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Biochemical aspects of stuck and sluggish fermentation in grape must

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Recently a number of studies have focused on the factors responsible for the occurrence of stuck and sluggish fermentations. Results from these studies indicate that together with nutritional deficiencies and inhibitory substances, technological practices could lead to such situations. This review explains, from a biochemical point of view, the influence of nutritional deficiencies, inhibitory substances and technological practices on yeast cell development and physiology and the fermentation process.

Keywords: stuck and sluggish fermentation; must; wine; yeast; enology; *Saccharomyces cerevisiae*

Introduction

One of the very important objectives during most winemaking processes is the achievement of complete alcoholic fermentation, so that the residual fermentable sugar in the wine is less than 2–4 g L^{-1}. The completion of the fermentation then allows the winemaker to begin the finishing operations, and more importantly, allows the wine to be stored under conditions of restricted contact with air, avoiding the resulting destructive oxidation reactions. Furthermore, complete alcoholic fermentation may help avoid problems not only with acetic acid bacteria but also with lactic acid bacteria, which could metabolise residual sugars to increase volatile acidity and also in the formation of abnormal esters and perhaps alter the pattern of diacetyl formation [69].

The current literature which relates to stuck or sluggish fermentation is substantial. Factors such as high initial sugar content [55], vitamins or nitrogen substrate deficiencies [1,40,72,88], anaerobic conditions [98], high ethanol content [22], excessive clarification of the must [34], presence of toxic fatty acids [33,102] and high concentrations of volatile acidity [50] have all been considered to be the cause of fermentation problems. The actual study of stuck and sluggish fermentation is therefore often rendered very difficult due to the multiple factors which could lead to a decrease in fermentation rate and the possibility of synergistic effects.

Despite the large body of literature concerning the cause of stuck or sluggish fermentation there are no reports which describe the relationship between the factors which lead to protracted fermentation and the biochemical mechanisms responsible for the decrease in fermentation rate. Therefore, the intent of this review is to focus on both factors and biochemical mechanisms responsible for stuck and sluggish fermentation in grape must.

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Nutritional deficiencies

Nitrogen deficiency

In some cases stuck or sluggish fermentation would appear to be caused by insufficient levels of assimilable nitrogen [1,40,88]. Nitrogen content in grape juice ranges from 60 to 2400 mg N L^{-1} [71], depending on grape variety, viticultural region, berry maturation and winemaking process [37]. For these reasons many researchers have attempted to quantify the requirement of wine yeast for assimilable nitrogen [43]. Various studies have shown that a minimum of 120–140 mg N L^{-1} is required to produce a normal fermentation rate. In a recent study Jiranek *et al* [43] demonstrated that nitrogen utilization was also influenced by the presence of air and initial glucose concentration. A low initial level of nitrogen acts by limiting growth rate and biomass formation of yeast, resulting in a low rate of sugar catabolism [14,65,66,88].

Upon exhaustion of the nitrogen source in the medium, a drastic decrease in sugar transport activity is observed [19,56] which may account in part for the inhibition of CO_2 production. This sugar transport inactivation is triggered by the arrest in protein synthesis linked to the lack of nitrogen source. While the half-life of the bulk proteins is greater than 70 h, the half-life of the glucose transport process is about 5 h. Thus complete inactivation of glucose transport occurs about 50 h after ammonium depletion [88,90]. To avoid glucose transport inactivation, it is necessary to maintain a high rate of protein synthesis which can be supported by an ample ammonium supply, before the depletion of assimilable nitrogen. The decrease in carbon dioxide evolution rate, which is related to lack of ammonium source, could also be explained by two other possible mechanisms. The first one involves the deactivation of the key enzyme, phosphofructokinase. It has been demonstrated that ammonia is an allosteric activator of phosphofructokinase [77]. The second mechanism is also related to the deactivation of phosphofructokinase. With a depletion in the supply of ammonia, the signalling pathway induced by the presence of fermentable sugar is also directly impacted, leading to decreased CO_2 evolution rates [96].

Oxygen deficiency

Metabolism of yeast in fermentation is dependent on the presence of dissolved oxygen at the beginning of the fermentation. During the fermentation, dissolved oxygen is rapidly consumed by the oxidases naturally present in the must and by the yeast. Decrease in oxygen availability results in an inhibition of fatty acid and sterol biosynthesis in the yeast [10,25] and consequently a decrease in biomass production and rate of glycolysis. Biosynthesis of monounsaturated fatty acyl residues in *S. cerevisiae* proceeds from the saturated residue in a reaction which involves NADPH and molecular oxygen. Under anaerobic conditions, Andreasen and Stier [9] observed an inactivation of the oxygen-dependent desaturase. Sterol biosynthesis in yeast also requires oxygen; for example, the cyclization of squalene which allows lanosterol synthesis to occur is oxygen dependent [78].

In a recent study, we have shown that deprivation of oxygen during fermentation of grape must induces qualitative and quantitative changes in the lipid composition of the cell [2]. We observed a rapid decrease in unsaturated fatty acid level during fermentation of must and, at the same time, an increase in squalene levels, which indicated an inhibition of sterol biosynthesis. When fermenting wort or grape juice is aerated, enhanced yeast growth and viability are observed. The increase in viability at the end of alcoholic fermentations, which comes from the aerobic formation of special sterol and fatty acids, resulted in their being named 'survival factors' [54,99]. Undoubtedly they are also used as growth factors, but their importance is more dramatically demonstrated at the ethanol concentrations normally associated with fermentation of high sugar media such as grape juice or high gravity wort. The greater survival rate of yeast when fermenting wort is aerated is probably associated with a plasma membrane enriched in unsaturated fatty acids and ergosterol [2,62]. Evidence of the direct role of unsaturated fatty acids and sterols in the observed increase in cell viability during fermentation was provided in an experiment in which sterols and unsaturated fatty acids were added to the wort [25]. Enrichment of the plasma membrane lipids of anaerobically grown *S. cerevisiae* with linoleic acid proved even more effective than supplementation with palmitoleic acid for the enhancement of cell viability in the presence of ethanol [98]. Thus, oxygen deficiency is responsible for sluggish fermentation as a consequence of the inhibition of lipid biosynthesis which results in decreased ergosterol and unsaturated fatty acid content, decreased biomass production and yeast viability. Alteration in plasma membrane lipid composition also results in changes in ethanol tolerance in the yeast cell. This relationship between lipid composition and ethanol tolerance will be discussed in more detail below.

To prevent the adverse effects due to lack of oxygen, winemakers often aerate the must and according to Sablayrolles and Barre [83], production of biomass and cell viability are maximal when oxygen is added at the end of yeast growth. This greater viability is due to an increase in ethanol tolerance associated with lipid composition in the plasma membrane and with decreased levels of toxic fatty acids.

Oxygen deprivation affects toxic fatty acid production, ie the production of octanoic and decanoic acid. We have shown that the level of these acids decreases during aeration of the must [2]. In the presence of oxygen, acyl-CoA synthesis is greater than under anaerobic conditions [70]. The presence of acyl-CoA inhibits fatty acid biosynthesis by inhibiting acetyl-CoA carboxylase [47] and fatty acid synthetase [94] which are directly linked to the lower level of toxic fatty acids when oxygen is present in the must.

The above effects of oxygen deprivation help explain the importance of addition of small amounts of sulfur dioxide to the must immediately after the grapes are crushed. The sulfur dioxide is inhibitory to oxidase enzymes naturally present in the must, especially the polyphenol oxidases. If these enzymes are not immediately inhibited, they will scavenge most of the dissolved oxygen (which is already very low because of its natural low solubility). Furthermore, the practice of aeration, mentioned above, will also not be effective unless these enzymes have been inhibited [17].

Mineral deficiency

Magnesium is important for many metabolic and physiological functions in yeast [103]. Magnesium is involved in cell integrity, generally by stabilizing nucleic acid, proteins, polysaccharides and lipids. Mg^{2+} also plays a key role in metabolic control, growth and cell proliferation. For greater detail, the review of Walker [103] is useful.

Although there have been no investigations to evaluate the role of Mg^{2+} during the alcoholic fermentation of grape must, the importance of this element in the fermentation process will be described since numerous studies report the influence of magnesium on the alcoholic fermentation of molasses or glucose (in minimal or complex media) [29,44,103,104]. Therefore it seems likely that magnesium constitutes an important factor of must fermentations.

Magnesium is indispensable to the glycolytic pathway since it is required for hexokinase and phosphofructokinase activity and the decarboxylation of pyruvate. This element is also involved in the activation of the alcohologenic enzymes. Thus magnesium plays a central role in ethanol production. There is a direct relationship between magnesium availability and fermentation kinetics. Limited Mg^{2+} availability is responsible for decreased yeast growth and fermentative activity [24,29,46]. Addition of magnesium in the fermentation medium results in enhanced ethanol production. This may be explained by the fact that magnesium stabilizes membrane structure [29]. Dombek and Ingram [29] demonstrated that increasing magnesium concentration in the medium resulted in a two-fold increase in ethanol production, which could be explained by a prolonged exponential growth phase in yeast resulting in increased cell mass, rate and yield of ethanol. These authors suggested that Mg^{2+} could reverse the inhibitory effects of ethanol. Supplementation of Mg^{2+} could counteract ethanol-induced leakage and consequently restore metabolic activity.

Unfortunately, data concerning Mg^{2+} levels in grape must are scarce. In white grape must, Walker [103] reported a concentration of 58 mg L^{-1}, Amerine *et al* [8] reported 10–25 mg L^{-1} and Eschnauer [31] reported 60–140 mg L^{-1}

in wine. It is interesting that Kunkee and Bisson [51] noted that the optimum concentration of Mg^{2+} ions to restore viability in yeast (5 mg L^{-1}) was less than that naturally found in vinification media. Further work related to Mg^{2+} utilisation by *S. cerevisiae* and Mg^{2+} interaction with wort constituents during fermentation of grape must is needed.

Vitamin deficiency

Some sluggish fermentations appear to be associated with insufficient availability of vitamins [72]. Literature concerning vitamin deficiency in grape must is rare and studies have focused essentially on thiamine levels which may range from 150 to 750 μg L^{-1} [76]. Although *S. cerevisiae* is able to synthesise thiamine, lack of thiamine in the fermentation medium may lead to sluggish fermentation. In a recent study, Bataillon *et al* [11] have shown that the observed decrease in thiamine levels in must could result from a rapid assimilation by wild yeast. They observed that a contaminant wild-type yeast population could deplete thiamine from the medium in a few hours, leading rapidly to stuck or sluggish fermentations. Musts are naturally contaminated with wild yeast populations; thus enological practices which delay the inoculation of the must with non-wild-type *S. cerevisiae* could affect the fermentative activity.

Thiamine is cleaved, and its biological activity destroyed, by sulfur dioxide (that is by disulfite ions). This cleavage can lead to fermentation difficulties when especially high concentrations of sulfur dioxide are employed or when grape musts (or concentrates) are stored for long periods of time at nominal concentrations of sulfur dioxide [17].

Inhibitory substances

Ethanol

Accumulation of ethanol during the fermentation of sugars by yeasts can lead to inhibition of the fermentation process itself and cause other unfavourable effects in yeast cells. Although ethanol 'accumulates' inside the cell during alcoholic fermentation, the best evaluations seem to show that the intracellular and extracellular concentrations of ethanol are comparable [51]. The biochemical bases for these events have been widely studied. Figure 1 summarises the main effects of ethanol on the cell.

It is well known that ethanol inhibits yeast growth [42,97] and viability [98]. Among the different transport systems utilised by *S. cerevisiae*, ethanol has been shown to inhibit the general aminoacid permease [20] and the glucose transport system [57,61,67,75,87]. It should be noted that up to 8.5% ethanol (v/v) does not change the glucose uptake velocity or the activity of key glycolytic enzymes. However, the fermentation rate is reduced by about 50% [75]. Conversely to the previous report, Leao and Van Uden [57] and Mauricio and Salmon [61] demonstrated that ethanol inhibits sugar transport activity. In a recent study Zamora *et al* [105] reported that sugar transport inactivation by ethanol showed different patterns depending on whether the high or low affinity transport system was investigated. At 5–15% (v/v) ethanol, altered activities in the low affinity transport system are seen. With the high affinity transport system, an increase in inhibition was observed with increasing ethanol concentration. The observed decrease in CO_2 production during enological fermentation is in part due to a decrease in sugar transport efficiency. This latter event results from catabolite inactivation and non-competitive inhibition of the sugar transport system by ethanol [87,88,105].

Ethanol also inhibits proton fluxes [21,48,58]. The ethanol-induced increase in the rate of proton influx decreases the transmembrane proton gradient, possibly resulting in the uncoupling of electrogenic processes and subsequent growth inhibition [58]. We and others have recently shown that the plasma membrane ATPase responsible for the creation of the electrochemical gradient, was activated when cells were grown in the presence of ethanol [4,81]. This adaptation mechanism is supposed to counteract increased cytoplasmic acidification induced by subsequent exposure to ethanol [4]. A decrease in plasma membrane H^+ATPase activity during the late exponential growth phase of *Saccharomyces cerevisiae* cultures has been reported [100]. The plasma membrane H^+-ATPase is responsible for the creation of an electrochemical gradient which constitutes the driving force for nutrients. A decrease in fermentation rate could be expected subsequent to the decrease in nutrient uptake. However it is not known if the decrease in fermentation is related to the decrease in ATPase activity, since there are currently no systematic studies on the evolution of ATPase activity during fermentation under enological conditions.

The presence of ethanol also affects the plasma membrane of *S. cerevisiae*; the damage caused results in altered membrane organisation and permeability [41,57,59,98]. Ethanol interacts with membranes, possibly by entering the hydrophobic interior and thereby increasing the polarity of this region, thus permitting the free exchange of polar molecules and weakening the hydrophobic interactions. Ethanol stress is known to produce changes in the lipid composition of the yeast plasma membrane [5,6,26,51,57,59,64,86,98], including the saturation level and chain length of unsaturated fatty acids, thereby resulting in a modification of membrane fluidity [5,6,45,59]. An increase in unsaturation index correlated well with an increase in ethanol tolerance and increased viability of *S. cerevisiae* [4,12]. Uptake of glucose and amino acids was also less affected by ethanol in yeast enriched with unsaturated fatty acids [97]. Increased viability when oxygen is added to the fermentation medium is due to enhanced synthesis of unsaturated fatty acids and sterols, the so-called 'survival factors' (see above), which allow greater ethanol tolerance and minimise the detrimental effects of ethanol. It is clear that a decrease in fermentation rate is related to ethanol formation, when one takes into account all the related effects of ethanol in the fermentation medium. Depending on the ethanol tolerance of the yeast, a high level of ethanol may quickly lead to stuck or sluggish fermentation.

Toxic acids

Medium chain fatty acids: Numerous factors favour stuck or sluggish fermentation as previously described.

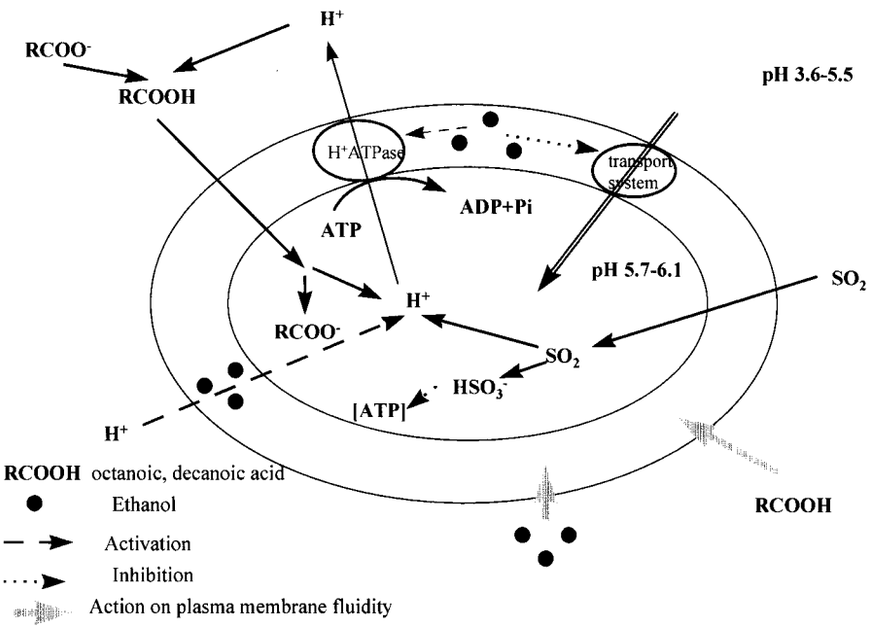

Figure 1 Mechanisms of action of inhibitors present in wine on yeast metabolism.

However, in a rich medium fermenting under satisfactory physico-chemical conditions, the cell yeast cycle may decline, with a consequent decline in fermentation activity until complete arrest of the process. In this case, it is clear that inhibition results from modification of the medium due to yeast metabolism. Apart from ethanol, medium chain fatty acids constitute another type of fermentation inhibitor produced during alcoholic fermentation [53]. According to Taylor and Kirsop [95], the medium chain fatty acids excreted into the wort during alcoholic fermentation by *S. cerevisiae* are intermediates in the biosynthesis of long chain fatty acids. The amount of fatty acids released into the fermentation medium is dependent on the yeast strain, medium composition and fermentation conditions (temperature, pH aeration) [2,46,49]. Nordstrom [68] and Freeze *et al* [32] have described medium chain fatty acids as antimicrobial components. With concentrations of up to 114 and 46 μM of octanoic and decanoic acid respectively, the specific growth rate of *S. cerevisiae* decreased as an exponential function of the fatty acid concentration [102]. Decanoic acid caused rapid cell death at its inhibitory concentration [39] of 46 μM. These fatty acids also decreased the specific thermal death rate of *Saccharomyces bayanus* [85] and stimulated ethanol-induced leakage of amino acids and unknown compounds (with a 260 nm-absorbing capacity) from *S. cerevisiae* [84].

Fatty acid activity is highly dependent on the pH of the medium: toxicity increases as pH decreases, indicating that the undissociated molecule is the most toxic, decanoic acid being more inhibitory than octanoic acid [102]. Stevens and Servaas Hofmeyer [91] showed fatty acids with shorter chain lengths exerted their effect by acting as proton carriers across the yeast plasma membrane, thereby reducing the intracellular pH and disrupting the proton gradient. These weak-acid preservatives enter the cell by simple diffusion, and once in the cytoplasm they rapidly dissociate into ions thus releasing protons and acidifying the cytoplasm (Figure 1). Decanoic acid, for example, enhanced the passive H^+-influx across the plasma membrane. In a recent study, we reported [3] that decanoic acid induced an alteration of the plasma membrane by increasing fluidity which may explain the observed increase in proton influx. The direct inhibitory effects of medium chain fatty acids on yeast cell growth account for the observed reduction of fermentation rate when high levels of medium chain fatty acids are present in the fermentation medium. It is possible that the medium chain fatty acids act in direct synergy with ethanol [85], to further slow the fermentation process.

In a recent study Zamora *et al* [105] described the effect of decanoic and octanoic acid on glucose transport. Both acids inhibit the high and low affinity hexose transport system, with high affinity transport being more sensitive to acidic effects. The inhibitory effect of decanoic acid was greater than octanoic acid. From their results they concluded that the inhibition of the high affinity transport system by medium chain fatty acids could be responsible for stuck or sluggish fermentation, since when sugar concentration is around 10 g L^{-1}, the high affinity transport system is responsible for sugar uptake [16,63].

Acetic acid: Another end product of alcoholic fermentation, acetic acid, also enhances the toxicity of ethanol with respect to growth, fermentation and viability in wine yeasts (Figure 1). The mode of action of acetic acid resembles that of the medium chain fatty acids [23]. Depending on extracellular pH, acetic acid enters the cell by simple diffusion, where it dissociates [73]. In an attempt to explain the role of acetic acid in the decrease in fermentation rate, Pampulha and Loueiro-Dias [74] have studied two possible inhibition mechanisms: either acidification of the cytoplasm (pH dependence of enzymatic activity) or the action of acetic acid directly on transport or enzymatic

activities. These authors reported that enolase was the most affected by acetic acid which resulted in alteration of glycolysis. It should be noted that many stuck and sluggish fermentations are caused by the formation of high concentrations of acetic acid. This can occur during improper transport of mouldy grapes from vineyards to the wineries, allowing the premature commencement of alcoholic fermentation of the released juices and subsequent acetification by acetic acid bacteria [101]. It can also occur from improper treatment of freshly crushed must (usually in red wine vinification with unacceptably high initial pHs of >3.5), and where the must is neither inoculated with wine yeast nor treated with sulfur dioxide, allowing a very rapid growth of the so-called 'ferocious' (but indigenous) *Lactobacilli* resulting in high concentrations of acetic acid, rather than lactic acid [17]. Another possible source of elevated concentrations of acetic acid, probably not enough to give marked inhibition of wine yeast activity but enough to give a marked sensory defect, can be simultaneous alcoholic and malolactic fermentations [52]. Wine regions with musts of low nitrogen content seem to be especially sensitive to this type of spoilage. In new wine-growing regions, simultaneous alcoholic and malolactic fermentations are quite usual, with no adverse effects [17].

Effects of sulphites

Sulphites have been used for centuries in the sterilisation of wine vessels, and are now used during wine making to prevent oxidation of the must or the wine by the atmosphere [13]. Sulphite is highly toxic to microorganisms. Wine bacteria and native yeasts and moulds are very sensitive to sulphite action, while yeasts routinely employed for fermentation are less sensitive [89]. The antimicrobial action of sulphite in aqueous solutions depends on pH, temperature and time of exposure. Sulfite exists in solution in three forms (SO_2, HSO_3^-, SO_3^{2-}), the proportions of which depend on the pH value. At low pH, sulphite exists predominantly as molecular SO_2 and at higher pH values largely as sulphite ions (SO_3^{2-}) [18]. Molecular SO_2 is 500-fold more active on yeast than the other forms of sulphites (HSO_3^-, SO_3^{2-}) which explains why the compound is particularly effective against yeasts present in the must where the pH is in the range 3.0–3.5.

Sulphite is added at various stages during winemaking but mainly to the must before alcoholic fermentation to control the growth of undesired species. Addition of SO_2 to the must should be tightly controlled; the dose used should inhibit the growth of undesired species but allow the development of fermentation yeast. It is well known that yeast resistance to SO_2 varies with the species [93]. Thus, high levels of SO_2 in must could be responsible for delayed or stuck fermentation [80], although this is rare nowadays. The importance of the addition of sulfur dioxide to inhibit polyphenol oxidases and to prevent the complete depletion of oxygen, has already been mentioned. We will summarise below the molecular mechanisms by which SO_2 affects microbial cells. Further information may be found in the review of Rose and Pilkington [82].

Sulfite toxicity to yeast is largely dependent on the level of SO_2 accumulation in the cell. Macris and Markakis [60] reported that uptake of SO_2 by *Saccharomyces cerevisiae* occurred by active transport but, according to Stratford and Rose [92], SO_2 enters the cell by simple diffusion. Thus the rate of sulphite transport should play an important role in sulphite toxicity [92], and lower membrane fluidity will facilitate diffusion across the plasma membrane [18]. *S. cerevisiae* accumulates SO_2 very rapidly and at equilibrium, intracellular sulphite concentrations are many times greater than those in the suspension. This can be explained by the dissociation of SO_2 to the bisulphite anion HSO_3^- and H^+ (due to the greater pH in the cell), thereby allowing further diffusion into the cell (Figure 1).

Once inside the cell, sulphites cause a rapid decrease of the intracellular ATP level. According to Hinze *et al* [38], the depletion in ATP is the decisive event causing cell death. Mechanisms of cell death by sulphite are still unknown, though *in vitro* reactions of sulphite with certain molecules such as proteins, coenzymes and metabolites have been demonstrated [82]. It is known that sulphites react with NAD^+/NADP, and also cleave thiamine and disulphide bridges of proteins. In our system (must fermentation), addition of an acetaldehyde and SO_2 mixture induced a switch-over from alcoholic fermentation to glycerol fermentation [80]. SO_2 is known to affect many enzyme systems. Inhibition could result from conformational changes, interaction with active sites or with cofactors [82]. We have already mentioned the importance of the cleavage of thiamine by high concentrations of disulfite or in long-term storage of grape juice and grape juice concentrate.

Enological practices

Must is the raw material of winemaking. White wine vinifications are usually conducted on clarified musts. The common clarification treatments used are sedimentation (settling), clarification with separators, filtration or centrifugation. When a clarification process is too intense, decreased fermentation rate and biomass production are the results [28,30]. The clarification of grape must causes a large decrease (40–100%) in fatty acid content (C_{12} to C_{18}) [15,27], in sterol content [28] and in macromolecules (15–50%) [36] often related to sluggish alcoholic fermentation [27,35,36]. Clarification increases acetic acid and medium chain fatty acid production [7] which inhibit fermentation activity [30]. This phenomenon is probably related to the absence of long chain fatty acids in clarified must, in which yeasts activate fatty acid synthesis from pyruvic acid via acetyl-CoA. Under anaerobic conditions acetyl-CoA accumulates and is hydrolysed to yield large amounts of acetic acid [27]. It has been reported [7] that insoluble materials in grape musts may also adsorb fatty acids and stimulate yeast growth through more efficient elimination of carbon dioxide [34].

Conclusion

The study of the factors responsible for the occurrence of stuck and sluggish fermentation is receiving increased interest because of the economic impact of fermentation problems. As reported in this review, many factors such as vitamin, magnesium, nitrogen and oxygen deficiencies or the

presence of ethanol, toxic fatty acids, acetic acid or sulphites may be involved. The effects related to these factors are numerous and include decrease in pH, inhibition of key enzyme activities, and alteration of the plasma membrane. These may induce decreases in the metabolism of the yeast cell and consequently decreases in biomass production, cell viability and fermentation rate. Furthermore, occurrence of stuck or sluggish fermentation could be the result of interactions of these factors. This explains why many studies are conducted in synthetic media or media which try to mimic the must. However, extrapolation from such studies should be done with caution, since it has been previously shown in the case of sugar transport activity that rates of uptake were significantly different when determined in rich medium or in must [79].

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