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Avoidance of oxidative-stress perturbation in yeast bioprocesses by proteomic and genomic biostrategies?

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ABSTRACT

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Aims: Bioprocess oxidative stress caused by many reactive oxygen species (ROS) can lead to largely irreversible perturbation of yeast bioprocesses. These include the production of proteins derived from recombinant DNA yeast technology (aerobically grown *Saccharomyces cerevisiae*). These proteins include rennin, amyloglucosidases (glucamylases), interferons, interleukins, insulin, monoclonal antibodies, tissue plasminogen activators (t-PA), sexually transmitted disease antigens, and measles, mumps and rubella antigens, growth hormones, somatotropin, blood clotting factors VIII and XIII. In addition, there may be a demand for severe acute respiratory syndrome–coronavirus antigens, hepatitis A, B and C viral-selected antigens, HIV retroviral antigens, influenza antigens, trypanosomal antigens, and foot and mouth disease antigens. Prevention of oxidative stress has been achieved by application of antioxidant redox metalloenzymes such as superoxide dismutases (containing Cu/Zn cytosolic, Mn mitochondrial and Fe bacterial) glutathione peroxidases (and other Se-containing proteins and enzymes such as the thioredoxins), catalases (Fe-containing), cytochrome *c* peroxidases (Fe-containing), ceruloplasmins (Cu-containing), metallothionines (these cysteine thiol-rich proteins bind ions of cadmium and mercury) and tyrosinases (Cu-containing).

Methods and Results: ROS are generated inadvertently by single metal valency couples such as FeII/FeIII and by FeIII/FeV present in 2700 (including 57 human) isoforms in cytochromes P450 mixed-function oxidases (EC 1·14·14·1; O_2 : mono-oxygenase NADPH/NADH requiring). In addition, mixed-metal couples such as valency unmatched forms in CuI/FeII and FeIII/MnIV can recycle electrons. Moreover, proteins/protein chaperone couples can recycle electrons, often where futile-recycling systems have been instigated. Furthermore, oxidized membrane phospholipids (R) can form ROOH (lipid hydroperoxides) and ROH (lipid alkoxides) that can generate ROS through Fenton chemistry (iron-catalysed) chain reactions. Utilization of chain-breaking antioxidants such as vitamin E (α-tocopherol) in the lipid phase and vitamin C (ascorbate) in the aqueous phase can terminate these ROS-producing reactions.

Conclusions: The main significance of the study is that proteomic strategies of relief from bioprocess perturbation by ROS of yeast fermentations (used to manufacture proteins required in the food and therapeutic bioindustries) may become possible through addition of selected proteins (including metalloenzymes). The main impact of the study is that the utilization of genetically modified (GM) yeast produced by recombinant DNA technology genomic strategies could circumvent the bioprocessing problems that otherwise result from the bioprocess perturbations: this is as a result of oxidative stress caused by ROS, which is avoidable by deployment of appropriate antioxidants such as vitamins E, C and D (and antioxidant proteins and enzymes often of microbial origin via recombinant DNA technology).

Keywords: antioxidants, bioprocesses, enzymes, metalloenzymes, micro-organisms, ROS, yeasts.

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INTRODUCTION

Where biomolecular injury is caused by reactive-oxidant molecular species, such as reactive oxygen species (ROS and toxic metals, see Tables 1 and 2), redox chemistry plays a dominant role in pathogenesis, through the onset of outcomes resulting from biomolecular injury (see Figs 1–4). In relation to this, many metals are able to perform futile one-electron recycling between couples formed between appropriate valency states (Wiseman and Woods 2002). Furthermore, hydrogen radical abstractions can occur that can trigger a chain reaction in unsaturated phospholipids of oxidized membranes (both periplasmic membranes and those of cytoplasmic organelles) that releases breakdown products (when heated with FeII/ascorbate) such as malondialdehyde that are appropriate for bioassay by the thiobarbituric acid (TBARS) procedure (Halliwell and Gutteridge 1999).

Furthermore, the likelihood of superoxide anion-free radical ($\cdot O_2'$) generation produces an enhanced risk of biomolecular injury as a manifestation of the biohazard of particular metal contamination, both of the environment and in the body. The essential role of oxygen gas, which has built up in the atmosphere as a result of the photosynthetic activity of green plants over a 3·5-billion-year period is paramount in ROS production. The toxic nature of oxygen (Abele 2002), although essential for aerobic life on earth, has been subject to amelioration of toxicity through evolution of antioxidant enzymes that have developed over the last 250 million years. Moreover these antioxidant enzymes in the body, such as superoxide dismutase, glutathione peroxidase and catalase, augment the role of dietary antioxidants such as vitamins E and C (Wiseman *et al.* 2000; Wiseman and Woods 2002).

Furthermore, the use of DNA recombinant forms may confer unexpected technological advantage in bioprocesses in cases where novel proteins, such as biocatalyst enzymes and cells, are introduced into previously unfavourable environments. Where these introductions are associated

Table 1 Toxic and nontoxic metals that may be detectable in yeast-bioprocess feedstocks (see Table 4)

Metal	Some possible valency states	Toxicity in the diet
Selenium	SeII/SeVI	Toxic at 500 μg day^{-1}; essential at 50 μg day^{-1} so is a benemin metalloid (beneficial at minimal dose)
Iron	FeII/FeIII/FeV	Bioaccumulates
Copper	CuI/CuII	Bioaccumulates
Tin	SnII/SnIV	Limit of 5 ppm in canned foods
Barium	BaII	Very toxic
Aluminium	AlII	Accumulates in brain
Arsenic	AsIII/V	Biocumulative poison (AsIII)
Mercury	HgI/HgII	Destroys cysteine (thiol) enzymes
Cadmium	CdII	Destroys cysteine (thiol) enzymes
Antimony	SbIII/V/VI	Destroys cysteine (thiol) enzymes
Lead	PbII/IV	Enzyme inhibitor
Nickel	NiII/III/V	Skin irritant and nongenomic carcinogen
Silver	AgI/II	Mildly toxic
Beryllium	BeII	Toxic
Magnesium	MgII	Can compete with CaII
Strontium	SrII	Can compete with CaII
Lithium	LiI	Can antagonize Na+
Plutonium	PuIV	Very toxic
Chromium	CrIII	All these metal ions are largely nontoxic in humans; but not necessarily so in bacteria of the nitrogen cycle (Butler 2002)
Zinc	ZnII	
Molybdenum	MoVI	
Cobalt	CoII	
Vanadium	VV	
Calcium	CaII	
Manganese	MnIV/MnVII	

Table 2 Multifunctional versus dysfunctional components of foodstuffs including yeasts: *Saccharomyces cerevisiae* used in brewing and baking (see Table 5)

Multifunctional component	Dysfunctional component	Main dietary origin
Phytoestrogens (isoflavones)	Ethanol/butanol	Soya flour Fermentation-produced alcoholic beverages
Polyphenols (flavones)		Tea/onions
Minerals including iron and selenium		Meat, bread and cereals
n-3 fish oils		Oily fish
	Mycotoxins such as patulin, ochratoxin and aflatoxin (see Table 5)	Fungi such as *Aspergillus flavus*
Polyunsaturated fatty acids		Vegetable oil
	Bacterial toxins such as *E. coli* O157 toxin	Bacteria such as *E. coli* O157 food poisoning see Table 5
Vitamins E, C and D, and other antioxidants		Fruits (C): cereals such as wheat germ (E), oily foods (E and D)
Vitamin B		Yeast bread products and in yeast-brewed beverages such as beer (Wiseman and Woods 2001)

Other food yeasts include: *Candida utilis*, *Candida tropicalis* and *Candida lipolytica*.
Saccharomyces ellipsoideus ethanol resistant is used in the manufacture of barley wines.

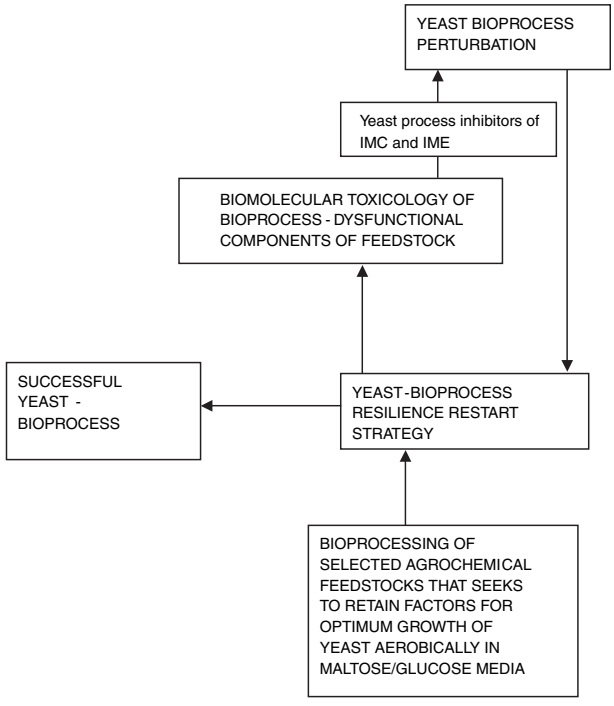

Fig. 1 Biomolecular injury that is responsible for bioprocessing perturbations of yeast bioprocesses: amelioration by bioprocessing resilience (IMC, immobilized cells; IME, immobilized enzymes)

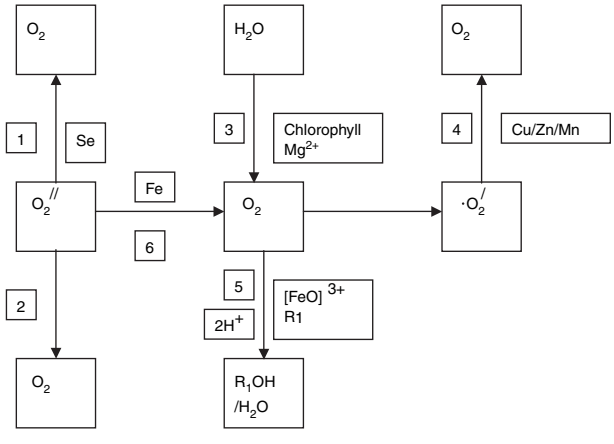

Fig. 2 Metalloenzymes that could be deployed to combat reactive oxygen species (ROS) in yeast bioprocesses. 1, SeII in glutathione peroxidases; 2, FeIII in catalases; 3, MgII in photolysis of water (light reaction in photosynthesis using chlorophyll supplies the oxygen); 4, CuII/ZnII/MnVII in superoxide dismutases; 5, FeII/FeIII/FeV $[FeO]^{3+}$ in cytochromes P450 mixed-function oxidases (R_1 is xenobiotic substrate); 6, FeII/FeIII in peroxidases)

with the biometabolism because of gut microflora of animals, dietary idiosyncrasy considerations are involved in the prediction of harmful outcomes. These predictions therefore need to be evaluated as part of the risk/benefit analysis

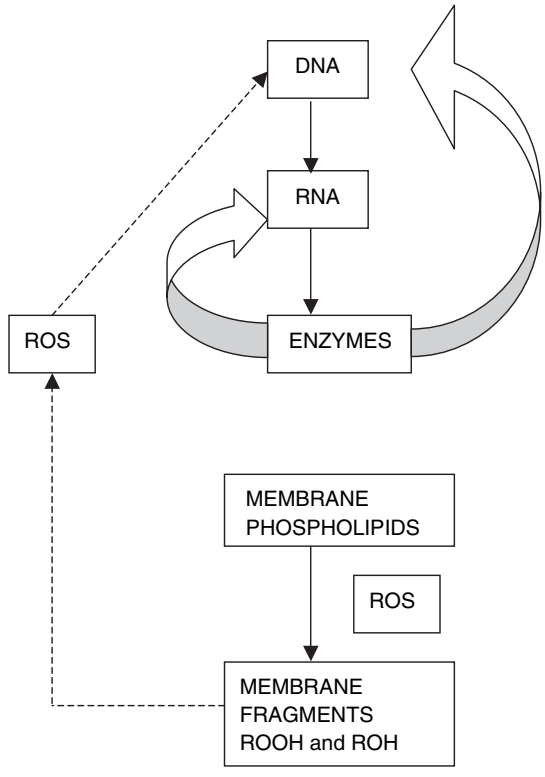

Fig. 3 ROS and the membrane fragments that they produce during oxidative stress as a cause of biomolecular injury to DNA, RNA and enzymes/proteins in yeasts and other micro-organisms. Protection of DNA and RNA by antioxidant enzymes (ROS, reactive oxygen species; ROOH, lipid hydroperoxides; ROH, lipid alkoxides)

associated with the necessary toxic hazard/risk recommendation by studies *in silico* and in real-lab (*in Petri*). There is a major interest in the possibility that bioactive compounds in foods from agriculture, particularly those in plant and microbially fermented foods, can protect against a wide range of diseases (Wiseman 1998). Furthermore, bioactive compounds can have gender-differentiated effects that are dependent also on a number of other factors including age, genomics and stage of reproductive development. Complacency may be the result of overconfidence in the prediction of 'nontoxicity', to bioprocess and to consumers, but failure to predict the likelihood of a disaster outcome with foodstuffs, has been manifest in the past. Examples include turkey X-disease caused by aflatoxin (a hepatotoxin and carcinogen) in groundnuts contaminated in storage by *Aspergillus flavus* (late 1940s Africa; see Tables 1 and 2); Spanish cooking oil syndrome caused by contamination of cooking oil with aniline (1970s); syndromes caused by forms of organic mercury produced by micro-organisms in waterways (1980s). Natural foodstuffs may also contain toxins (see Tables 3 and 4) and protectants (see Tables 5 and 6).

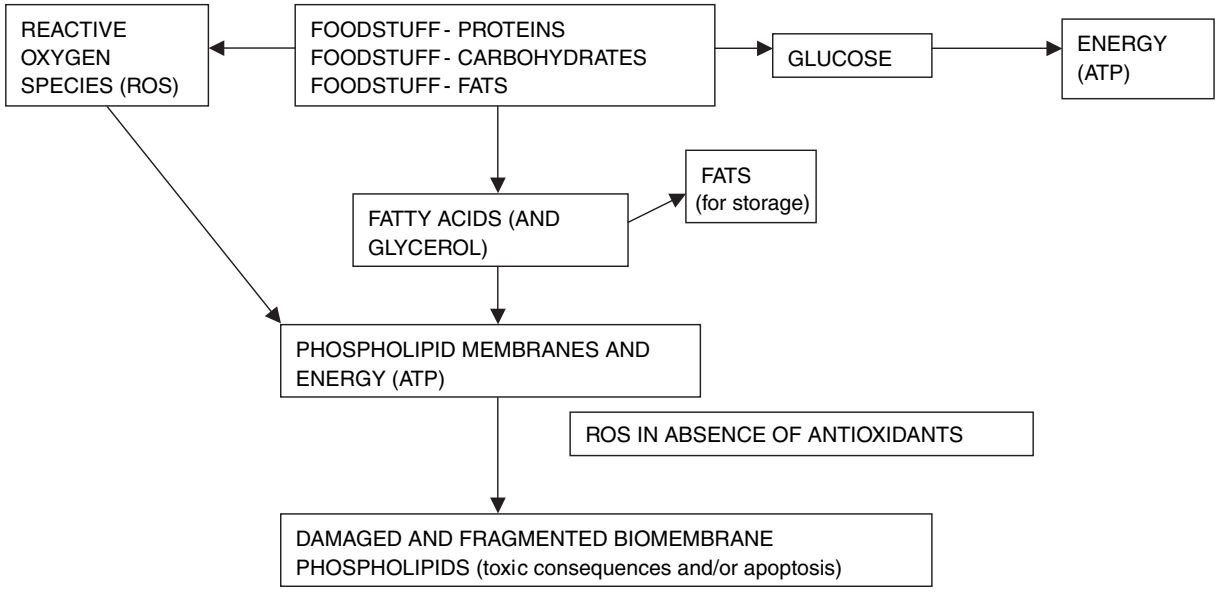

Fig. 4 Biomolecular outcome dysfunctionality caused by ROS toxicity (see Table 3) in yeasts and other micro-organisms (Antioxidants such as vitamins E and C chain terminate the formation of membrane-degrading ROS)

Table 3 Causes of biomolecular injury through oxidative stress in micro-organisms including yeasts (see Fig. 4)

Chemical species	Name
ROS ($\cdot O_2', \cdot OH, \cdot \Delta gO_2$)	Reactive oxygen species (FR)
RSS	Reactive sulphur species (FR)
RSeS	Reactive selenium species (FR)
RNS (NO_x)	Reactive nitrogen species (FR)
O_2	Oxygen (FR in $\cdot \Delta gO_2$ molecular singlet state – see above)
O_3	Ozone
Cl_2	Chlorine (bleaches)
RL	Redox ligands
X	Xenoestrogens
Y	Radionuclides (■-emitters)
Z	Redox dyes (such as phenol red)
M^n/M^{n+1}	Redox metal couples
RE	Redox enzymes such as cytochromes P450 in futile electron recycling when substrate is in short supply. The electron transport chain proceeds through NADH and redoxins in bacteria

FR, free radical.

Table 4 Prevention of biomolecular damage in bioprocessing through addition of antioxidant biochemicals, enzymes and chaperone proteins (see Table 1)

Antioxidant chemicals
Vitamins such as E and C are free radical production chain breakers
Phytoestrogens (Wiseman *et al.* 2000, 2002)
Free radical scavengers such as other free radicals
Reducing agents (low redox potentials)
Some proteins (thiol-containing for example)
Xenoestrogens such as bisphenol A, alkylphenols (octylphenols and nonylphenols)
Antioxidant enzymes
Catalases (attacks H2O2 by direct cleavage)
Cu/Zn, Mn or Fe (bacterial) superoxide dismutases (attacks $\cdot O_2'$) (Wiseman and Woods 2002)
Se-glutathione peroxidases (attacks H2O2 with peroxidation of glutathione)
Thioreductases (Se) remove peroxides
Peroxireductases (Prdx1) remove peroxides
Proteomic strategies with antioxidant chaperones coupling
Protein couples that protect one of the pair from ROS (positive or negative cooperativity may be observed here). The proteomics of the detailed understanding of protein : protein interactions in the cell can be progressed rapidly by separation techniques (e.g. 2D-SDS/PAGE, GC/MS and HPLC/MS)

Even mildly toxic metals must not be deliberately added to food bioprocesses even in protein or co-complexed form: for example the Ru metal analogue should not be employed instead of Fe (complexed in porphyrin protohaematin IX) in cytochromes P450 in redox bioconversions mimics in the food industry (Lewis 2001; Wiseman and Woods 2003a,b).

Molecular toxicology has provided a good mechanistic basis for prediction of future problems, and this approach is supported by the power of computer modelling on a wide range of unusual microbially produced chemicals that reach the body via methods of bioprocessing of foodstuffs that include solid-state fermentation. Injury to macromolecules,

Table 5 Toxins in bioprocess media that could contaminate the initial product prior to downstream processing (see Table 2)

Toxin or precursor	Example of source
Aflatoxin (causes liver damage, and is a very potent carcinogen)	Mouldy groundnuts – contaminated by *Aspergillus flavus* (aflatoxin is removed in oil manufacture)
Cyanogens (can form cyanide)	Untreated cassava almond nut kernels
Mutagens and carcinogens such as benzo(a)pyrene (cause damage to DNA)*	They form in burnt feedstocks and foods
Solanins (neurotoxins)	Skins of potatoes greened by light exposure
Patulin	Mouldy apples
Bacterial toxins	Foods contaminated with for example *E. coli* O157, *Salmonella* spp., *Shigella* spp.
Algal toxins	Red algal blooms
Ochratoxins	Mouldy feedstocks
Heavy metals such as mercury	Some feedstocks of microbial, plant and fish origin

*Benzo(a)pyrene can be activated to the ultimate (proximate) carcinogen by cytochromes P450 1A1.

Table 6 Nutraceuticals retained after food bioprocessing: metabolism by gut microflora

Protectant (additive)	Source in feedstock
Quercetin (flavonoid)	Onions and tea
Lycopene (carotenoid) removes singlet state molecular oxygen ($\cdot\Delta gO_2$)	Red tomato derived (Crozier *et al.* 1997)
Phytoestrogen (isoflavones) (Bowey *et al.* 1998; Wiseman *et al.* 2002)	Soya protein

Phytoestrogen isoflavones include the soya protein-derived glucones daidzin and genistin. These are degraded to the aglycone forms daidzein and genistein by gut microflora: and these initial products can be degraded further by other bacteria in the gut for example to equol derivatives and equol conjugates with glucuronic acid (excreted through the kidney). Fermented soya foods, such as miso or tempeh, contain mostly the unconjugated isoflavone aglycones (Wiseman *et al.* 2002).

relates to more than just structural damage that is sometimes obvious and predictable: functional damage may also follow.

Moreover, functional damage can be predicted *in silico*: injury therefore to catalytic proteins (enzymes) and honorary enzymes (many other proteins) may result in functional problems with nucleic acids and other biologically active biomolecules. Further investigation is needed on the interactions of macromolecules especially in micro-organisms prior to harnessing these into food bioprocessing.

PREVENTION OF TOXICITY IN FOOD BIOPROCESSING THAT UTILIZES YEASTS

Prevention of toxicity by safe choice (or GM control) of micro-organisms is to be recommended. Unfortunately, prevention is based upon prediction of toxic responses via risk-prediction analysis usually *in silico*. Cure or at least the 'successful treatment' of manifest bioprocess toxic responses may become the easiest option: these will then become a strategy to ameliorate the undesirable damage caused by biomolecular injury in yeast bioprocesses such as brewing (Wiseman 2003; Wiseman and Woods 2003a,b).

The recognition of characteristic responses of classes of cellular biomolecules, such as nucleic acids, proteins and phospholipids to injury was developed in the twentieth century. Nevertheless, a more holistic approach can advance further our understanding of the behaviour, of these macromolecules under oxidative stress. A web of interactions is evident amongst biomolecules in micro-organisms, disturbance of such biomolecular webs by toxic substances is often the basis of the toxic manifestations seen as loss of homeostasis in pure cultures of micro-organisms. Furthermore, stability of microbial species in mixed cultures has been attributed to relatively few species in a web of interaction: perturbation to the web by external forces may be subjected to compensatory restoration of the 'equilibrium position' amongst the microbial participants.

Moreover, supply of metabolites is attributable to enzyme action within the living micro-organisms. For example, atmospheric nitrogen-fixing (using the nitrogenase system) by soil organisms such as *Azotobacter vinelandii* (aerobic) or *Clostridium pasteuranum* (anaerobic) are controlled by metabolite supply. In the latter case, supply of pyruvate allows a chemostat interpretation of growth kinetics, mediated by the CoA pyruvate ferredoxin oxidoreductase-catalysed cleavage of pyruvate to acetyl phosphate (this allows a subsequent production of ATP) plus carbon dioxide. Such micro-organisms occupy an ecological niche that can be subjected to perturbation by the introduction of the end-product of nitrogen fixation (ammonia). Linking species webs, are therefore a reflection of the underlying enzyme-catalysed webs of biochemical metabolites that make up both the cellular interior milieu of organisms and the associated biochemical mixtures in the growth and maintenance media, especially in scale-up fermentation bioreactors.

Immobilized enzyme utilization complicates the biochemical mixes present, and make reductionist approaches in prediction of expected biochemical balance more risky, especially if an unidentified microbial mix is employed as the bioindicator in pilot plant studies. Toxicity to one species-web, may however favour stability of a competing species web: the presence of known (or unknown) yeast-

bioprocess antitoxicants (these are often antioxidants against ROS), however, lower the accurate prediction of final outcome in the balance equilibria observed with mixed cultures of micro-organisms including bioprocess yeasts, and their enzymes used in bioprocessing (Tucker and Woods 1997).

The dilemma faced by molecular toxicologists for biohazard prediction lies, therefore, in an identification of chemostasis based upon a relatively few named species webs. Resilience as opposed to growth perturbation may be predicted wrongly *in silico* for some mixed cultures of micro-organisms: it is essential, therefore, that real-lab (*in Petri*) studies are also undertaken. Species bioindicators and molecular biomarkers should both be subject to continuous assessment by critical biomonitoring to predict such yeast-bioprocess perturbation as opposed to resilience outcomes.

ANTIOXIDANT/PRO-OXIDANT RELATIONSHIPS THAT RESULT IN BIOMOLECULE PROTECTION OR DAMAGE

Harmful free radicals (Halliwell and Gutteridge 1999), such as superoxide anion are produced in micro-organisms during aerobic respiration because of only partial reduction of some oxygen molecules in the electron transport chain. This is because of one-electron reduction of each atom of oxygen, instead of two-electron reduction to form water. Similarly, in many micro-organisms the electron transfer chain from NADH to water (with insertion of one oxygen atom into xenobiotic substrates) that uses cytochromes P-450 may display futile cycling, in the absence of substrate, to produce the superoxide anion ($\cdot O_2'$). Reduction of ROS, and also of reactive nitrogen species (RNS), is a priority for avoidance of production in particular microbial cultures susceptible to oxidative stress. For example, damage to biomolecules can occur by attack on phospholipid cytoplasmic and internal membranes, and also notably on DNA: and on the recombinant DNA of GM yeasts employed in manufacture of proteins such as insulin and interferon, intended for human therapy.

REDOX POTENTIALS OF ANTIOXIDANT : PRO-OXIDANT MOLECULAR COUPLES

All redox biochemicals, existing in their oxidized and reduced forms, constitute an oxidation/reduction couple, for instance, with polyphenols and some nutraceuticals (Ridgway and Tucker 1999). Each of such redox couples displays an experimentally determined redox potential (reduction oxidation potential) of a particular voltage under standard (defined) conditions of temperature and pressure (usually 25°C, pH 7·0, 1 atm). The standard redox potential scale (E_o^1) has good oxidizing agents (pro-oxidants) such as molecular oxygen at the top with the high value of +0·815 V, and the reducing agents (antioxidants) at the bottom. For example, NADH is at −0·320 V (the scale passes through zero) and a voltage difference of 1·135 V therefore is associated with electron flow from NADH to O_2 (in oxygen-utilizing respiratory chains). This potential difference is normally sufficient to allow the production of a total of at least three ATP, each one at a particular step in the respiratory chain via known carriers by proton pumping through the inner membrane of yeast mitochondria. For instance, in interconversion of Fe(III) : Fe(II) the latter loses one electron during its oxidation to the FeIII (3+) form and thus these two forms of iron are pro-oxidant (Fe^{3+}) and antioxidant (Fe^{2+}), respectively, but with the extra involvement of perferryl Fe^{5+} in the cytochrome P450 mechanism via $[FeO]^{3+}$ the oxonium cation (Lewis 2001; Williams *et al.* 2003).

CONCLUSIONS

Fe^{3+}/ascorbate is commonly employed to generate Fe^{2+}, ostensibly the antioxidant form of the iron III : iron II couple to catalyse the breakdown of phospholipid liposomal membrane model systems. Fe^{2+} reacts through Fenton chemistry (Halliwell and Gutteridge 1999) with lipid hydroperoxides (ROOH) or lipid alkoxides (ROH) in the phospholipid mixture after storage in an atmosphere that contains oxygen, to form these peroxyl ($ROO^{\bullet}$) and alkoxy ($RO^{\bullet}$) free radical derivatives of phospholipids. These free radicals initiate a chain reaction of further attack of the phospholipids via degradation reactions that produce large amounts of these free radical species (Wiseman *et al.* 2000; see Figs 3 and 4).

Avoidance biostrategies against ROS and RSS in many oxidatively stressed bioprocesses that utilise yeasts (and other micro-organisms) are essential where human therapeutic proteins are expected to be produced in high yield. These proteins intended for therapy include insulin, inter ferons, interleukins, growth hormone and blood clotting factors (VIII and XIII). Moreover, the renewed demand for antigens of viruses such as hepatitis, severe acute respiratory syndrome (coronaviruses), foot and mouth disease, and HIV retroviruses, has established a greater demand for nonperturbation of yeast bioprocesses by ROS (Wiseman 2003) by development of stable phenotype despite variation in gene deletion (Stearns 2003).

Moreover, antioxidants, natural and synthetic, are being developed to supply the correct level of yeast-bioprocess resilience (Wiseman 2003) (see Tables 4–6) through a range of genomic and proteomic strategies.

Furthermore, the coupling in yeast bioprocess of thioredoxin (Se) as an electron donor for peroxiredoxins (these use catalytic cysteine–cysteine residues to remove peroxide) can be viewed as an useful extension to other antioxidant enzymes which could overload the yeast bioprocess with unwanted metals such as Cu and Zn (see Table 1). Nevertheless, Se-supplementation could be viewed as health benefiting where soils are deficient in this metalloid antioxidant component of glutathione peroxidases (Rayman 2002).

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