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Sodium Meta Bisulfite and pH Tolerance of *Pleurotus sajor caju* Under Submerged Cultivation

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ABSTRACT

To develop a procedure for submerged cultivation of the edible fungus *Pleurotus sajor caju*, we investigated the organism's tolerance to sodium meta bisulfite and acidic pH levels. These factors were evaluated as means of controlling bacterial and/or fungal contamination. Trials were conducted in 500 mL Erlenmeyer flasks that contained 200–300 mL of a 3% glucose, 0.5% yeast extract medium, and either 0-0.225% SMB (wt/wt) added, or initially adjusted to pH levels between 1.8–6.5. Inoculated flasks were incubated 7–10 d at 30°C and 200 rpm in an environmental shaker, with samples removed daily to determine mycelial dry weights. Results showed that SMB levels up to 0.05% significantly lengthened the lag phase (from 27 to 79 h) but had no effect on productivity. Maximal productivity varied between 0.054–0.057 g/L/h, whereas overall productivity was 0.042–0.045 g/L/h. Biomass concentrations ranged from 7.1–8.4 g/L. Higher SMB levels rapidly reduced productivities and yields, eventually inactivating the inoculum above 0.1% SMB. In one instance the SMB tolerance of *P. sajor caju* was increased to 0.075% by repeatedly exposing the organism to sublethal levels of the chemical; however, this trait was not maintained in later trials. Bacterial contaminants were detected at SMB concentrations of 0.02–0.07%, while fungal contaminants were found up to 0.125% SMB. Thus it appears that SMB might be useful in controlling bacterial contamination, but may not be as effective

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against fungal contaminants. The optimum pH range in terms of lag phase length, biomass yield, and productivity was 4.5–5.5, however, in certain trials *P. sajor caju* still exhibited good growth parameters at pH levels as low as 3.8–4.0. pH levels below 3.8 and above 5.8 greatly reduced both growth rates and yields. Acidic pH levels (3.8–4.5) were also effective in controlling the majority of bacterial and fungal contaminants encountered. Therefore low pH or perhaps a combination of low pH and SMB should be useful in developing a large scale system for submerged cultivation of *P. sajor caju*.

Index Entries: *Pleurotus sajor caju;* sodium meta bisulfite; pH; tolerance; submerged cultivation.

INTRODUCTION

Initial development of techniques for submerged cultivation of fungal mycelium took place in the 1940s and 1950s (*1–3*). Since then researchers have used these techniques for production of mycelium for the following purposes:

1. As a source of protein and vitamins (*4–9*);
2. As flavoring agents (*2,5,7,10*);
3. As inoculum or spawn for mushroom fruitbody production (*5,8,11,12*);
4. As a support or carrier for immobilized cells or enzymes (*6*);
5. For treatment of organic wastes (*8,9,13,14*); and
6. For physiological studies of fungal metabolism (*8,9*).

Many excellent reviews detail the development of these processes and summarize the various aspects of submerged culture of fungal mycelium (*2,8,9,11,15,16*).

Submerged culture techniques have been successfully used for fungal production of citric acid (*15*) and various antibiotics (*17*), but large scale production of the mycelium itself, for the purposes noted above, has yet to be widely developed (*2*). Reasons for this range from unfavorable economics of certain processes, to lack of flavor or consumer acceptability of mushroom mycelium/pellets used in foods (*2*). Perhaps most surprising, however, is that liquid spawn has not found wider use as a replacement for grain spawn (*2*) in mushroom production. This, in spite of lower labor requirements (*12*), faster production rates, and reduced costs (*11*) for liquid spawn production.

The resistance to switch from grain spawn to liquid spawn has been largely a matter of tradition, however, other contributing factors have also been noted. The longer shelf life of grain spawn and the minor nutritional contribution the grain substrare provides growing mushrooms are two commonly cited advantages (*2*). Meanwhile, other investigators have

observed physiological differences between the use of grain and liquid spawn. For example, Humfeld and Sugihara (*18*) and Szuecs (*19*) noted that strains of *Agaricus campestris* grown in liquid culture were unable to establish themselves on compost. Kurtzman (*12*) postulated that this lack of growth may have been owing to the physiological shock of abruptly changing the concentration and source of nutrients when liquid culture mycelium was used to inoculate a solid substrate. An additional explanation was that nutrients in the liquid medium promoted contamination by other microbes present in the nonsterile compost. On the other hand, in current studies with the oyster mushroom *Pleurotus sajor caju,* we have successfully used liquid spawn to inoculate pasteurized wheat straw (*20*). In addition to faster inoculum production rates with liquid spawn, we have found that liquid spawn colonized the substrate as rapidly as grain spawn.

The major problem we encountered with use of liquid spawn was contamination during the submerged cultivation process. Others have previously noted the importance of maintaining the desired fungi in pure culture throughout the production cycle (*12,21*). The great potential for bacterial and/or fungal contamination of submerged cultures is primarily a result of the relatively slow growth rates of many cultivated fungi, in relation to bacteria and certain rapidly growing opportunistic fungi. Most studies address this problem by simply noting that equipment, media, and oxygen supply must be thoroughly sterilized prior to use (*21*). Relatively large inoculum levels of up to 20% (v/v) have also been utilized to give the desired organism an initial numeric advantage over potential contaminants (*10,21*). This technique, however, can reduce mycelial yields if high concentrations of inhibitory metabolites are transported into the fermentor with the inoculum (*22*).

Strict aseptic techniques are often sufficient to control contaminants in lab- and pilot-scale fungal fermentations, but they often become more costly and less practical or effective as processes are scaled-up to industrial size (*21*). As a result, various fungal antibiotics have been developed to control bacterial contaminants during submerged fermentation. For example, penicillin and streptomycin are often used in ethanol fuel production plants to control acetic and lactic acid bacterial contamination of yeast fermentations. The same antibiotics have also been used to suppress contaminating bacteria during submerged culture of mushroom mycelium (*2*). Use of such antibiotics is, however, becoming more restricted as studies link long-term use of antibiotics to the development of resistance in pathogenic bateria. In addition, processes that produce materials that will directly enter the food chain are closely regulated as to utilization of antibiotics (*23*). Consequently, one of the most serious limitations to industrial scale production of fungal mycelium remains that of contamination control.

To develop a procedure which would permit contaminant-free submerged cultivation of *P. sajor caju,* we evaluated the organism's tolerance

to sodium meta bisulfite and acidic pH levels. Previous studies with the yeast *Saccharomyces cerevisiae* have determined that either sodium meta bisulfite (SMB) (*24*), potassium meta bisulfite (PMB) (*25,26,27*), or acidic pH levels (*28,29*) could be used effectively to control bacterial contamination. We therefore theorized that either SMB or low pH might be effective in controlling contamination during submerged cultivation of *P. sajor caju*, provided the organism was sufficiently tolerant. In the trials reported herein, we evaluated the effects of SMB and reduced pH on the growth of *P. sajor caju*.

METHODS

A culture of *Pleurotus sajor caju* (WC 537) was obtained from the Pennsylvania State University mushroom culture collection for use in this study. For long-term storage (3–6 mo) the organism was grown on potato dextrose agar slants, covered with sterile mineral oil, and refrigerated at 3–5°C. Working cultures were grown and stored under similar conditions, except that slants were not covered with mineral oil and cultures were transferred at 4–6 wk intervals.

Inoculum used in the submerged fermentation trial was prepared by aseptically transferring 1–2 mm square segments of colonized agar (from working culture slants) into 250 mL Erlenmeyer flasks containing 100 mL of a 3% glucose, 0.5% yeast extract broth (*6,30*). Flasks were incubated for 5–8 d at room temperature on a New Brunswick table top shaker set at 150 rpm. Final biomass concentrations in the inoculum generally ranged from 5–6 g/L (dry basis). Inoculum was refrigerated for up to 2 wk prior to use. Dijkstra et al. (*11*) previously observed that inoculum age (up to 8 wk old) had no effect on mycelial growth curves.

Submerged fermentation trials, evaluating the effects of sodium meta bisulfite (SMB) and pH on the growth of *P. sajor caju*, were conducted in 500 mL Erlenmeyer flasks which contained 200 mL of the 3% glucose, 0.5% yeast extract medium. To achieve the desired SMB concentration, sterile SMB crystals were dissolved in the medium prior to inoculation. In other trials, the pH of the medium was adjusted using sterile 6*N* H_2SO_4. Flasks were then aseptically inoculated with 2 mL of the previously grown inoculum broth using wide mouthed 10 mL pipets. Inoculum pellets were used whole (not macerated) to more closely simulate conditions that would likely exist in an industrial scale production system. Flasks were incubated 7–10 d in a New Brunswick environmental shaker set at 30°C and 200 rpm (*13,31*).

Cultures were examined on a daily basis for pellet size and the presence or absence of bacterial/fungal contaminants. The average pellet size range for each flask was estimated to the nearest mm by visual observation (*11*).

Bacterial contamination was readily apparent when fermentation broths showed dramatically increased turbidity over a short period of time. Fungal contamination was suspected when mycelial growth showed atypical morphologies and/or growth rates. In either case, suspect cultures were Gram stained (*32*) and plated onto nutrient agar and potato dextrose agar plates, which were incubated 24–48 h at 30°C. The resulting cellular and colonial morphologies allowed us to both confirm the presence of contaminants and to postulate as to their probable identities.

The final parameters evaluated during submerged cultivation of *P. sajor caju* were pH and mycelial dry weight. Representative samples of the broth from each flask (10 mL) were aseptically collected on a daily basis using wide mouthed pipets. Following pH analyis, the samples were processed to determine mycelial dry weight. The procedure used was similar to that previously described by other investigators (*6,10,14,31*). Mycelium was separated from culture fluid by filtering through oven dried, preweighed Whatman No. 1 filter paper. Pellets were then washed with 50 mL water. After drying at 80°C to a constant weight and cooling in a desiccator, the filter paper was again weighed to determine mycelial dry weight.

RESULTS AND DISCUSSION

Numerous investigators have noted the difficulty of obtaining representative data on fungal growth rates (particularly those that form discrete pellets) owing to the problem of taking uniform samples from nonhomogeneous culture broths (*2,11*). To overcome this problem, some researchers have utilized series of identically treated flasks that were completely harvested at different incubation times (*11*). Although useful for trials in which relatively large pellets (5–10 mm dia.) are obtained, this technique is highly dependent upon the assumption that all flasks are treated equally. An alternative technique, usable when pellets are smaller than 5 mm dia., is to repeatedly sample culture broths at regular time interals (*6,11*). This method reduces the impact of variability between flasks, but it is dependent on sample uniformity. We chose the latter method for collection of growth rate data, since our combination of inoculum level (1%, v/v), culture volume (200 mL), agitation rate (200 rpm), and strain (*P. sajor caju* WC 537) produced pellets generally in the range of 2–5 mm (*15,33,34*). This, coupled with the use of wide-mouth pipets (4 mm dia. openings), allowed us to collect representative samples from culture vessels throughout the incubation period.

To determine the effects of sodium meta bisulfite (SMB) on the growth and productivity of *P. sajor caju*, ten SMB levels, ranging from 0–0.225% (wt/wt), were first evaluated. The initial pH in these flasks ranged from

6.0–6.3 at the lower SMB concentrations to pH 5.5–5.7 at the higher concentrations. At the end of the incubation period (7–10 d) pH values ranged from 5.5–6.0 to 4.5–5.0 in the low and high SMB flasks, respectively. Since these pH levels were well within the range commonly cited in the literature as optimum for submerged cultivation of *Pleurotus* spp. (*5,7,9, 10,35*), we believe they had no significant effect on the growth rate data discussed below.

Figure 1a presents the results of these trials, and the data represents the average of five such trials. As the SMB concentration was increased *P. sajor caju* took progressively longer to adapt to increased SO_2 levels, hence, the lag phase rose from 27 h at 0% SMB to 96 h at 0.1% SMB. We believe the relatively long lag phases observed were at least partially owing to the use of whole inoculum pellets instead of dispersed mycelium. Nevertheless, the trend towards longer lag phases does indicate an inhibitory effect of SMB on *P. sajor caju*. Higher SMB levels (>0.1%) completely inhibited growth and eventually killed the inoculum.

The results also indicate that SMB levels up to 0.05% had little effect on either biomass yield, overall productivity, or maximum rate productivity (productivity during the exponential phase of growth). However, as SMB concentrations were further increased to 0.1%, biomass yield, and overall and maximum rate productivity each showed dramatic reductions. Above 0.1% SMB the organism failed to grow, thus giving productivities and biomass yields of zero (data not shown).

The yields and productivities we obtained at SMB levels up to 0.05% are generally similar to those previously reported for *P. ostreatus*. Yields ranging 5–7.5 g/L and productivities ranging 0.02–0.05 g/L/h have been obtained on substrates such as malt extract (*36*), peat extract (*10*), beer wort (*35*) and sugar beet molasses (*5*). Yields and productivities were lower on ethanol (*7*) and fermentation sludge liquor (*14*) media and higher on glucose/minerals (*5*), and trub press liquor (*14*) media. Litchfield (*2*) noted that biomass yields vary widely among fungi and among the same organism grown on different substrates.

In previous studies with the yeast *Saccharomyces cerevisiae* we observed similar trends for the effects of SMB (*24*) and potassium meta bisulfite (PMB) (*25*) on growth and fermentation rates. However, in these trials the yeast was able to withstand SMB and PMB levels of up to 0.30 and 0.35%, respectively, with no apparent deleterious effect. Higher levels of either chemical proved increasingly inhibitory, and eventually toxic. Wick (*27*) used SO_2 levels of 0.0125% to control contamination in ethanol fermentations, with no adverse effects on either yeast growth or metabolism. In comparison to these studies, our results indicate *P. sajor caju* is only moderately tolerant to SMB.

To further explore the effects of SMB on *P. sajor caju* and more accurately pinpoint the organism's level of tolerance to SMB, we conducted a second series of fermentation trials in which SMB levels of 0–0.08% (in

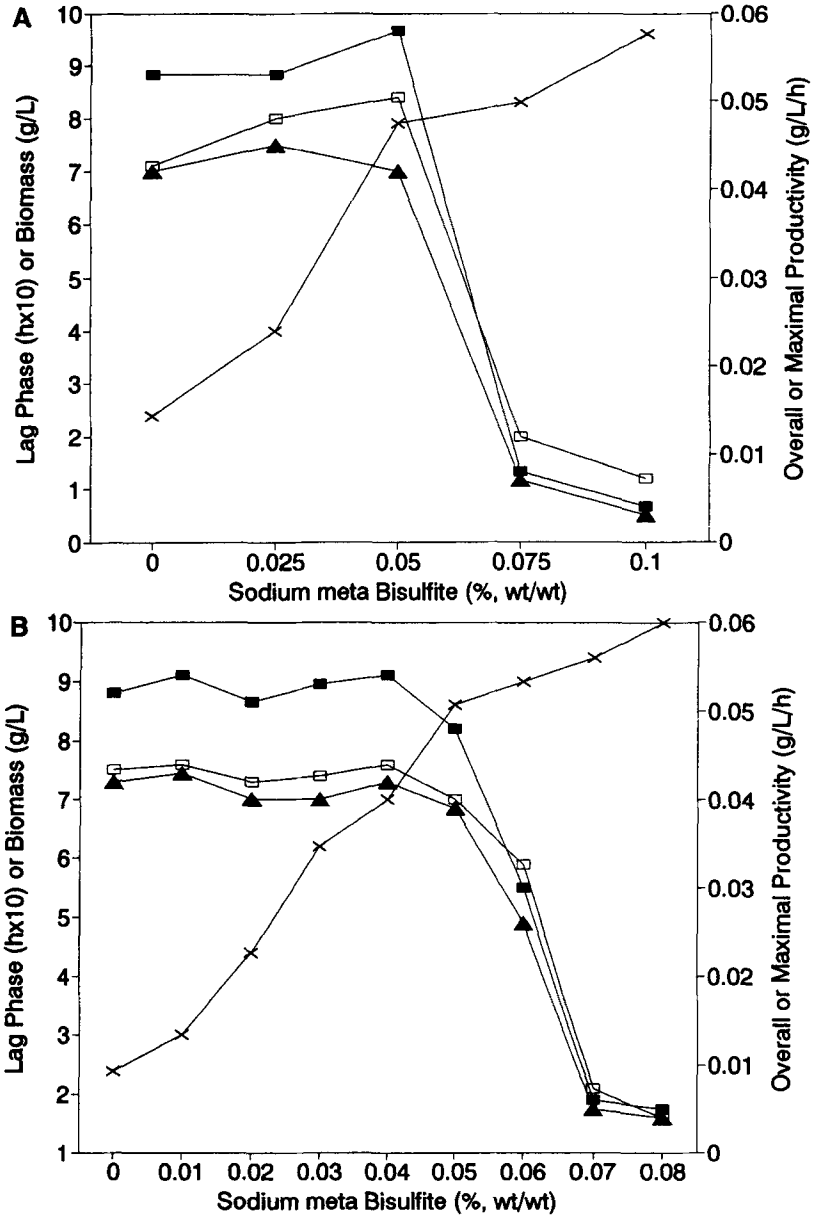

Fig. 1a,b. Effect of SMB on the length of lag phase, biomass concentration, and productivity of *P. sajor caju*. Symbols are: (X) length of lag phase; (▲) biomass concentration; (■) maximal productivity, and (□) overall productivity.

the average of six such trials. Although presenting an expanded description of the effects of SMB on *P. sajor caju,* this data also confirmed the findings of Fig. 1a. Especially important, however, are the results shown for the range of 0.05–0.07% SMB. This appears to be the transitory range over which SMB causes significant changes in the growth and productivity of *P. sajor caju*. Over this range the biomass yield and both overall and

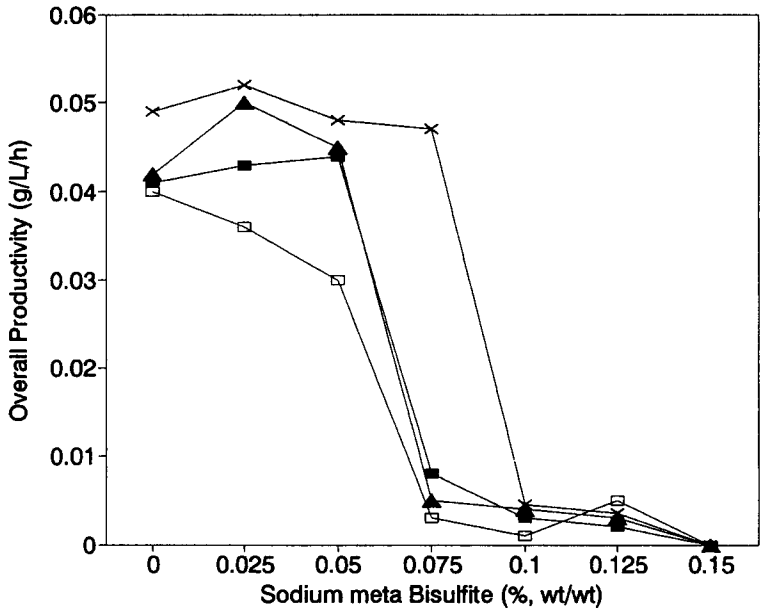

Fig. 2. Acclimation of *P. sajor caju* to increasing levels of SMB. Symbols are: (■) first trial, (▲) second trial, (X) third trial, (□) fourth trial.

maximum rate productivity fell by 87–88%. From this data one can conclude the maximum tolerance of *P. sajor caju* to SMB is approx 0.05%.

In the final series of SMB trials we sought to determine if the tolerance of *P. sajor caju* to SMB could be increased by repeated exposure to sublethal levels of the chemical. In these trials 10 SMB levels, ranging 0–0.225%, were used. Freshly grown inoculum was used for the first trial. Inoculum for subsequent trials was the most SMB tolerant culture from the previous trial, used immediately after that trial had been ended. In most cases this was the 0.05% SMB culture. Results (Fig. 2) show that overall productivity was relatively similar for the first two trials of this series. This data closely agrees with the information previously presented in Figs. 1a and b. In the third trial of this series, however, the organism showed a marked increase in tolerance to SMB, with productivity maintained at 0.046 g/L/h out to 0.075% SMB. This represented a 657% increase in productivity over previous trials at that SMB level. At SMB levels of 0.1% and higher, however, productivity fell back to 0.005 g/L/h or less. Mycelium from the 0.075% SMB flask was grown on potato dextrose agar slants, labeled as SMB-tolerant, and refrigerated.

Because of unavoidable circumstances it was not possible to immediately transfer the 0.075% SMB culture to yet another set of flasks to see if SMB tolerance could be increased even more. However, after approximately three weeks the refrigerated SMB-tolerant strain was grown up in glucose/yeast extract broth and used to inoculate a fresh set of SMB flasks. Results from this trial, also shown in Fig. 2, were very disappoint-

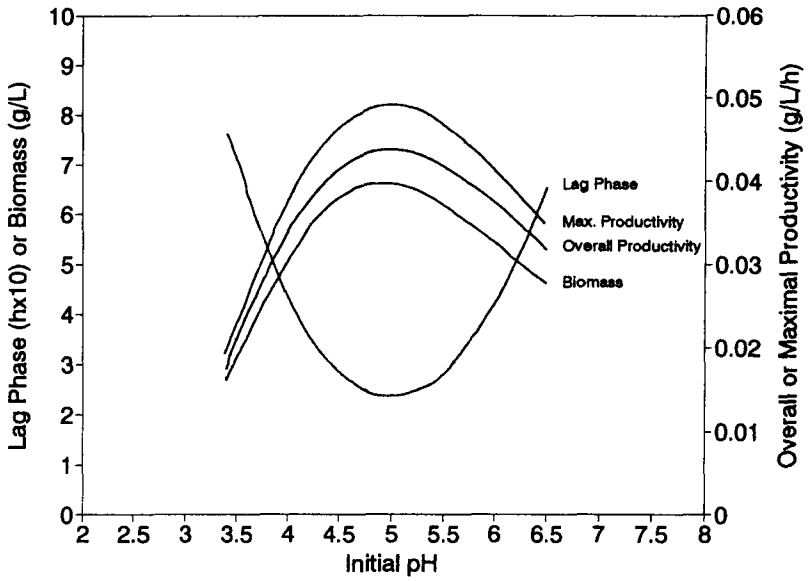

Fig. 3. Effect of pH on the length of lag phase, biomass concentration, and productivity of *P. sajor caju*.

ing; productivities of the SMB adapted strain were significantly lower than those of the cultures used in the three previous trials. For example, in comparison to the previous (third) trial, reductions in productivities ranged from 33–35% at 0.025% SMB to 96% at 0.075% SMB. Further trials with this SMB adapted strain gave productivities in the same reduced range. Moreover, attempts to repeat the SMB adaptation of *P. sajor caju* using the initial strain were also unsuccessful.

In spite of these findings, which seem to indicate that increased SMB tolerance is a transient property, our results, nevertheless, do show that the organism possesses the potential to withstand relatively high SMB levels. It may be possible through the combined use of adaptation/selection and mutagenic treatment to more permanently incorporate SMB tolerance into the organisms physiology. This potential will be explored in future trials.

The second objective of this study was to determine the tolerance of *P. sajor caju* to acidic pHs. Thirteen separate fermentation trials were conducted, each consisting of ten flasks initially adjusted to pH levels ranging from 1.8 to 6.5. In low-pH flasks that exhibited little or no mycelial growth, pH remained relatively constant during incubation, while in flasks with good mycelial growth, pH gradually rose between 0.5–2 U. Figure 3 provides the results of these trials, with length of lag phase, mycelial yield, and productivity data regressed over the continuous variable, pH. As is shown, the effect of pH on each of these four variables was quite consistent with an optimal pH range of 4.5–5.5. The minimal lag

Table 1
Coefficients of Polynomial Equations Describing Effects of pH on *P. sajor caju* Growth Parameters[a]

Growth parameter	Regression coefficients			Correlation coefficient
	a	b	c	
Lag phase	18.66	−187.77	496.19	0.87
Biomass yield	−1.15	11.92	−24.28	0.73
Overall productivity	−0.007	0.078	−0.160	0.72
Maximum productivity	−0.008	0.088	−0.180	0.71

[a]Polynomial equation used $y = ax^2 + bx + c$.

phase time and maximal biomass yield and productivities obtained at these pH levels were similar to those we previously observed while determining SMB tolerance. A number of other studies have previously reported that pH in the range of 5–6.5 was optimal for various *P. ostreatus* strains, with no growth observed below pH 3.0 (*5,7,9,10*). Our results are in general agreement with this, however it appears that *P. sajor caju* may be tolerant of slightly lower pH levels.

Table 1 lists the coefficients of the polynomial equations which describe each of the regression lines in Figure 3, along with the correlation coefficients, which ranged from 0.71–0.87. Evaluation of the raw data used to construct these lines indicates that in certain trials, high biomass yields and productivities were maintained down to pH levels of 3.8–4.0. Therefore, it is likely that *P. sajor caju* could be readily adapted to grow consistently at such pH levels.

Although the primary purpose of this experiment was to determine the SMB and pH tolerance of *P. sajor caju*, a secondary objective was to establish the general range of SMB and pH tolerance that might be expected of naturally occurring contaminants. Such information would be critically important to predict the effectiveness of utilizing either SMB or low pH in a fermentation system. Throughout this study, various bacterial and fungal contaminants developed in approx 9–11% of the experimental flasks. We feel this high level of contamination, which seemed to occur in a random manner, was caused in large part by the fact that flasks were briefly exposed to possible contamination during the daily samplings, in spite of the use of a laminar flow hood. Other contributing factors, previously noted, were likely the long lag phase and slow initial growth rates of *P. sajor caju*. This problem with contamination, while a nuisance in lab scale experiments, would represent the potential for significant losses in pilot- and industrial-scale production processes (*21*). Moreover, it is likely that such processes would be even more subject to contamination due to the difficulties of maintaining sterility and aseptic conditions in a production-sized facility.

Table 2
Characteristics of Bacterial and Fungal Contaminants that Developed in *P. sajor caju* Cultures

Characteristics of contaminants	SMB range[a] (%, wt/wt)	pH Range[b]
Bacteria		
Gm+cocci	0–0.02	5.25–6.39
Gm+rod	0–0.05	4.77–6.15
Gm−rod	0–0.075	—
Fungi		
Yeast	0–0.075	4.5 -6.15
Trichoderma spp.	0–0.125	—
Fusarium spp.	0–0.05	—
Rhizopus spp.	0–0.02	—

[a]Sodium meta bisulfite range over which the contaminant was detected.
[b]pH range over which the contaminant was detected.

Flasks that became contaminated during the study were removed and the contaminating organisms evaluated microscopically and after growth on potato dextrose agar. Table 2 lists the Gram stain reactions of bacterial contaminants and the genera of fungal contaminants (as determined by colonial and cellular morphology), along with the SMB and pH ranges over which the organisms occurred. Bacterial contaminants isolated in this study had SMB tolerance ranging from 0.02–0.075%. This low tolerance of certain bacteria to SMB is consistent with the findings of Wick (*27*), who used 0.0125% SMB to control ethanol fermentation contaminants. On the other hand, Gibbons and Westby (*24,25*) have previously encountered bacterial contaminants able to tolerate up to 0.15% SMB and 0.20% PMB. However, these bacteria were associated with a root crop—fodder beets— and otherwise would likely not be a problem in fermentation processes. Therefore, SMB shows good potential for use in controlling bacterial contamination in *P. sajor caju* submerged cultivation. Fungal contaminants isolated in this study proved to be somewhat more tolerant of SMB than the bacteria. For example, a wild yeast and a *Trichoderma* isolate were tolerant of 0.075 and 0.125% SMB, respectively (Table 2). With respect to the lower tolerance of *P. sajor caju* to SMB (0.05%), these ubiquitous fungi present the more serious limitation for use of SMB to control contamination in large scale production.

Bacterial and fungal contaminants were isolated from flasks with pHs ranging from 4.5–6.39. While more acidophilic or acidotolerant bacteria and fungi exist, the organisms isolated in this study are likely typical of airborne contaminants which might be encountered in a fermantation plant. *P. sajor caju* was relatively tolerant of pH levels down to 3.8–4.0

with minimal loss of mycelial yield or productivity, therefore, use of acidic pH appears to be a promising option for contaminant control.

SUMMARY

This study evaluated the SMB and pH tolerance of *P. sajor caju* grown on a glucose-yeast extract medium under submerged cultivation. *P. sajor caju* was tolerant of SMB concentrations of up to 0.05% and pH levels down to 3.8–4.0. Biomass yields of 7–8 g/L and overall productivities of 0.040–0.045 g/L/h were observed under these conditions. The SMB level of 0.05% was largely effective against the bacterial contaminants we encountered, but it was ineffective against fungal contaminants. On the other hand, *P. sajor caju's* tolerance of acidic pH levels was useful in controlling both bacterial and fungal contamination. It may be possible, through the combined use of SMB and low pH, to create conditions even more effective for controlling bacterial/fungal contamination.

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