Laboratory-Scale Studies of the Impact of Oxygen on Mashing

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ABSTRACT

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An assessment of the impact of oxygen and hydrogen peroxide on mashing and wort parameters has been made on a laboratory scale. Oxygen has been stridently eliminated by using an anaerobic chamber during mash analysis. Additionally the relative importance of proanthocyanidin species has been assessed by comparing the behaviour of "conventional" malt and a malt produced from a low proanthocyanidin variety. It seems that oxygen and peroxide act independently in causing the oxidation of thiol-containing materials and polyphenols in mashes and that oxygen is not primarily exerting its impacts through the intermediacy of peroxide. The removal of thiols (presumably at least in part through the production of disulphide bridges between proteins) and of polyphenols (presumably via polymerisation) both contribute to increased wort turbidity and decreased rates of wort separation after mashing. Three inhibitors (nordihydroguaiaretic acid, ethylenediamenetetraacetate and potassium cyanide) have been employed in an attempt to differentiate between enzymic and non-enzymic events and also to identify whether lipoxygenase and peroxidase are catalysing key events. Whilst it seems that peroxidase has a key role in catalysing the oxidation of polyphenols by H_2O_2, it does not appear that either peroxidase or lipoxygenase is involved in the removal of measurable thiol. Nonetheless a significant proportion of the thiol elimination is likely enzyme-catalysed. We have been unable to demonstrate the production of hydroperoxides in mashes, but added hydroperoxide is undetectable, which suggests that these materials are either lost by onward conversion or by adsorption onto spent grains.

Key words: Enzyme, hydroperoxide, inhibitor, mashing, oxygen, peroxide, polyphenol, thiol.

INTRODUCTION

There is an increased conviction on the part of many that the achievement of enhanced flavour stability of beer demands not only the elimination of oxygen from the finished product but also the avoidance of oxygen ingress throughout the brewing operation[31]. It has even been suggested that oxidative events occurring during malting are to the detriment of flavour shelf life downstream[37], although typically for an area that attracts tremendous controversy and debate these claims have been refuted by others[38]. There is rather more agreement that oxidation in the brewhouse, through promoting insolubilisation, benefits colloidal stability downstream[6].

The successful and unequivocal demonstration of the impact which upstream events have on downstream stability is by no means straightforward. The substances responsible for aged character in beer, notably a series of carbonyl compounds[25], tend to have low flavour thresholds and so display their impact when present in very small quantities. Accordingly relatively small variations in the level of these molecules may have a profound impact on perceived character and these variations may constitute little more than batch to batch "noise". Despite the fact that brewing processes have never been so controllable, it is extremely difficult to regulate operations so precisely as to eliminate the subtle differences which will be manifest in these carbonyl compounds. Thus the tendency is for there to be tremendous variability in the results obtained in such experiments.

Another major problem is the reporting of sensory data. Meilgaard[26] has registered his "despair" at the paucity of properly described tasting protocols in the vast majority of papers covering areas such as flavour stability. Furthermore, as we have stressed recently[18], it is the sensory dimension that is a far more meaningful yardstick of staling than chemical analysis of products.

We suggest that it is most useful experimentally to dissect the process into unit stages and investigate, on a manageable scale, the impact that oxygen has on them. It is possible to criticise very small-scale wort production operations, for example in terms of excessive opportunities for oxygen ingress[7]. However, if oxygen can be demonstrated to be without effect on a bench scale, this certainly would suggest that it wouldn't be an issue in the larger commercial scale operations where opportunities for oxygen ingress are substantially less.

In considering the impact of oxygen in mashing we draw attention to a number of interactive processes that have been proposed to occur (Fig. 1). Of special interest in the context of staling is the oxidation of the unsaturated fatty acids (UFA) to produce hydroperoxides, which it is believed degrade in a series of steps to carbonyl compounds. The oxidation of the UFA may occur enzymically, through the agency of lipoxygenase (LOX,[36]) or non-enzymically[11]. There are those who strongly advocate the importance of the former route, whilst others question its relative importance in comparison to the impact of radi-

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cal-based oxidation. Regarding the latter, ground state oxygen is relatively unreactive, but becomes more potent when converted to activated species such as superoxide or perhydroxyl (O_2H), peroxide (H_2O_2) or hydroxyl ($OH^{\bullet}$)[8]. This activation is promoted by transition metal ions such as iron and copper[23], although it has also been suggested that the reaction of thiol-containing proteins with oxygen leads to the formation of hydrogen peroxide, H_2O_2[30]. The cross-linking of the proteins (gel proteins?[27]) and perhaps pentosans via diferulate bridges[34] is understood to lead to retarded wort separation through its involvement in teig formation.

Barley malt contains enzymes which protect against the accumulation of superoxide (superoxide dismutase, SOD[5]) and peroxide (catalase[32]), but their significance during mashing has been questioned[9]. Hydroxyl radicals, the most reactive species known, can be scavenged non-enzymically by polyphenols, and oxidation of the latter is a forerunner to polymerisation and the formation of coloured materials and of complexes with protein, leading to haze and turbidity. Polyphenols may also be oxidised by H_2O_2 in reactions catalysed by peroxidases (POD[21]). To add to the complexity, polyphenols may also inhibit LOX[16]. Billaud and Nicolas[15] have reviewed the enzymology of oxido-reductases in malt.

Clearly the situation is complex, with a myriad of possible interactions. In this study we have sought to investigate the likely extent to which these various reactions occur and to gain an understanding of the relative importance of enzymic and non-enzymic reactions. As tools in this work we have included an experimental protocol which allows rigorous anaerobiosis and also a series of enzymic inhibitors. We have also employed two types of malt: a typical ("traditional") lager-style malt and one made from a low-proanthocyanidin barley. This has allowed us also to gain an appreciation of the relative significance of polyphenols in the "economy" of oxygen-dependent processes in mashing, it being recognised that the single most important "sink" into which oxygen flows in beer is the polyphenol fraction[33].

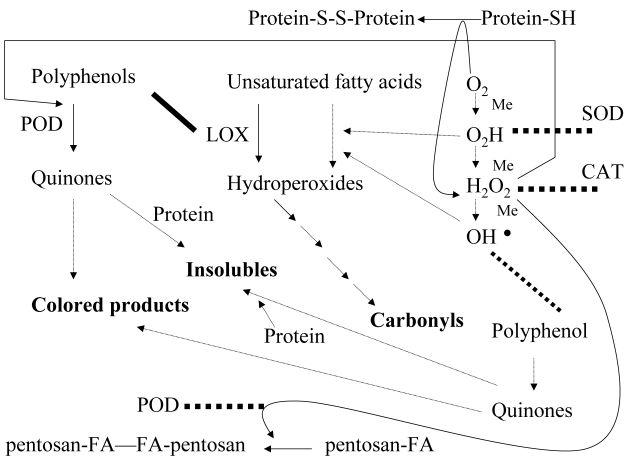

Fig. 1. Proposed interactions involving oxidative events in mashing. LOX = lipoxygenase; POD = peroxidase; SOD = superoxide dismutase; CAT = catalase; FA = ferulic acid.

MATERIALS AND METHODS

The "conventional" malt was a blend of Klages and Harrington varieties and was supplied by the Great Western Malting Company. The low-proanthocyanidin variety was Clarity, generously supplied by the Crisp Malting Group Ltd., U.K. Denatured malt was prepared as described elsewhere[10] and mashed with an addition of alpha amylase (Sigma #232-565-6). All malts were milled in a Buhler Miag cone-type mill at a measured gap setting of 1.23 mm immediately prior to mashing.

A Canongate CM-3 Mashing Bath was used for all mashing experiments. Mashes were prepared by the method of Buckee[17] with modifications. All mashes consisted of 50 g of milled malt combined with 150 g of deionised water. Where mashes are designated "anaerobic," the water was also deaerated by boiling for 15 min. Milled malt was placed in the mash cups a minimum of ten min in advance of the start of the mash during which time grist for anaerobic mashes was sealed within the mash beaker and continuously sparged with nitrogen gas until completion of the mash. All mashes were prepared at 65°C. Upon completion, mashes were immersed in an ice bath for cooling and the ice baths removed to a refrigerator where they were held at 4°C pending analysis. Anaerobic mashes were held cold and under vacuum prior to analysis. Analysis of all mashes was undertaken within 24 h of refrigeration at 4°C. Analysis of all aerobic mashes took place under normal, atmospheric conditions on a laboratory bench. Analysis of anaerobic mashes took place in a large, sealed chamber supplied with nitrogen gas and referred to throughout this work as the "anaerobic chamber" (Fig. 2). A positive pressure of nitrogen gas was maintained on the chamber and the chamber was purged and flushed with nitrogen weekly. All reagents destined for use in the anaerobic chamber were vacuum deaerated prior to use.

Determination of the filterability of wort and wort clarity were performed as described by Clarkson et al.[19]. Prior to filtration, mashes were adjusted to 450 g for a final dilution ratio of 1:8 with cold, deionised water (deaerated and deionised water for anaerobic mashes was held cold and under vacuum until use). Ahlstrom's Grade 609 filter paper was used for all mash filtrations. Filtration rate was defined as the quantity of wort collected in 30 min. Relative wort haze was defined as the absorbance of the wort sample measured at 660 nm against a water blank.

Total polyphenols in wort were measured according to the standard method of the American Society of Brewing Chemists[3] except the total assay volume was reduced from 25 mL to 3.125 mL by proportionate adjustment of all components.

Wort peroxide values were by a method modified from that of Shantha and Decker[35]. After adjusting the weight of the mash to 450 g, a 10 mL sample was withdrawn using a wide-mouth pipette. The sample was centrifuged at 3000 × *g* for five min. A 200 µL aliquot of supernatant was added to a second centrifuge tube containing 9.8 mL of 7:3 (v/v) methanol/chloroform. The contents of this tube were vortexed and centrifuged for 10 min at 3000 × *g*. A 5 mL aliquot of the resultant supernatant was withdrawn to a clean test tube, 25 µL of ammonium thiocyanate added and the solution vortexed before adding 25 µL of iron (II)

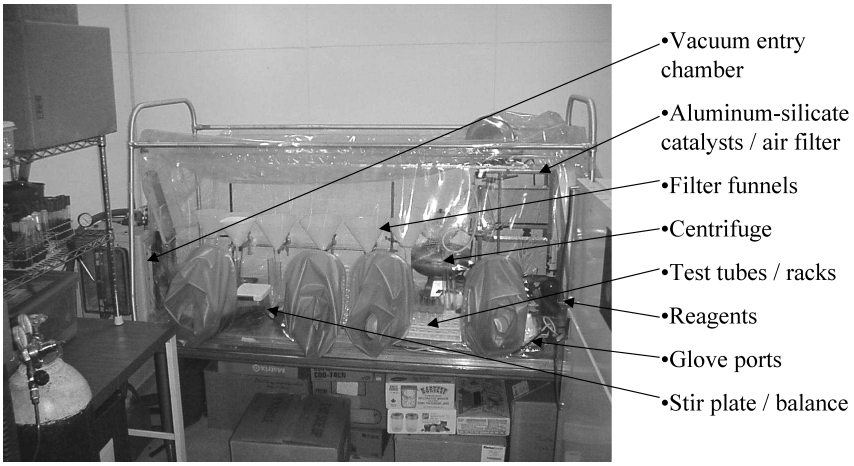

Fig. 2. The anaerobic chamber.

chloride and re-vortexing. The prepared sample was held for 10 min in subdued light before reading the absorbance at 500 nm. Results are expressed as hydroperoxide equivalents based upon construction of a standard curve prepared with increasing concentrations of cumene hydroperoxide (Sigma #C-0524).

Determination of free thiols in wort was by a modified version of the method of Muller[29]. To 2.4 mL of 0.1 M phosphate buffer (pH 7.5) was added 600 µL of filtered wort prior to vortexing. 120 µL of 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) was added and the sample was re-vortexed and held for 15 min before reading the absorbance at 412 nm. A standard curve was prepared using reduced glutathione and values are reported as "glutathione equivalents".

Some mashes included additions of hydrogen peroxide immediately upon mash-in just following the grist hydration. Nordihydroguaiaretic acid (NDGA), ethylenediamenetetraacetate (EDTA) or potassium cyanide (KCN) was also added where indicated to mashes immediately upon mash-in at final concentrations in the mash of 0.1 mM, 0.5 mM, and 25 mM respectively. Solutions of inhibitors were vacuum deaerated prior to addition to anaerobic mashes.

POD was assayed as described by Clarkson et al.[20] and LOX according to Biawa and Bamforth[13].

RESULTS AND DISCUSSION

Selection of enzyme inhibitors

We reasoned that if it were possible to identify inhibitors that act specifically against the enzymes LOX and POD then we would have a valuable tool to differentiate between the action of the two enzymes and also aid in identification of the relative importance of enzymic and non-enzymic reactions.

NDGA, which originates in the creosote bush, is well known as an inhibitor of LOX[2,28] and we had anticipated using it as a "marker" for this enzyme. However we find that it is even more potent against POD (Fig. 3a). There is evidence that it can inhibit cyclo-oxgenases[12], phospholipase A2[14] and cytochrome P-450[1]. Accordingly, and contrary to supposition by many, NDGA is perhaps not as discriminatory a tool for identifying LOX involvement as is generally assumed to be the case.

KCN also inhibits POD, but it activates LOX (Fig. 3b). The converse situation occurs with EDTA: it is a very weak inhibitor of LOX, but activates POD (Fig. 3c).

On this basis we surmise that if an event is largely blocked by NDGA, somewhat by KCN, but activated by EDTA, then this would suggest an involvement for POD. However if a reaction was partially inhibited by NDGA and EDTA, but promoted by KCN, then we might suppose that LOX is involved. If the inhibitors are without impact then this would be consistent with the event being non-enzymic, although a profound interference by the chelating agent EDTA might be indicative of a non-enzymic process involving transition metal ions.

It must be stressed that effects by these inhibitors/activators will only be apparent if the event has not reached completion. For example, if a reaction catalysed by LOX proceeds very rapidly and reaches completion well within the time frame under investigation, then an inhibitor that does not block the enzyme absolutely will not be seen to have any impact because the reaction will still have occurred, albeit more slowly. Enzymes influence the **rate** of reactions, not the final equilibrium achieved. Only NDGA at concentrations exceeding 50 µM has a near total inhibitory impact.

Thiol levels

A disproportionate level of free thiols was measured in wort from the anaerobic mash of the low proanthocyanidin malt (Fig. 4). Thiol levels were much less in wort from the aerobic mash of this malt, indicating that there was a strong opportunity for oxygen to react with the thiol-containing compounds. There were also more thiols in the anaerobic mash from the "conventional" malt, but differences were much less than for the low proanthocyanidin malt. It appears that the elimination of available thiols occurs rapidly. Comparing the aerobic and anaerobic mashes with the low proanthocyanidin malt, the wort collected at time zero has already reached its lowest value and does not decline further through the time course of mashing. It seems likely that the thiol level is lowered rapidly during the mash filtration operation and/or the

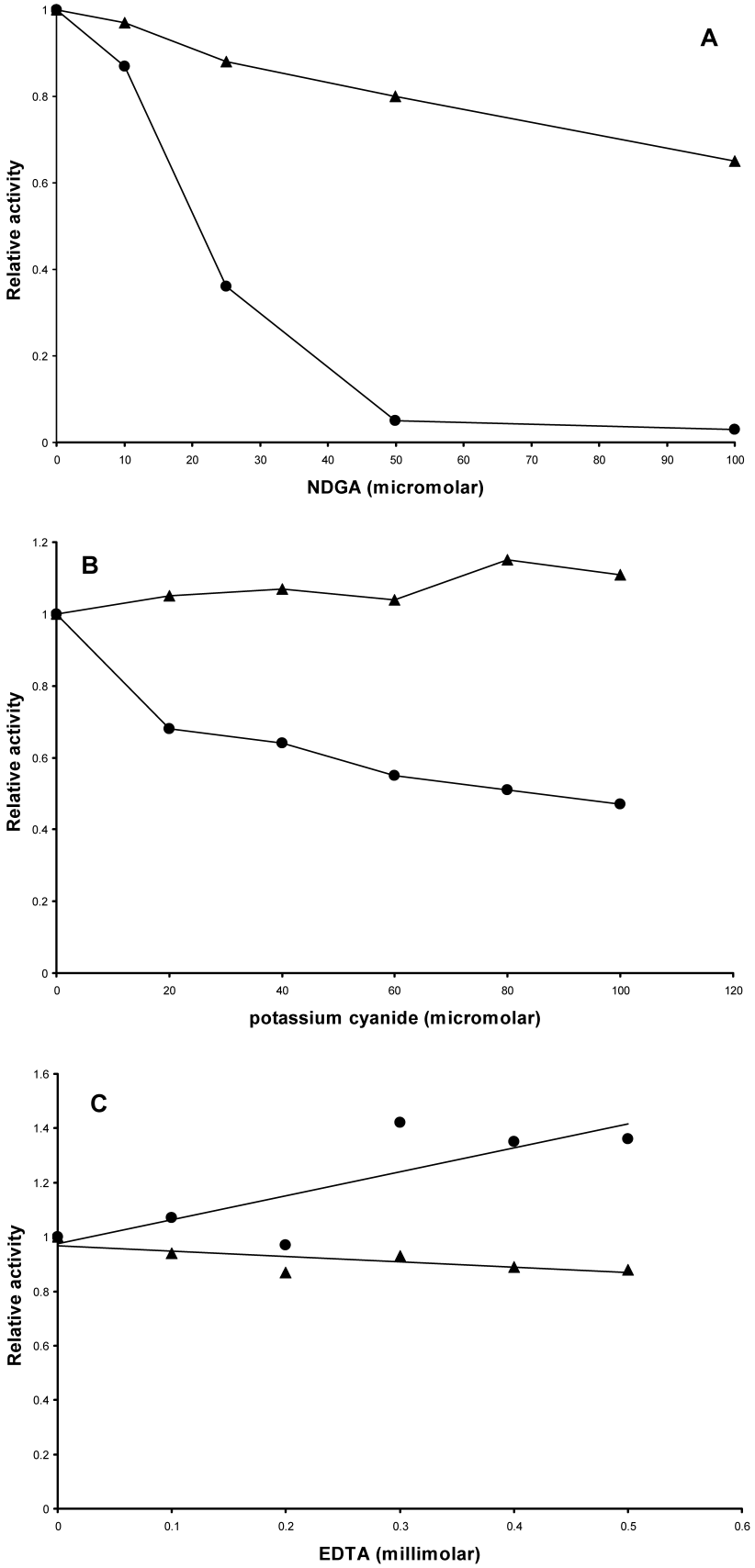

Fig. 3. (A) The impact of nordihydroguaiaretic acid (NDGA) on activity of peroxidase (●) and lipoxygenase (▲). (B) The impact of potassium cyanide (KCN) on activity of peroxidase (●) and lipoxygenase (▲). (C) The impact of EDTA on activity of peroxidase (●) and lipoxygenase (▲).

time that elapses between stopping the mash and analysing the wort.

Whilst the model shown in Fig. 1 invokes the generation of H_2O_2 by reaction of thiols with O_2, it is well known that H_2O_2 will also react with thiols[39]. Fig. 5 illustrates the impact that H_2O_2 has on thiol levels in worts from aerobic and anaerobic mashes of both types of malt. In this experiment for the samples lacking peroxide, less difference was observed between the thiol levels from the two malts for the aerobic and anaerobic mashes than described above, although the trends were the same. More importantly it seems that H_2O_2 equalises differences between the mashes and leads to very low thiol levels throughout. 20 mM H_2O_2 is sufficient to maximise the effect. This begs the question of whether the action of O_2 (as illustrated in Fig. 5) is largely through the intermediacy of H_2O_2, which must arise in a manner different from the oxidation of thiols, perhaps via activation of oxygen by trace levels of metal ions. Another possibility is that H_2O_2 is acting via POD (or another enzyme) to oxidise polyphenols, which duly polymerise and interact with proteins to precipitate them out, thereby reducing thiol level. If this was the case one would expect to see that agents which inhibit POD interfere with thiol removal (leading to higher thiol levels) and vice versa for activators of POD. In fact Fig. 6 shows that NDGA has no substantial effect, whereas EDTA slightly increases thiol levels. We infer that POD is not involved in the thiol-H_2O_2 interaction. (Cyanide could not be evaluated as it interferes by masquerading as –SH in the assay.)

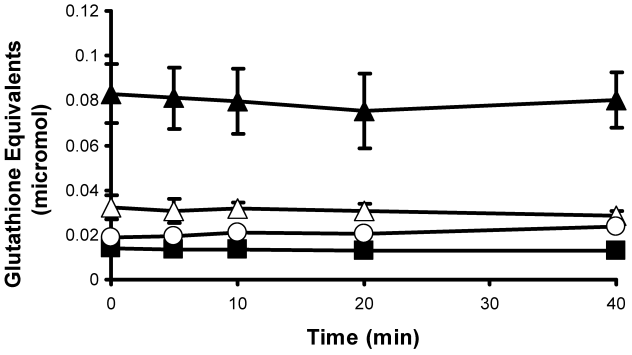

Fig. 4. The impact of aerobiosis and anaerobiosis on thiol levels in worts. Standard malt, ■ aerobic; △ anaerobic. Low proanthocyanidin malt, ○ aerobic; ▲ anaerobic.

When malt was denatured there was a significant downturn in the levels of free thiols measurable in wort (Fig. 7). In part this may reflect the removal of thiols by precipitation – e.g. the difference between the thiol levels in control and denatured sample under anaerobic conditions. We infer that the difference between free thiols seen in denatured samples under aerobic and anaerobic conditions indicates the relative contribution of non-enzymic cross-linking of thiols, which is substantially less than the enzyme-catalysed cross-linking (difference in thiol levels in control malts subjected to aerobic and anaerobic mashing). We note from Fig. 6 that none of the inhibitors exhibits this magnitude of an effect, suggesting that neither POD nor LOX is primarily involved in the loss of thiols. It must be stressed that the thiols being detected may include low molecular weight and high molecular weight species – the former will include glutathione. As NDGA does not impact the thiol level it seems unlikely that LOX is catalysing the cross-linking of thiol groups, although this capability has been reported[4]. Enzymes that may be involved here may include sulphydryl oxidase, glutathione oxidase, glutathione peroxidase and phospholipid-hydroperoxide glutathione peroxidase.

Polyphenol levels

Key substrates for POD in mashing are polyphenols. As would be expected, there are substantially more polyphenols in worts derived from the "conventional" malt than the low proanthocyanidin malt (Fig. 8). The finite

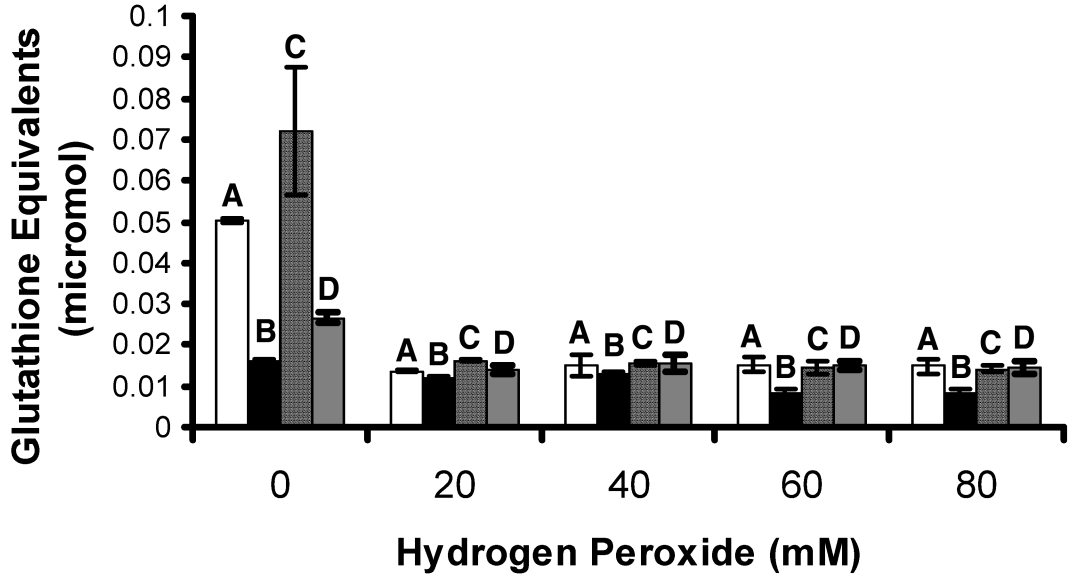

Fig. 5. The impact of hydrogen peroxide on thiol levels in wort. Standard malt: anaerobic (A); aerobic (B). Low proanthocyanidin malt: anaerobic (C); aerobic (D).

level of polyphenolic material in the latter malt represents those polyphenols other than the haze-forming proanthocyanidins[22]. Soluble polyphenol levels in wort were lower when mashing of the conventional malt was under aerobic conditions, consistent with the oxidative polymerisation, insolubilisation and removal of proanthocyanidins. Differences were maximal within 10–20 min of mashing, but amounted to no more than 15% of the total polyphenol. The presence of oxygen had no impact on the polyphenolic species residual in the low proanthocyanidin malt.

H_2O_2 had a much more pronounced effect on the polyphenol levels for the conventional malt than did oxygen (Fig. 9), consistent with the greater ability of peroxide as compared to oxygen to react with polyphenols, especially when catalysed by POD. H_2O_2 (20 mM) gave maximal lowering of polyphenols, indicating that this level of peroxide is ample to saturate the POD. (The K_m value of peroxidase for H_2O_2 in crude extracts of barley is 57 μM[20]: an enzyme typically operates at maximal rate at $10 \times K_m$, and 20 mM represents a concentration 35 times higher than this). In all instances polyphenol levels were lower in worts mashed under aerobic conditions, which would suggest that a proportion of polyphenol removal is not via POD/H_2O_2 action. Comparison of Figs. 8 and 9 suggests that much of the polyphenol depletion under aerobic conditions is a result of this non-POD dependent action. Perhaps it reflects polyphenol eliminated by interaction with protein that is oxidatively cross-linking through thiol groups. H_2O_2 has no impact on polyphenol levels in low proanthocyanidin malt mashes, suggesting that no polyphenols susceptible to POD action are present in such malt.

As seen in Fig. 10, NDGA and cyanide (which inhibit POD) elevate somewhat the residual levels of polyphenol in aerobic mashes, whereas EDTA (which activates POD) lowers the levels of polyphenol in those worts. This would tend to suggest a role for POD in the removal of polyphenols even in the absence of added hydrogen peroxide,

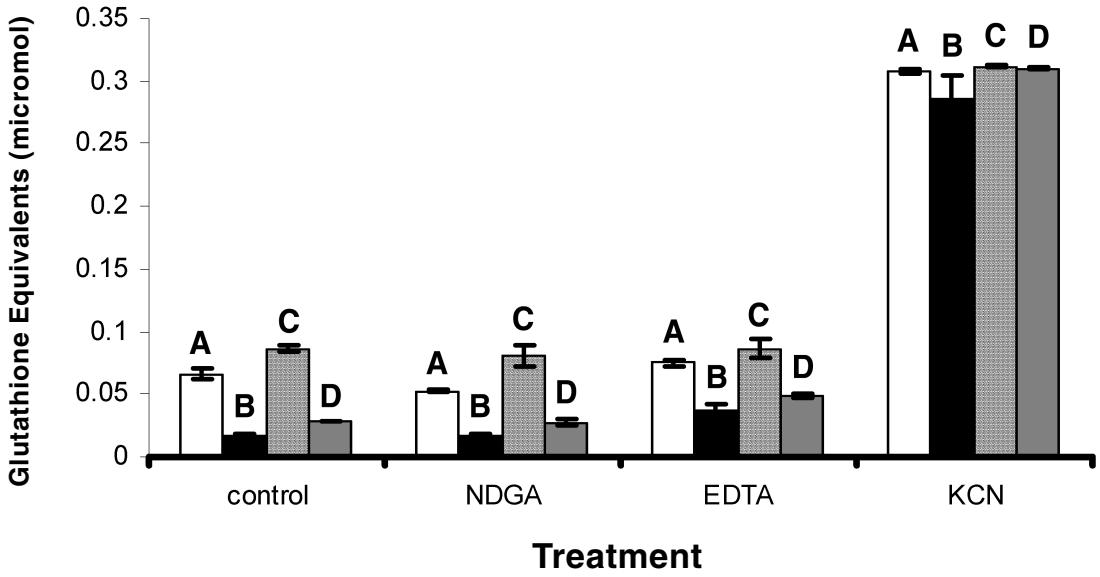

Fig. 6. The impact of hydrogen peroxide on thiol levels in wort after mashing in the presence of inhibitors. Standard malt: anaerobic (A); aerobic (B). Low proanthocyanidin malt: anaerobic (C); aerobic (D).

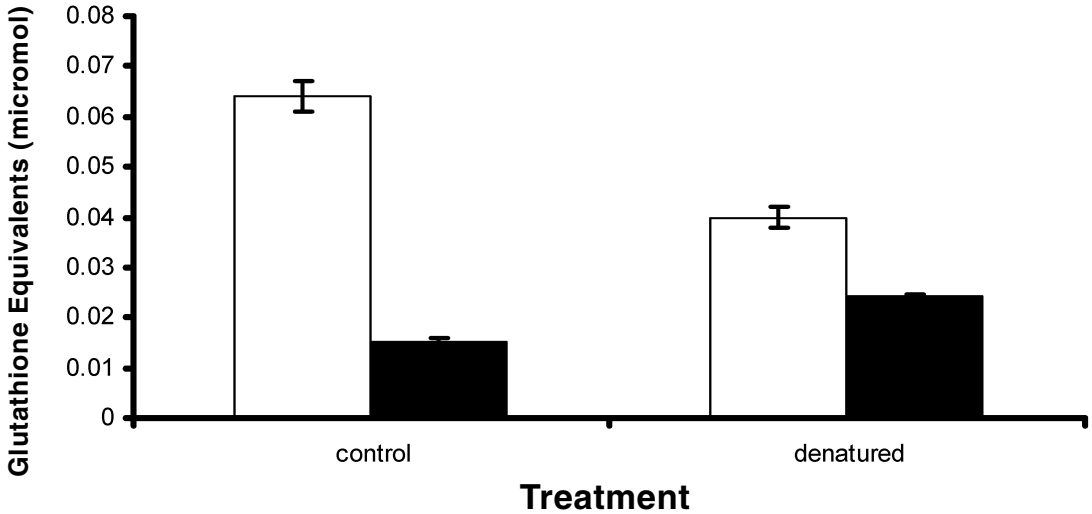

Fig. 7. The impact of denaturing malt prior to mashing on thiol levels in wort. Anaerobic (white bars); aerobic (black bars).

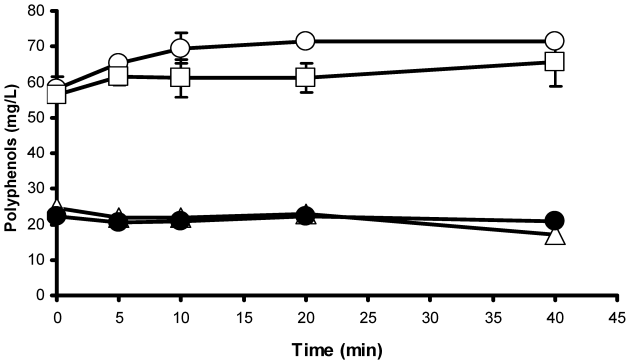

Fig. 8. The impact of aerobiosis and anaerobiosis on polyphenol levels in worts. Standard malt: ○ anaerobic; □ aerobic. Low proanthocyanidin malt: △ anaerobic; ● aerobic.

although the data does imply that the greater part of the impact of aerobic conditions is not exerted through H_2O_2/POD.

Turbidity

In the absence of added peroxide, worts produced under aerobic conditions are substantially more turbid than those made under rigorous anaerobic conditions (Fig. 11). This is much more prevalent for the conventional malt, highlighting the greater importance of proanthocyanidins over thiol compounds in causing turbidity. At the lower addition rates for H_2O_2 there is a large increase in haze, especially under aerobic conditions. This suggests a role for both H_2O_2- and O_2- dependent reactions in the insolubilisation – perhaps oxygen is involved in the thiol-thiol bridging of proteins. Clearly such bridging cannot in itself

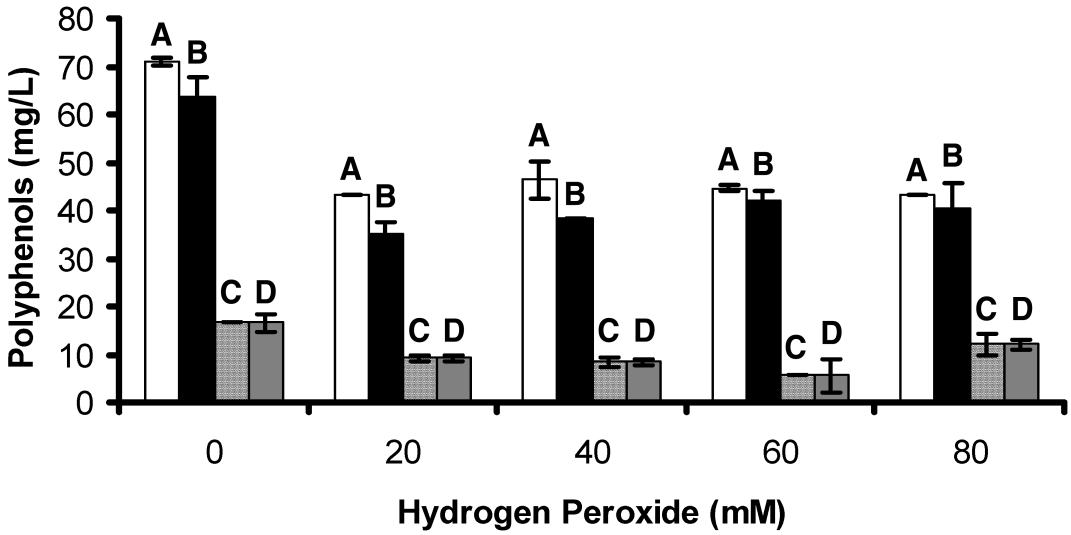

Fig. 9. The impact of hydrogen peroxide on polyphenol levels in wort. Standard malt: anaerobic (A); aerobic (B). Low proanthocyanidin malt: anaerobic (C); aerobic (D).

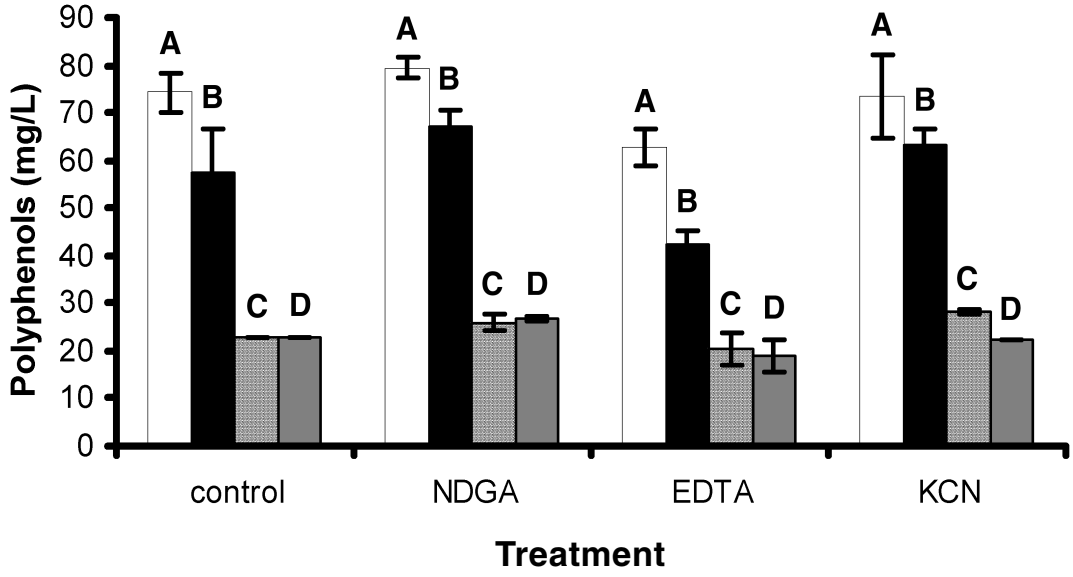

Fig. 10. The impact of inhibitors on polyphenols levels in worts. Standard malt: anaerobic (A); aerobic (B). Low proanthocyanidin malt: anaerobic (C); aerobic (D).

lead to a substantial increase in turbidity, for haze values were far lower for the worts from low proanthocyanidin malt mashes, despite their elevated levels of oxidisable thiol groups (Fig. 4).

As the H_2O_2 concentration is increased in anaerobic mashes there is a progressive increase in turbidity, whereas increasing the H_2O_2 over 20 mM in the aerobic mashes lowers the turbidity levels. One possibility is that if the opportunity for cross-linking of proteins is reduced (anaerobic conditions) then the availability of oxidised polyphenols becomes limiting. It needs re-stating however that the peroxidases are already saturated at 20 mM H_2O_2, which implies that the progressive impact on haze of higher concentrations is via stoichiometric rather than kinetic effects. In terms of the progressively lower turbidity in worts produced under aerobic conditions as the H_2O_2 is increased, this is likely to be a reflection of the increased molecular size of agglomerates of oxidised polyphenols and proteins which are so insoluble as to be trapped with the spent grains during filtration.

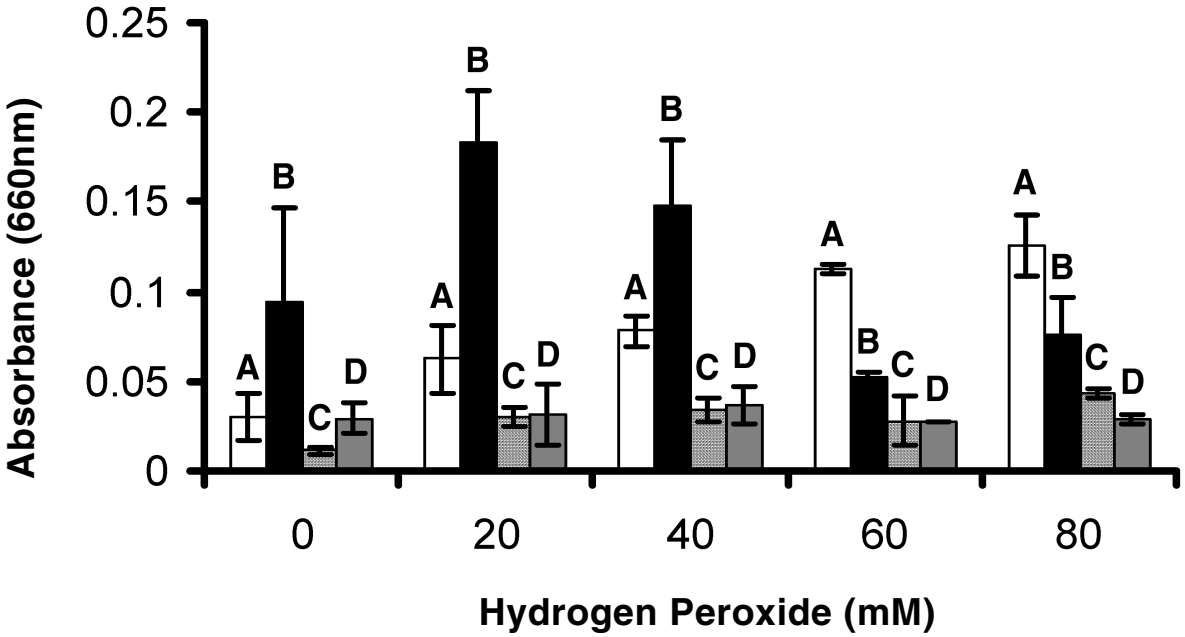

Fig. 11. The impact of aerobiosis/anaerobiosis and hydrogen peroxide on haze levels in wort. Standard malt, anaerobic (A); aerobic (B). Low proanthocyanidin malt: anaerobic (C); aerobic (D).

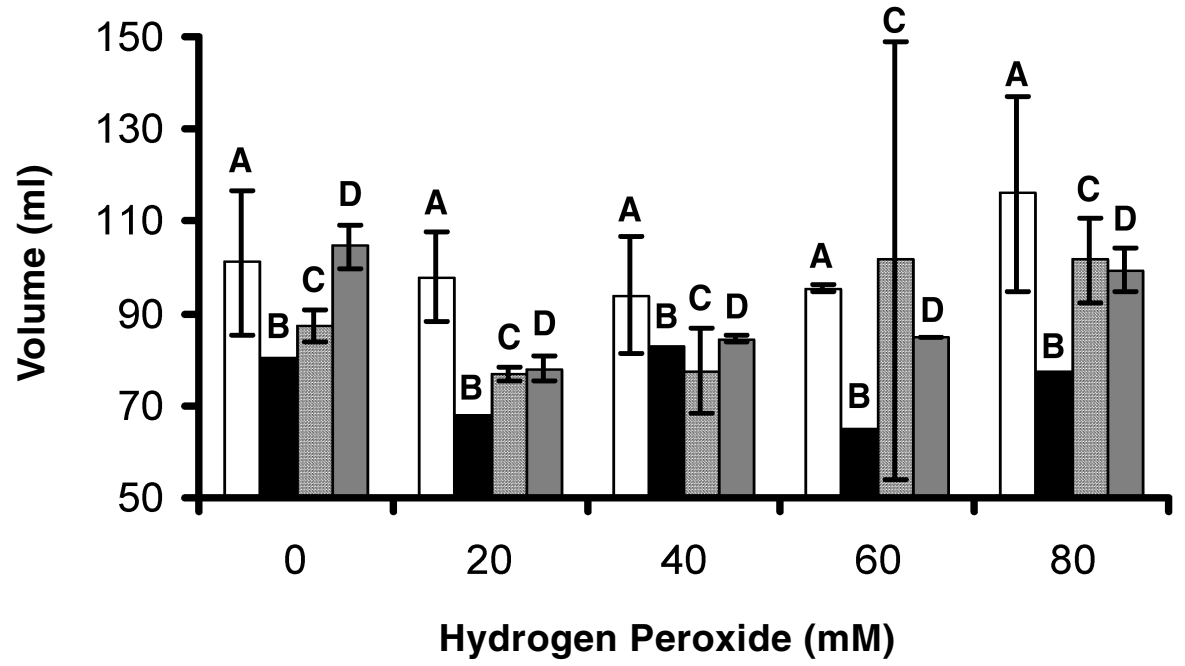

Fig. 12. The impact of aerobiosis/anaerobiosis and hydrogen peroxide on wort separation rates. Standard malt: anaerobic (A); aerobic (B). Low proanthocyanidin malt: anaerobic (C); aerobic (D).

The turbidity factor precluded us from making reliable assessment of colour in the present trials.

Wort filtration

The variation in filtration rates was extremely high (Fig. 12), making it difficult to draw firm conclusions. However in all instances with the conventional malt, wort collection was slower for aerobic mashes. In the absence of added H_2O_2, filtration was actually slower for the aerobic mash of the low proanthocyanidin malt, possibly as a result of the cross-linking of proteins. At very high H_2O_2 concentrations the situation was reversed, whilst there is also some indication that H_2O_2 does tend to have a bigger impact on the filtration of mashes of the conventional malt than does aerobiosis. Again, though, a combination of aerobiosis and H_2O_2 seems to have the biggest negative impact on wort filtration, as might be predicted if both cross-linking through thiol proteins and insolubilisation consequent to polyphenol polymerisation are both involved in rendering a mash bed of decreased permeability.

Hydroperoxides

In no experiment have we been able to detect finite levels of hydroperoxides emerging from mashes. We have used a relatively simple colorimetric procedure, using cumene hydroperoxide as a standard reference. Wort had a slight impact in lowering the response (Fig. 13), nonetheless we were confident in our ability to measure 10–20 nmol of hydroperoxide. This compares favourably with chromatographic methods described by others[24]. In control mashes to which cumene hydroperoxide was added, we were unable to recover the addition in the wort. This suggests either that the hydroperoxides are binding with spent grain components or are being converted to non-hydroperoxide materials during mashing. Because we were unable to recover added hydroperoxide we are not in a position to say whether hydroperoxides are, or are not, produced during mashing of these malts under the conditions studied. However Kobayashi et al.[24] found that hydroperoxides did decrease in level during mashing and wort separation.

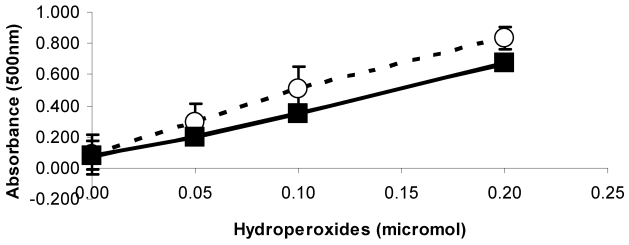

Fig. 13. Calibration plots for hydroperoxide levels. ○ Cumene hydroperoxide in water. ■ Cumene hydroperoxide in wort.

The only positive detection of hydroperoxides was that of H_2O_2, which is measured in this assay (Fig. 14). Interestingly this was only detected in sizeable quantities in those mashes containing in excess of 60 mM H_2O_2, and then especially in the mashes of low proanthocyanidin malt, particularly the aerobic ones The most peroxide that can be recovered in any instance amounted to less than 3% of the amount of hydrogen peroxide added. We infer that the H_2O_2 added to mashes is essentially entirely removed, only a proportion of which can be by interaction with polyphenols and thiols.

CONCLUSIONS

Thiol species are substantially lowered if mashes are aerobic. This is at least partly enzyme-catalysed, though not by LOX or POD. Peroxide also lowers thiol levels in mashes. Only a limited amount of polyphenol can be eliminated in mashes by reaction with molecular oxygen. Rather more polyphenol is removed in mashes in the presence of hydrogen peroxide and it probably represents a different population of polyphenol to that

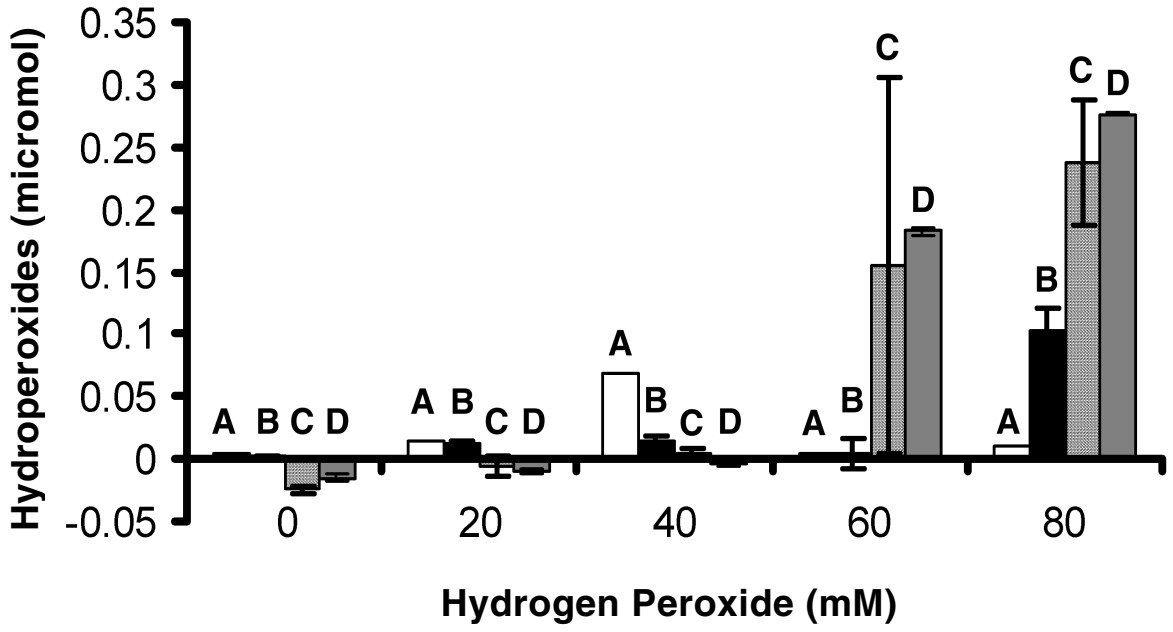

Fig. 14. Impact of added hydrogen peroxide on measurable peroxide levels in wort. Standard malt: anaerobic (A); aerobic (B). Low proanthocyanidin malt: anaerobic (C); aerobic (D).

removed in the presence of oxygen. Relatively low concentrations of peroxide exert a maximal effect, consistent with the low Michaelis constant that POD has for H_2O_2. Oxygen and peroxide also seem to act independently in respect of the production of insoluble components during mashing. Hydroperoxides cannot be detected in worts collected under aerobic or anaerobic conditions. However, as added hydroperoxide could not be detected, it seems that any hydroperoxide produced during mashing is likely eliminated or binding irreversibly to spent grain material. H_2O_2 added to mashes is very substantially consumed, yet only a proportion can be accounted for through reaction with thiols or polyphenols.

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