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Combined Effects of Sulfites, Temperature, and Agitation Time on Production of Glycerol in Grape Juice by *Saccharomyces cerevisiae*†

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Analysis of variance was used to evaluate the simultaneous effects of strain, incubation temperature (15 to 25°C), agitation time (0 to 24 h), and initial sulfite concentration (100 to 300 ppm) on glycerol production in grape juice by *Saccharomyces cerevisiae*. Fourteen strains were studied to determine their growth patterns in the presence of sulfites and ethanol. Baker's yeast strains were more sensitive to sulfite than wine strains, and little growth occurred at initial sulfite levels greater than 150 ppm. Sensitivity to sulfite increased with increasing levels of ethanol. Three strains exhibiting the best growth in the presence of sulfites and ethanol were selected for interaction studies. Fermentations were carried out until the solids content had decreased to less than 6°Brix, which was the point that glycerol content became stable. For the three strains used, the greatest level of glycerol production was observed in the presence of 300 ppm of sulfite for most incubation temperatures and agitation times. There was significant interaction between the strain, incubation temperature, and agitation time parameters for glycerol synthesis, and a response surface method was used to predict the optimal conditions for glycerol production. Under static conditions, the highest level of glycerol production was observed at 20°C, while incubation at 25°C gave the best results when the cultures were agitated for 24 h. Response surface equations were used to predict that the optimum conditions for glycerol production by *S. cerevisiae* Y11 were a temperature of 22°C, an initial sulfite concentration of 300 ppm, and no agitation, which yielded 0.68 g of glycerol per 100 ml.

Glycerol is the wine constituent that is derived from yeast fermentation that is usually present in greatest abundance after ethanol and carbon dioxide. It is nonvolatile and does not contribute to wine aroma (5), but does contribute to the smoothness and viscosity of a wine (11). Glycerol concentrations vary between 1 and 10 g/liter (7, 9, 10, 12, 16).

Traditional microbiological studies on the effects of different parameters on the synthesis of metabolites are performed by varying one parameter at a time. With such a procedure, many factors have been found to influence glycerol production by *Saccharomyces cerevisiae*, including strain, inoculation level, aeration, temperature, sugar content, and sulfite content. In a study of 23 different yeast strains, considerable variation was observed within the species *S. cerevisiae*; extreme values of 4.4 and 11.6 g of glycerol formed per liter were obtained (14). When inoculum size was studied, the highest level of glycerol formation was observed when high cell populations (up to 10^8 cells per ml of juice) were inoculated (14, 20). Aeration also influences glycerol production, and greater glycerol yields are obtained with fermentation flasks that are agitated (14). For one yeast strain, the level of glycerol production increased from 6 to 13 g/liter when the culture was agitated. An increase in the temperature of fermentation increases the glycerol yield (12, 16). With regard to substrate concentration, glycerol synthesis was greater when the grapes were more mature (i.e., when the sugar content was higher) (12). It can be hypothesized that sugar concentration influences osmotic pressure, and many authors have observed that glycerol is produced in response to solute stress in *S. cerevisiae* (1, 4, 8, 17). Addition of even a small amount of SO_2 (100 ppm) can produce a significant increase in glycerol formation (16).

Although a considerable amount of information is available concerning individual factors influencing glycerol production, little information is available concerning the possible interactions between the parameters. The aim of this study was to investigate the simultaneous effects of strain, agitation time, initial sulfite concentration, and incubation temperature on glycerol synthesis by *S. cerevisiae* in grape juice fermentations by using analysis of variance and response surface methods.

MATERIALS AND METHODS

Microorganisms. Cultures of wine and baker's yeasts were obtained from the Institut Technologie Alimentaire of Saint Hyacinthe (strains Y27 and Y28) or from the Lallemand company collection (Lallemand, Inc., Montréal, Québec, Canada). All 13 strains used (Table 1) were confirmed as *S. cerevisiae* strains by using API strips (API, Plainview, N.Y.). The reference strains were maintained at 4°C on YM slants covered with sterile mineral oil and were transferred to fresh medium once a year. Mother cultures were kept in YM broth and transferred twice a week by using 1% inocula. Inocula for grape juice fermentations were produced in YM broth on a Labline gyratory shaker operated at 100 rpm and 25°C for 36 h. Flasks were incubated at 4°C overnight, after which the supernatant was decanted and the cells were resuspended in grape juice. The decantation was carried out to prevent any carryover of ingredients of YM broth into the

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TABLE 1. Strains of *S. cerevisiae* used in this study

Strain[a]	Type
Y3	Baker's accelerated fermentation yeast for bakeries
Y4	Baker's yeast for active dry yeast products
Y5	Wine yeast with killer factor
Y6	Wine yeast, can be used in champenoise process
Y7	Wine yeast, champenoise process, has killer factor
Y8	Beer yeast
Y9	Wine yeast, flocculant
Y10	Wine yeast sensitive to killer factor
Y11	Wine yeast
Y12	Wine yeast, champenoise process
Y13	Wine yeast
Y14	Wine yeast, champenoise process
Y15	Wine yeast sensitive to killer factor
Y27	Wine yeast, for Bourgogne type
Y28	Wine yeast, for Bourgogne type

[a] Most strains were obtained from Lallemand, Inc.; the exceptions were strains Y27 and Y28, which were obtained from the Institut Technologie Alimentaire of Saint Hyacinthe.

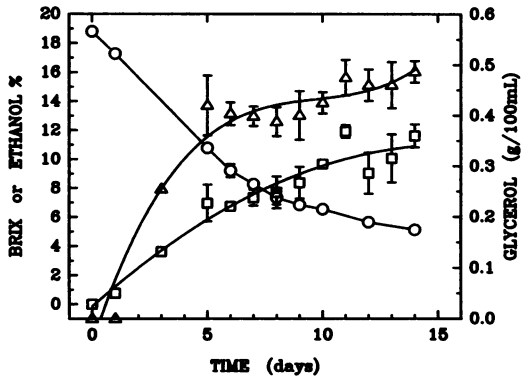


FIG. 1. Soluble solids content (Brix) (○), ethanol concentration (in grams per 100 ml), and glycerol concentration (△) during grape juice fermentation by *S. cerevisiae* Y7 at 20°C in the presence of 150 ppm of sulfites with no agitation.

grape juice. The viable cell population was determined by direct microscopic counting by using methylene blue (0.1%) as a vital stain. The inoculation procedure was standardized to provide an inoculation level of 2×10^6 to 6×10^6 CFU/ml of juice.

Yeast selection. Yeast strains were evaluated for their ability to grow and produce glycerol in grape juice supplemented with up to 300 ppm of sulfites and 15% ethanol. Growth at temperatures ranging from 15 to 25°C and under three agitation protocols (0, 12, or 24 h of agitation at 100 rpm) was determined by measuring optical density at 560 nm with a model DU-7 spectrophotometer (Beckman, Irvine, Calif.).

Media and reagents. Concentrated Beaujolais grape juice (Niagara Vine Products, Ltd., Sainte Catharines, Ontario, Canada) was diluted to a soluble solids content of 20°Brix (degree Brix is a measure of the density of a sugar solution, 1°Brix is equivalent to 1% [by weight] sucrose in a solution, and this parameter is related empirically to density) and was heated at 68°C for 15 min. After cooling to 20 to 25°C, sulfites in the form of $K_2O_5S_2$ were added to the juice, and the pH was adjusted, if necessary, to 3.70 with 3 N tartaric acid (Fisher, Montréal, Québec, Canada).

In yeast selection studies, 95% ethanol (Anachemia, Montréal, Québec, Canada) was added to the juice in order to obtain an ethanol concentration of 15% (vol/vol).

Fermentations. Aliquots (115 ml) of grape juice were distributed into 125-ml flasks and inoculated with 2×10^6 to 6×10^6 CFU of the appropriate yeast per ml. The flasks were capped and put in the proper incubators. When aeration was called for, the flasks were shaken at 150 rpm for the required time period (12 or 24 h). The production of glycerol by selected yeasts was studied by varying three parameters, temperature (15, 20, and 25°C), sulfite concentration (100, 150, and 300 ppm), and length of aeration (0, 12, and 24 h), according to a split-split-plot design. The whole design matrix was replicated twice. The musts were sampled for glycerol production when the soluble solids content had decreased to between 5 and 6°Brix. Preliminary studies showed that the concentration of glycerol produced in the final wine was stable for up to 15 days for the three strains used once this solids level had been reached (Fig. 1). Some samples were frozen at −20°C after centrifugation, since it

TABLE 2. Effects of sulfite and ethanol concentrations on the growth of *S. cerevisiae* strains in grape juice, as estimated by optical density at 560 nm following 72 h of incubation at 30°C

Strain	Optical density at 560 nm under the following conditions:											
	No sulfites				150 ppm of sulfites				300 ppm of sulfites			
	No ethanol	8% Ethanol	12% Ethanol	15% Ethanol	No ethanol	8% Ethanol	12% Ethanol	15% Ethanol	No ethanol	8% Ethanol	12% Ethanol	15% Ethanol
Y3	1.12	0.49	0.43	0.06	0.08	0.05	0.03	0.03	0.04	0.03	0.05	0.06
Y4	1.10	0.87	0.18	0.05	0.06	0.05	0.01	0.01	0.04	0.06	0.03	0.04
Y5	1.14	0.94	0.25	0.55	0.61	0.24	0.01	0.03	0.61	0.23	0.07	0.05
Y6	1.02	0.58	0.28	0.10	0.24	0.04	0.01	0.01	0.61	0.23	0.07	0.05
Y7	1.05	0.79	0.51	0.20	0.45	0.01	0.01	0.02	0.36	0.06	0.05	0.04
Y8	1.08	0.84	0.58	0.02	0.04	0.04	0.04	0.02	0.03	0.04	0.06	0.04
Y10	0.98	0.85	0.31	0.21	0.06	0.02	0.04	0.04	0.04	0.05	0.06	0.06
Y11	1.03	0.89	0.52	0.10	0.50	0.42	0.09	0.03	0.51	0.09	0.04	0.03
Y12	0.73	0.29	0.02	0.06	0.05	0.04	0.04	0.03	0.03	0.03	0.03	0.07
Y13	1.12	0.79	0.71	0.14	0.54	0.12	0.03	0.04	0.47	0.04	0.04	0.05
Y14	0.96	0.69	0.54	0.10	0.42	0.20	0.04	0.02	0.35	0.04	0.03	0.03
Y15	1.03	0.85	0.31	0.07	0.06	0.05	0.04	0.05	0.04	0.05	0.05	0.06
Y27	0.96	0.95	0.53	0.15	0.05	0.06	0.04	0.03	0.04	0.06	0.04	0.03
Y28	0.85	0.51	0.14	0.01	0.04	0.03	0.03	0.02	0.03	0.04	0.04	0.05

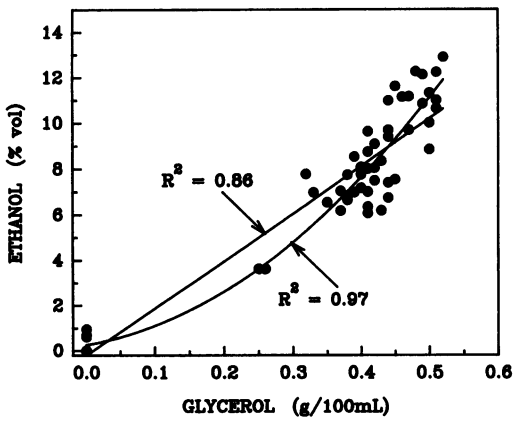


FIG. 2. Relationship between glycerol and ethanol concentrations in samples of grape juice fermented by *S. cerevisiae* Y7.

was found that glycerol analysis was not influenced by prior freezing and storage at −20°C for at least 21 days (data not shown).

Sample preparation. Fermented samples were immediately centrifuged at 3,000 × *g* for 20 min to remove the cells and were frozen at −20°C. It was not necessary to centrifuge the crude juice prior to freezing. On the day of the analysis, an internal standard (1% ethylene glycol) was added to a measured volume of each thawed sample, and the preparation was filtered through a 0.45-μm-pore-size membrane. The filtrate was further cleaned by passing it through a SepPak C_{18} cartridge (Waters Associates, Milford, Mass.) and was immediately cooled at 4°C in a high-performance liquid chromatography (HPLC) automatic sampler (Bio-Rad Laboratories, Richmond, Calif.).

HPLC analysis. The HPLC analysis was carried out by using the method of Schneider et al. (18). A 20-μl portion of a sample or standard solution was injected into a Bio-Rad liquid chromatograph equipped with a refractive index monitor. Sugars and acids were analyzed by using a 30-cm Aminex HPX-87H column (Bio-Rad). The column was operated at 65°C. The eluent used was 0.001 N sulfuric acid at a flow rate of 0.8 ml/min. The peaks were analyzed with a model 3392A integrator (Hewlett-Packard, Avondale, Pa.).

Statistical analyses. Analysis of variance was used to determine the simultaneous effects of strain, agitation time, initial sulfite concentration, and incubation temperature on glycerol production. The variances of glycerol production were analyzed by using a split-split-plot design with agitation time-temperature as the main unit. This design was used because of randomization restrictions; it was not possible to completely randomize the runs because of equipment limitations (agitation and incubation units). Hence, the main units consisted of fixed levels of agitation time and incubation temperature which are called AT block below. The type of strain constituted the subunits, and the initial sulfite concentration formed the subsubunits. Frequently, one factor (e.g., water bath temperature) cannot be changed as rapidly as other factors (e.g., solution concentration). In a split-split-plot design, the levels of one or more factors (whole-plot factors) are kept at fixed values for a set of experimental units while the levels of the remaining factors (subfactors) are randomized over the set of experimental units. Then, the levels of the subplot factor are kept at a fixed value for a set of experimental units while the levels of the remaining factors (subsubfactors) are randomized over the set of experimental units. Split-plot designs may be used when the levels of whole factors require larger amounts of experimental material for each experimental unit or when a more involved procedure is required to change whole-plot factor levels than to change subfactor levels.

TABLE 3. Analysis of variance to determine the effects of agitation time, incubation temperature, strain, and initial sulfite concentration on glycerol production by *S. cerevisiae*

Source of variation	DF[a]	Mean square	F	P[b]
Main units				
Repetition	1	0.183	2.30	0.1303
AT block[c]	8	0.056		
Error A	8	0.024		
Subunits				
Strain	1	0.005	1.77	0.1983
AT block × strain	16	0.007	2.56	0.0288
Error b	18	0.003		
Subsubunits				
Sulfite	2	0.011	4.38	0.0186
AT block × sulfite	16	0.002	0.83	0.6439
Strain × sulfite	4	0.001	0.31	0.8685
AT block × strain × sulfite	30	0.002	0.95	0.5535
Error	43	0.002		
Total	148			

[a] DF, degrees of freedom.
[b] *P*, probability of a significant effect. All statistical tests were performed at an α level of 0.05.
[c] AT block, agitation-temperature block (see Materials and Methods).

The assumptions underlying the linear model of analysis of variance were tested and verified. Some outliers had to be removed from the analysis. In these cases, special care was taken in the computation of F test results and the least-squares means and standard errors. Significant effects and/or interactions were partitioned into linear and quadratic components by using orthogonal polynomials in order to develop empirical models, using the response surface method. The response surface method is a collection of mathematical and statistical techniques that are useful for analyzing problems in which several independent variables influence a dependent variable or response, and the goal is to optimize this response. The adequacy of response surface models was tested for lack of fit. All statistical operations were carried out by using software obtained from SAS, Cary, N.C.

RESULTS AND DISCUSSION

Strain selection. Increasing the ethanol or sulfite concentration reduced the growth of all strains (Table 2). However, marked differences were observed between strains. As a whole, baker's yeast strains were more sensitive than wine yeast strains to both ethanol and sulfites. The inhibitory effects of ethanol and sulfites were additive. Thus, growth inhibition by ethanol was more marked as the sulfite concentration increased.

Since it is well known that sulfite concentration increases glycerol production (6), the following three strains that exhibited good growth in the presence of sulfites were selected for further study: strains Y7, Y11, and Y13. Selec-

TABLE 4. Partitioning of the agitation-temperature-strain interaction in linear and quadratic components

Contrast	DF[a]	Mean square	F	P[b]
Strain 1 main effects				
Agitation linear	1	0.005	1.95	0.1793
Agitation quadratic	1	0.006	2.38	0.1403
Temp linear	1	0.029	11.10	0.0037
Temp quadratic	1	0.008	3.23	0.0889
Strain 1 interactions				
Agitation linear × temp linear	1	0.007	2.81	0.1107
Agitation linear × temp quadratic	1	0.013	5.04	0.0376
Agitation quadratic × temp linear	1	0.036	14.08	0.0015
Agitation quadratic × temp linear	1	0.000	0.08	0.7793
Strain 2 main effects				
Agitation linear	1	0.013	4.95	0.0391
Agitation quadratic	1	0.006	2.38	0.1406
Temp linear	1	0.186	71.89	0.0001
Temp quadratic	1	0.036	13.77	0.0016
Strain 2 interactions				
Agitation linear × temp linear	1	0.006	2.42	0.1371
Agitation linear × temp quadratic	1	0.065	25.22	0.0001
Agitation quadratic × temp linear	1	0.005	1.79	0.1980
Agitation quadratic × temp quadratic	1	0.000	0.13	0.7274
Strain 3 main effects				
Agitation linear	1	0.000	0.01	0.9286
Agitation quadratic	1	0.003	0.10	0.7641
Temp linear	1	0.040	15.30	0.0010
Temp quadratic	1	0.014	5.50	0.0307
Strain 3 interactions				
Agitation linear × temp linear	1	0.007	2.78	0.1126
Agitation linear × temp quadratic	1	0.050	19.34	0.0003
Agitation quadratic × temp linear	1	0.000	0.17	0.6885
Agitation quadratic × temp quadratic	1	0.005	1.76	0.2015

[a] DF, degrees of freedom.
[b] *P*, probability of a significant effect at an α level of 0.05.

tion was based not only on growth in the presence of various ethanol and sulfite concentrations, but also on the ability of the strains to grow well at temperatures ranging from 15 to 25°C (data not shown).

Glycerol production in time. The glycerol and ethanol production curves were slightly different. Little production of glycerol occurred after 5 days of incubation, but ethanol production, although of quadratic nature, seemed more constant (Fig. 1). As Rankine and Bridson (16) have suggested, glycerol production occurred mostly at the early stage of fermentation, but it was not stoichiometrically coupled to ethanol production. Indeed, the quadratic relationship found between glycerol and ethanol contents was more significant than the linear relationship (Fig. 2), which would have been the most significant relationship if production of glycerol and production of ethanol were directly coupled. Similar results were obtained with strains Y11 and Y13 (data not shown). In a study of the effect of NaCl on glycerol production by *Pichia farinosa*, Vijaikishore and Karanth (19) found that lower glycerol yields were always accompanied by higher ethanol and biomass yields. Therefore, glycerol and ethanol, although both derived from glycolysis, seem to be subject to different control systems.

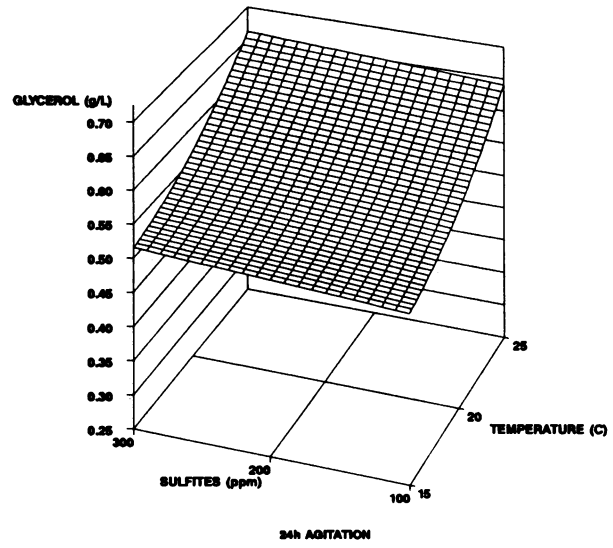
Main effects. The blocks themselves (agitation time and incubation temperature) did not have a significant effect on glycerol production ($P = 0.1303$) (Table 3). However, as discussed below, the AT block-strain interaction was significant ($P = 0.0288$) (Table 3). This means that agitation and temperature did have an effect which varied as a function of the strain used.

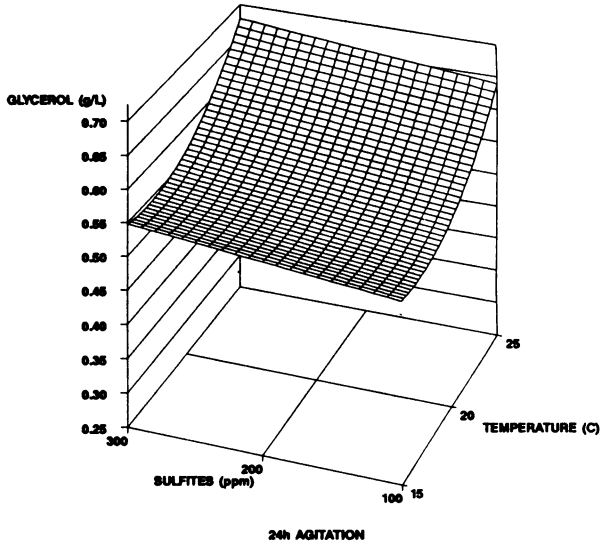
The initial sulfite concentration had a significant effect on glycerol production ($P = 0.0186$) (Table 3). Both the linear and quadratic effects of sulfite concentration on glycerol production were significant. Our data reveal that the increase in glycerol production was greater when the sulfite level increased from 200 to 300 ppm than when the sulfite level increased from 100 to 200 ppm. This is consistent with the results of various studies (3, 12) which showed that the initial sulfite concentration had little effect in the concentration range of from 0 to 200 ppm. The strain used had no significant effect on glycerol production ($P = 0.1983$) (Table 3). This finding apparently contradicts the results of previous studies that demonstrated marked strain effects (15, 16, 19). It thus appears that the selection process used in this study, which was based on growth in the presence of ethanol and sulfites, allowed us to obtain strains that had comparable glycerol production patterns.

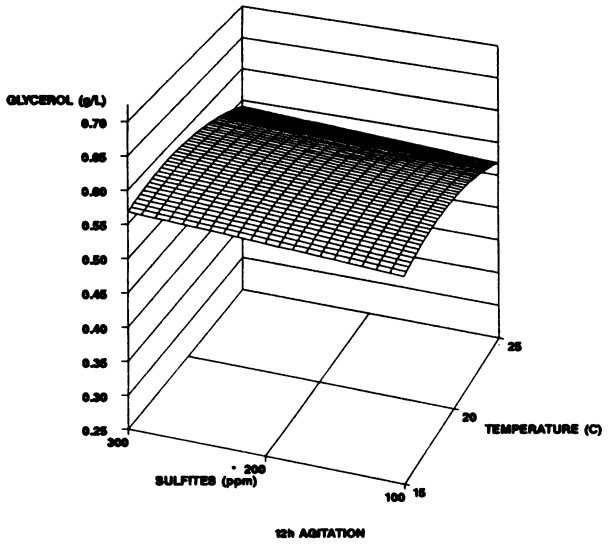
Interactions between AT block, strain, and sulfite concentration on glycerol production. The overall AT block-strain-sulfite interaction was not significant ($P = 0.5535$) (Table 3). This indicates that no matter which agitation rate and temperature were used, the initial sulfite concentration influenced the three strains in the same way.

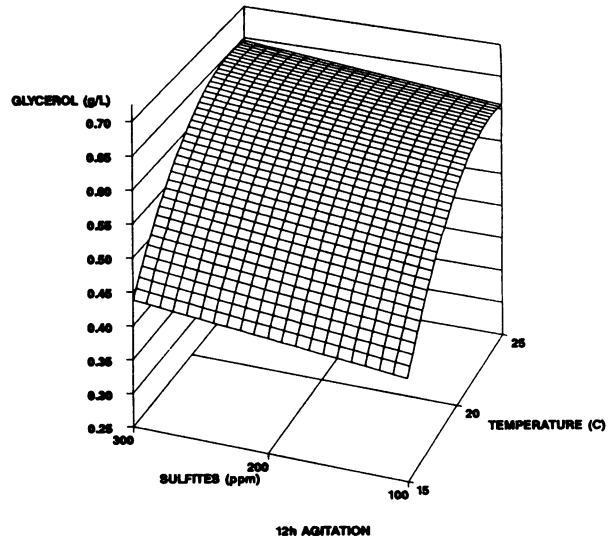
The strain-sulfite interaction was not significant ($P = 0.8685$) (Table 3). Thus, the effect of the initial sulfite concentration was the same for each strain.

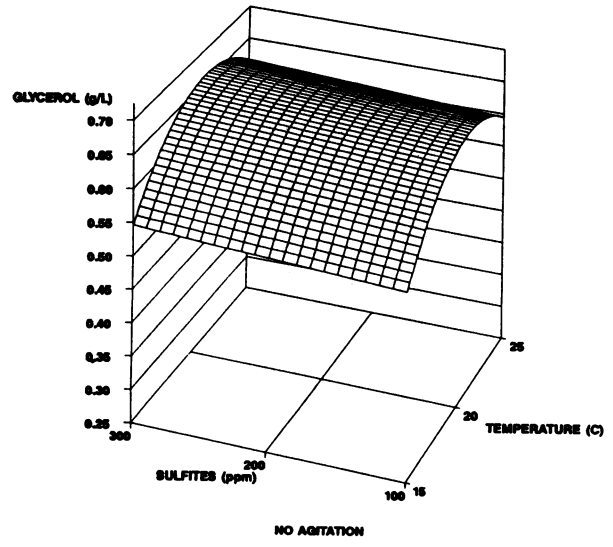
The AT block-sulfite interaction was not significant ($P = 0.6439$) (Table 3). This suggests that whatever agitation time and incubation temperature were used, the initial sulfite concentration effect on glycerol production was the same.











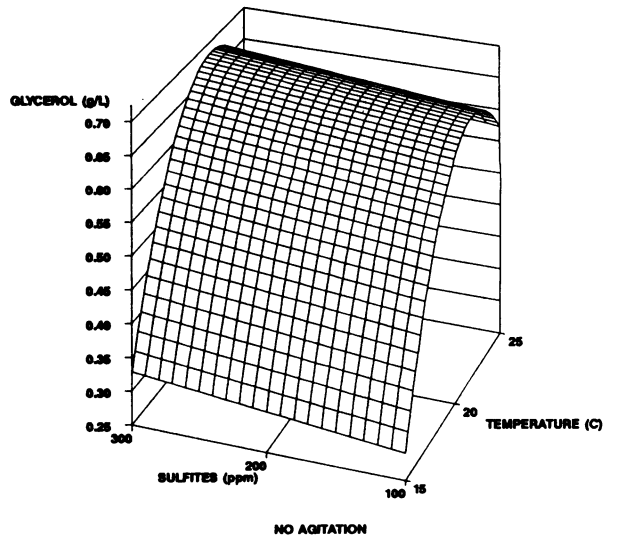


FIG. 3. Response surfaces showing the effects of agitation time (0, 12, or 24 h), incubation temperature, and initial sulfite concentration on glycerol production by *S. cerevisiae* Y7.

The AT block-strain interaction was significant (P = 0.0288) (Table 3), and an additional study of the effects of agitation and temperature on glycerol production was performed with each strain (Table 4). Since the combined effects of agitation and temperature differed among strains, response surfaces were produced for each strain (Fig. 3 through 5). Figures 3 through 5 show that glycerol production was greatest at temperatures higher than 20°C, thus confirming the results of Ough et al. (12). However, the optimum temperature for glycerol production varied depending on the agitation time. Increasing the agitation time tended to increase the predicted optimum temperature for glycerol production for all three strains (Fig. 3 through 5), but different relationships were observed. The observation that the optimum temperature for glycerol production is influenced by other experimental conditions might explain why conflicting results appear in the literature (12) since one-factor-at-a-time experimental designs were used in previous studies. Rankine and Bridson (16) also observed that an interaction between strain and temperature affected glycerol production, although this finding was not confirmed by a statistical analysis. Both agitation and temperature potentially influence the level of dissolved oxygen in the medium, but in opposing ways. Thus, greater agitation should increase the level of dissolved oxygen, while higher temperatures reduce the solubility of oxygen in the medium. The temperature-agitation interaction raises the question of the importance of the dissolved oxygen level in glycerol production. Oura (13) suggested that the utility of glycerol synthesis is based on a need to maintain the redox balance in the cell; factors, such as respiration, that divert acetaldehyde or pyruvate from ethanol synthesis, create an NAD deficiency in the glycolysis pathway and promote the glycerol pathway to regenerate NAD. The fact that CO_2 sparging influences glycerol synthesis (2) and the fact that glycerol appeared to be produced early in the fermentation (Fig. 1 and 2), when dissolved oxygen levels are potentially higher, support the assumption that oxygen level plays a role in glycerol synthesis.

It must also be said that glycerol could be metabolized aerobically (6) and that the values obtained might reflect not only expression of synthesis, but also partial utilization, particularly in the latter stage of fermentation when sugar levels become low.

Predicted values. The object of this study was to determine whether interactions between parameters occurred and not to optimize glycerol production. Nevertheless, the polynomial regression models developed enabled us to estimate optimal values, but the resulting data were not subjected to confirmatory fermentation analysis.

In all cases, the highest glycerol levels were obtained with an initial sulfite concentration of 300 ppm. With strain Y7 the highest level of glycerol production was predicted to be 0.66 g/100 ml at 25°C with 24 h of agitation. The corresponding values for strains Y11 and Y13 were 0.68 g/100 ml at 22°C with no agitation and 0.64 g/100 ml at 20.3°C with no agitation, respectively.

Since the glycerol concentration in wines can reach 1.14 g/100 ml (14), the strains used in this study can be considered only moderate glycerol producers. However, the levels reached are sufficient to influence the perceived sweetness of the wine (11).

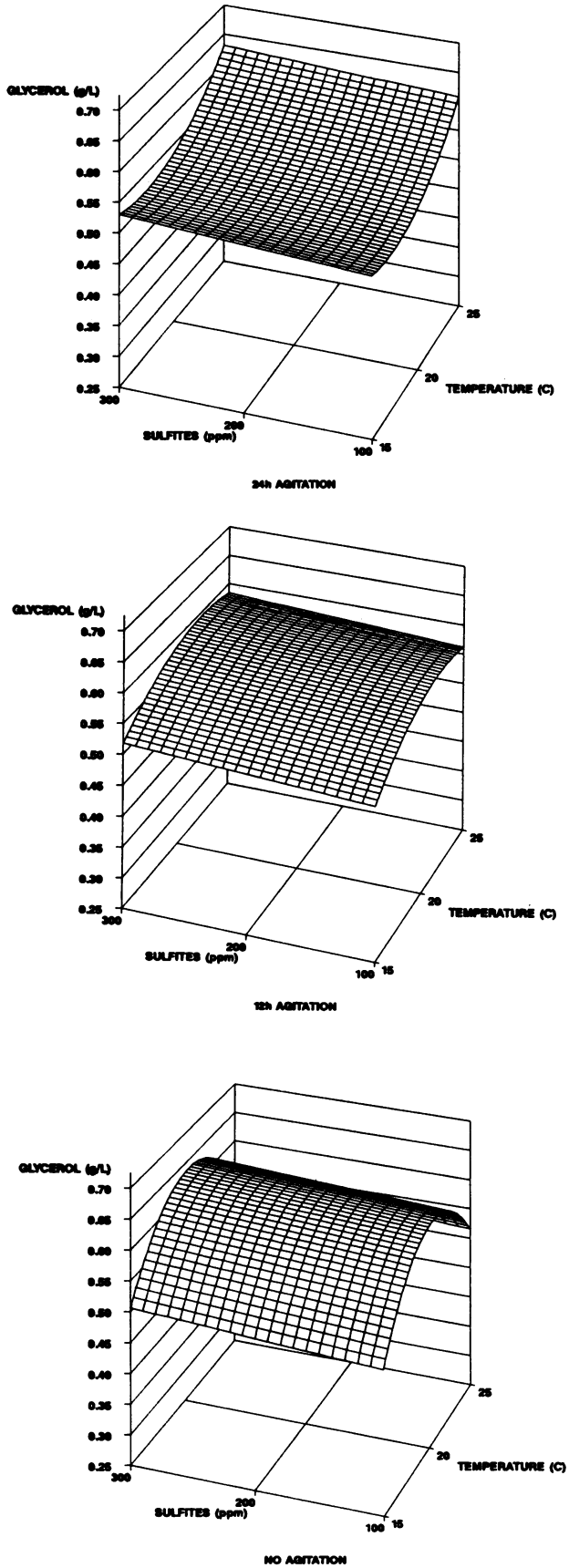


FIG. 4. Response surfaces showing the effects of agitation time (0, 12, or 24 h), incubation temperature, and initial sulfite concentration on glycerol production by *S. cerevisiae* Y11.

FIG. 5. Response surfaces showing the effects of agitation time (0, 12, or 24 h), incubation temperature, and initial sulfite concentration on glycerol production by *S. cerevisiae* Y13.

ACKNOWLEDGMENT

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