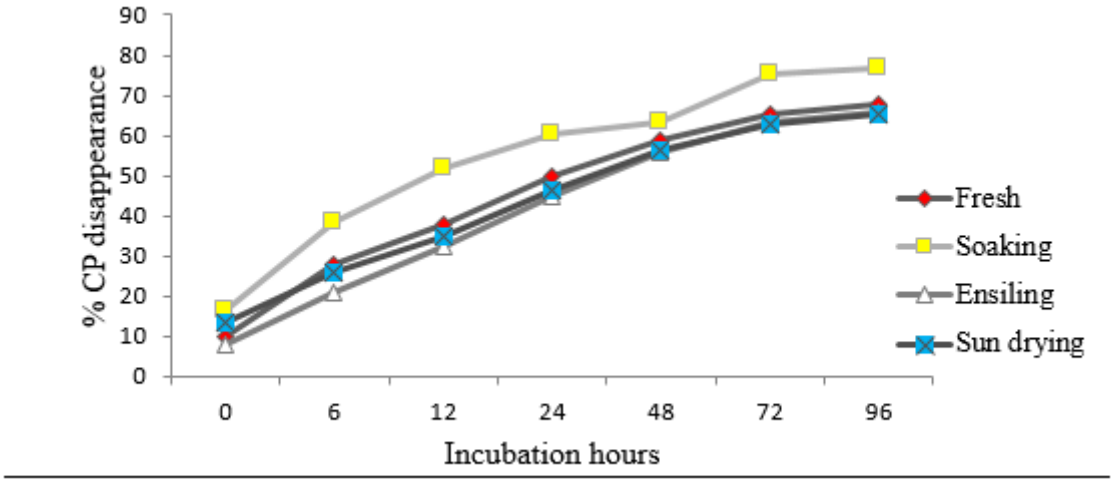

Figure 1

In situ CP disa*pp*earance characteristics of fresh Vs conserved WBG