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ADAPTATION OF THE TWEEN 80 ASSAY WITH A RESOLUTION V FRACTIONAL FACTORIAL DESIGN AND ITS APPLICATION TO RANK *Ophiostoma fungi* WITH WOOD EXTRACTIVE DEGRADING CAPABILITIES

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ABSTRACT

Wood extractives in radiata pine are the source of the observed pitch problems in the pulp and paper industry. Various methodologies have been studied and used to reduce or eliminate their negative effects. A biological treatment with albino fungi of the *Ophiostoma* genus, fungi that degrade the lipidic components of extractives, has been proposed as a more environmentally friendly alternative for pitch degradation.

The current methods used to search for *Ophiostoma* albino strains with the highest degradation rates of wood extractives are labor intensive and require a large amount of resources. The Tween 80® Opacity test, an assay designed to measure lipolytic enzyme activity in filamentous fungi, was studied to verify the feasibility of its application as a methodology to rank *Ophiostoma* albino strains with deresination capabilities.

The Tween 80® Opacity Test was first characterized by implementing a non-replicated 2^{k-p} fractional factorial design of resolution V for a 5 factors with 16 treatments and then to study the effect of the fungus species on the lipolytic enzyme activity; a randomized one factor general factorial design was conducted. The incubation temperature; antibiotics presence; and Tween 80®, $CaCl_2$, and peptone concentrations were investigated in the first experiment. The *Ophiostoma* species effect was studied in the second experiment. In both experiments, the halo area size, which was formed by the fatty acid-calcium complex precipitate, was the response variable.

The results demonstrated the effectiveness of the Tween 80® opacity test to measure the lipolytic enzyme activity of *Ophiostoma* albino strains. Similarly, the incubation temperature and the concentrations of Tween 80®, $CaCl_2$, and bacteriological peptone had the highest statistically significant effect on the response variable. In addition, our data demonstrated that the albino strains from the specie *O. floccosum* exhibited the highest rate of lipolytic enzyme production.

Keywords: Factorial design, lipase enzyme activity, *Ophiostoma floccosum, Ophiostoma piceae, Ophiostoma piliferum,* pitch control.

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INTRODUCTION

Wood extractives, which are composed mainly of triglycerides, fatty acids, and sterols (Fengel and Wegener 1984), induce the formation of pitch deposits during the fabrication of mechanical pulp (Allen 1980, Martínez *et al.* 2000). Pitch deposition, among other problems, causes the breakage of paper during its manufacturing, the formation of dust during the printing process, and the increment of effluents toxicity (Farrell *et al.* 1997, Gutiérrez *et al.* 2009). In other areas of wood processing also increases drying times, promotes brown stain, and prevents the impregnation of wood (Miller *et al.* 1983, Flynn 1995, Matsumura 2001, Sundqvist 2002, Malkov *et al.* 2003).

The wood storage time, the use of chemical additives (Allen 1980, Back and Ekman 2000), and more recently, the use of enzymes and microorganisms have been proposed to reduce their effects (Blanchette *et al.* 1992, Fujita *et al.* 1992, Farell *et al.* 1993, Fischer *et al.* 1994, Gao *et al.* 1994, Wang *et al.* 1995, Rocheleau *et al.* 1998, Chen *et al.* 2001, Calero-Rueda *et al.* 2004).

The biological treatment of wood chips with albino strains from the genus *Ophiostoma*, which are fungi that degrade resin acids and triglycerides (Blanchette *et al.* 1992, Gao *et al.* 1994), is a known strategy that is used to reduce and/or eliminate the pitch formation problems during the production of mechanical pulp (Blanchette *et al.* 1992). Currently, Cartapip 97, which is also known as Sylvanex™ and is a fungal inoculum of the albino strain from the species Ophiostoma piliferum, is being commercialized to control the pitch problem in the pulp and paper industry (Farrell *et al.* 1993). Recent studies have suggested the use of Ophiostoma albino fungi, depending on the lumber final destination, as a potential approach to prevent the occurrence of sapstaining fungi in Radiata pine wood (Held *et al.* 2003, Navarrete and Sánchez 2010, Berrocal *et al.* 2012)

Each region throughout the world has its own sapstaining fungal diversity (Seifert 1993, Harrington *et al.* 2001); consequently, a customized regional biological treatment is needed. The search for new albino strains from the species *Ophiostoma floccosum* include, among other steps, the isolation of individual spores, the verification of its albino nature, the evaluation of their growth rate in solid and liquid media, their aggressiveness in the colonization of substrates, and quantification using solvent extraction of their pitch reducing capabilities (Held *et al.* 2003, Navarrete *et al.* 2005). The best albino strain preselection process, which is based on the best rate of mycelia growth on solid media, does not ensure the discovery of the optimal deresination fungus because this process assumes the fungus having a highest growth rate also has the best capabilities of reducing pitch (Navarrete and Sánchez 2005).

It is clear from these facts that a large amount of resources and number of experiments are needed if a statistically significant result is desired. Therefore, the development of an efficient screening and selection methodology to minimize the use of materials and resources would be advantageous for research in this area (Martínez et al. 2000, Martínez-Iñigo *et al.* 2000, Held *et al.* 2003)

The Tween 80® opacity test has been successfully used in the detection of lipase activity in pathogenic strains of the genus *Candida* (Slifkin 2000) in autolysates from filamentous fungal cultures of *Fusarium* sp., *Aspergillus* sp., *Penicillium* sp. (García-Lepe *et al.* 1997), and, more recently, in the selection of fungal species for the consumption of contaminating oils in petroleum spills in aquatic environments (Gopinath *et al.* 2005).

The triglycerides contained in the Tween 80® reagent are hydrolyzed by fungi due to lipase activity, releasing glycerol and fatty acids within the solid media, which contain calcium salts. The chemical reaction between the fatty acid and the calcium salts results in the formation of a white complex precipitate (Sierra 1957).

Because fungi from the *Ophiostoma* genus also exhibit significant lipase activity (Gao *et al.* 1994, Tamerler *et al.* 2001), we hypothesized that the Tween 80® opacity test may be used as a screening methodology to rank the *Ophiostoma* fungi pitch reducing capability.

Consequently, the objectives of this study were to a) verify the application of the Tween 80® opacity test for albino species from the genus *Ophiostoma* and (b) to adapt its use to study the bioreducing capacity of certain albino species from the genus *Ophiostoma*.

MATERIALS AND METHODS

Part I. Validation of the Tween 80® Opacity Test

Albino fungus for the Tween 80® validation assay

The albino strains used in this study were obtained from the Bioprocess and Biotreatment Laboratory Culture collection of the Department of Timber Engineering at the University of Bio-Bio (Table 1): PCF_2A3 and PCF_2A14 from the species *O. piceae*; PLF_2D8 and PLF_1 A7 from the species *O. piliferum*; and FLF_1A65 and FLF_1A18 from the species *O. floccosum*. These strains, which were stored at -84ºC, were transferred to culture plates containing malt extract and agar (MEA 1,5 % (w/v)) and were maintained at 4ºC until needed. The albino strains were previously obtained according to the methodology described by Held *et al.* (2003).

Experimental design

The opacity test characterization in Tween 80® media for the Ophiostoma albino strains was performed using a 5 factor, non-replicated, completely randomized resolution V, 25-1 fractional factorial design. A total of 16 treatments were conducted. The factors analyzed and their levels, which were selected from previous studies with Tween 80® in other microorganisms (García-Lepe et al. 1997, Slifkin 2000, Gopinath et al. 2005) were A: bacteriological peptone (8 and 20 gL^{-1}), B: Tween 80® (4 and 10 mL^{-1}), C: calcium chloride (0,1 and 1 gL^{-1}), D: temperature (18 and 25 ºC) and E: antibiotics (the presence or absence of chloramphenicol and streptomycin sulfate). The response variable for each of the 6 evaluated strains was the area of the halo formed by the calcium salts precipitate in the Tween 80® medium measured at 2, 5, and 8 days of growth. The run order of the randomized treatments is shown in table 2. Three replicates were performed for each treatment.

Statistical Analyses

An analysis of variance (ANOVA) was performed for each strain (6) and growth period (2, 5, and 8 days), resulting in a total of 18 ANOVAs. The experimental set up, the treatment-randomized run order, ANOVA, and model adequacy verification were conducted using Design Expert 8,0 software (Stat Ease Inc., Minneapolis, USA).

Tween 80® media and culture plate inoculation

The solid medium for each treatment was prepared with bacteriological peptone (Merck), calcium chloride (Sigma-Aldrich), Tween 80® (Sigma-Aldrich), and agar (Difco). The concentrations of the solutions, which were adjusted with distilled water according to the individual treatments, were sterilized at 121ºC for 20 min. The Tween 80® was sterilized separately and subsequently added to the media. The pH was adjusted to 6,0 using a solution of NaOH (1 M).

Petri dishes for each treatment were inoculated with a 5-mm diameter plug acquired from the actively growing margins of the tested strain; a total of 48 plates per strain were incubated according to the established conditions for each treatment.

Halo measurements

Each plate was placed on a polycarbonate sheet graduated in mm and the halo diameter, produced by the precipitated calcium salts, was measured at 2, 5, and 8 days of growth. A SMZ645 Nikon stereomicroscope set with a cold light source (Nikon Lamp NI-150) and a 20X magnification level was used. The diameter was used to calculate the area of the halo for each incubated fungus.

Part II. Quantification of lipase activity in solid Tween 80® media

Fungi species

A total of 30 albino strains, with 10 strains from each species, were studied (Table 1). The Petri dish inoculation and halo measurements of the precipitated calcium salts were performed as indicated in the previous section.

Experimental design

A single-factor experiment with three levels of the factor and n=10 isolates as replicates were performed. The factor studied was the *Ophiostoma* genus albino species and the *O. floccosum, O. piceae* and *O. piliferum* levels. The response was the area of the halo produced by the precipitated calcium salts in the Tween 80® media measured at 2 days of growth.

Statistical analyses

A single-factor ANOVA with a confidence level of 95% was developed. The treatment averages of the area of the halo of the calcium precipitate for each species were determined, and Fisher´s least significant difference (LSD) for pairwise testing of means was conducted. The experimental set up and the statistical analysis were performed using the Design Expert 8,0 software.

Preparation of culture media with Tween 80® and incubation conditions

The culture media composition, which was determined from the validation assays in Part I (Table 4 and Figure 1), were peptone (8 gL^{-1}), calcium chloride (1 gL-1), agar (15 gL^{-1}), and Tween 80®(4 ml/L^{-1}). The Tween 80® was sterilized at 121ºC for 20 min separately and subsequently added to the media. The pH was adjusted to 6,0 with 1 M NaOH. A total of 150 Petri dishes, with 5 dishes per strain, were inoculated with the 30 albino strains (Table 1) and incubated at 25ºC for 2 days.

Table 1. Albino strains from the genus *Ophiostoma* used in this study. Each strain corresponds to the individual spore isolated from a direct mating.

Nº	Specie	STRAIN	Nº	Specie	STRAIN	Nº	Specie	STRAIN
1	***O. piceae***	**PC F2 A3 ***	11	***O.piliferum***	**PL F2 B68**	21	***O.floccosum***	**FL F1 A18 ***
2	***O. piceae***	**PC F2 A9**	12	***O.piliferum***	**PL F2 B82**	22	***O.floccosum***	**FL F1 A55**
3	***O. piceae***	**PC F2 A14 ***	13	***O.piliferum***	**PL F2 C57**	23	***O.floccosum***	**FL F1 A65 ***
4	***O. piceae***	**PC F2 A29**	14	***O.piliferum***	**PL F2 D6**	24	***O.floccosum***	**FL F1 A94**
5	***O. piceae***	**PC F2 A35**	15	***O.piliferum***	**PL F2 D8 ***	25	***O.floccosum***	**FL F1 A 2**
6	***O. piceae***	**PC F2 A68**	16	***O.piliferum***	**PL F2 D88**	26	***O.floccosum***	**FL F1 A 7**
7	***O. piceae***	**PC F2 A70**	17	***O.piliferum***	**PL F2 C60**	27	***O.floccosum***	**FL F1 A 8**
8	***O. piceae***	**PC F1 A 32**	18	***O.piliferum***	**PL F1 A 1**	28	***O.floccosum***	**FL F1 A 9**
9	***O. piceae***	**PC F1 B 39**	19	***O.piliferum***	**PL F1 A 4**	29	***O.floccosum***	**FL F1 A 11**
10	***O. piceae***	**PC F1 C 23**	20	***O.piliferum***	**PL F1 A 7 ***	30	***O.floccosum***	**Fl F1 A 22**

(*): Strains selected for the Tween 80® opacity test validation study.

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RESULTS AND DISCUSSION

The quest for new environmentally friendly technologies to minimize the effect of pitch on the production process of mechanical pulp has led to the search for microorganisms with outstanding enzymatic and growth characteristics to improve the efficiency of these economic activities (Behrendt *et al.* 1995, Behrendt and Blanchette 1997, Held *et al.* 2003, Calero-Rueda *et al.* 2004, Farrell *et al.* 1993, Gao *et al.* 1994, Rocheleau *et al.* 1998). Although our group has been successful in finding and developing microorganisms with these capabilities, in particular, albino strains from the genus *Ophiostoma*, the task has been time consuming and costly (Navarrete and Sánchez 2005, 2010, Navarrete *et al.* 2008).

The opacity assay, which is also known as the radial diffusion assay for the detection of lipase activity in agar and initially described by Sierra (1957) appears to be a technique that may help to ease this task. This assay measures the ability of certain fungi to produce lipolytic enzymes that liberate fatty acids in a media enriched with an oily compound, which react with calcium ions, producing an insoluble precipitate around the area of inoculation. This methodology has been used for the detection of dermatophyte fungus and yeast species, especially through the presence or absence of a visible halo around the colony (Slifkin 2000). García-Lepe *et al.* (1997) used the diameter of the halo formed in this specific media to quantify the potential production of lipolytic enzymes from several autolysates of filamentous fungus and observed that *Aspergillus nidulans* was an important source of lipases. In addition, the size of the halo has previously been used to detect fungal species with remediation potential, e.g., for the filamentous fungi *Fusarium sp., Aspergillus spp., Trichoderma viride, Rhizopus stolonifer* and *Penicillium spp*., which were isolated from environments containing an abundant amount of oil wastes (Gopinath *et al.* 2005). In our study, we observed a correlation between the size of the halo and the lipolytic capacity of the genus *Ophiostoma* species and trends that may help to find a species and strain with this genus that more effectively prevent blue stain and/or reduce wood extractives.

Validation of the Tween 80® Test

To validate the application of the Tween 80® test to study the lipophilic activity of the *Ophiostoma* genus fungi, we used a factorial design instead of a one-factor-at-a-time experimental design, a frequently used approach to study fungal growth or metabolite production in solid medium. The former approach allowed us to find enough statistical evidence to discern the existence of interactions among variables. Unfortunately, in many studies, this methodology is not used (Czitrom 1999, Montgomery 2005, Berrocal *et al.* 2012, Wahid and Nadir 2013). Thus, a non-replicated 2^{k-p} fractional factorial design of resolution V, with 5 factors and 16 treatments was conducted. In table 2, the responses are plotted, which are the areas of the calcium precipitate of the albino strain of the species *O. floccosum* FLF_1A18, one of the six studied species, for each of the 16 treatments at 2, 5, and 8 days of growth.

Table 2. Fractional factorial experimental design matrix with their actual values for the adaption of the Tween 80® opacity test to strains of the genus *Ophiostoma* showing the area size halo response after 2, 5 and 8 days of incubation for the *O. floccosum* strain FLF_1A18.

Std	Run	Factor 1 A:Temp C	Factor 2 B:Antibiotic	Factor 3 C:Tween80® ml/L	Factor 4 D:$CaCl_2$ g/L	Factor 5 E:Peptone g/L	Incubation time Day 2(*) mm^2	Day 5 (*) mm^2	Day 8 (*) mm^2
12	1	25	yes	4	1	8	113	396	735
9	2	18	no	4	1	8	113	327	641
3	3	18	yes	4	0,1	8	75	327	553
4	4	25	yes	4	0,1	20	75	264	471
5	5	18	no	10	0,1	8	19	207	396
6	6	25	no	10	0,1	20	44	207	327
1	7	18	no	4	0,1	20	44	327	553
13	8	18	no	10	1	20	44	264	553
10	9	25	no	4	1	20	113	471	735
2	10	25	no	4	0,1	8	113	396	641
8	11	25	yes	10	0,1	8	75	264	327
16	12	25	yes	10	1	20	113	237	471
7	13	18	yes	10	0,1	20	0	157	396
11	14	18	yes	4	1	20	75	327	553
15	15	18	yes	10	1	8	44	264	471
14	16	25	no	10	1	8	113	327	471

(*) Each data point represents the average of three replicates.

Relative importance of individual factors

The ANOVA performed during the validation study for the 6 strains considered demonstrated that the total variability observed in the halo area size is explained, mainly in terms of the relative contribution of the factors and their interactions, by the concentration of calcium chloride, the temperature, and the concentration of Tween 80® (Montgomery 2005). In addition, the interaction of calcium chloride and peptone originally were statistically significant.

Table 3 demonstrates that the effect relative importance of the concentration of calcium chloride on the formation of the halo gradually decreases from 39,4% on day 2 to 17% by day 8, with an intermediate value of 35% by day 5. The initial importance of this factor can be explained, according Hoshino *et al.* (1991), Tamerler *et al.* (2001), and Kontkanen *et al.* (2006), by the inducing effect of the calcium ion (Ca^{2+}) on the microbial lipase activity associated with the processes of excretion of extracellular lipolytic enzymes. According to Hoshino *et al.* (1991), the metabolic pathway of secretion of these enzymes is controlled by the intracellular concentration of Ca^{2+} ions using the cellular signal transduction pathways for the activation of repressed lipase production pathways.

Table 3. Factors and interactions percent contribution to the total observed variability of the area size halo during the growth of albino strains from *O. floccosum* (**FLF_1 A18, FLF_1 A65**), *O. piceae* (**PCF_2A3, PCF_2A14**) and *O. piliferum* (**PLF_2 D8, PLF_1 A7**) at 2, 5 and 8 days of growth.

FACTOR	Day 2							Day 5							Day 8						
	PC F_2A3	PC F_2A14	PL F_2 D8	PL F_1 A7	FL F_1 A18	FL F_1 A65	Avg.	PC F_2A3	PC F_2A14	PL F_2 D8	PL F_1 A7	FL F_1 A18	FL F_1 A65	Avg.	PC F_2A3	PC F_2A14	PL F_2 D8	PL F_1 A7	FL F_1 A18	FL F_1 A65	Avg.
A-Temperature	45,5	23,7	18,6	32,7	30,3	13,8	27	0,82	0,52	11,6	0,2	18,3	4,8	6	7,6	33,2	19	10,1	0,07	6,6	13
B-Antibiotic	2,4	1,3	0,7	0,3	0,1	0,5	0,7	0,02	0,16	0,05	0,001	0,1	3,4	0,6	2,6	4,5	1,8	1,3	2,5	1,2	2
C-Tween80®	7,4	3,5	8,5	3,8	31,5	32,2	14	26,8	15,5	16,5	15,5	401	40,1	25,8	59,1	30	29,5	5,3	58,4	36,1	36
D-$CaCl_2$	28,4	54,5	53,9	51,9	18,9	22,5	39,4	43,5	46,6	35,2	44,7	20,9	19,1	35	11,6	0,3	12,9	42,5	23,8	10,7	17
E-Peptone	2,4	0,1	1,6	3,8	5,6	0	2,4	5,4	6,2	2	8,3	1,9	0,1	4	0,0	0,9	0,3	7,2	0,7	2,7	2
AB	1,7	0,1	0,01	0,3	0,02	0,9	0,2	0,4	0,5	6,9	8,8	1	0,4	3	0,9	1,6	0,02	0,1	0,01	4,8	1,2
AC	0,01	0,75	0,12	0,1	4,2	0,3	1,5	5,4	3,4	6,4	4,3	0,8	0,01	3,4	0,8	3	0,9	3,3	5,5	10,2	4
AD	0,01	0,1	1,1	0,3	0,1	3,5	1,3	0,2	15,5	0,2	0,3	3,6	12,9	5,5	0,5	7,8	20,8	5,3	4,8	5,5	7,5
AE	1,1	1,3	0,7	0,1	0,02	1,5	0,8	5,4	1,5	8,8	10,4	0,6	1	4,6	6,3	0,01	1,3	3,1	0,2	2,2	2,2
BC	0,01	0,1	0,3	0,5	0,07	2,1	0,6	0,3	0,0	3,9	1,6	0,6	5,8	2	0,4	0,9	2,1	0,3	0,3	0,4	0,7
BD	0,1	0,1	0,7	0	0,1	0,5	0,3	1,4	0,8	0,6	0,3	0,0	0	0,5	0,9	0,2	0,7	0,2	0,01	1,8	0,6
BE	1,1	0,4	0,01	0,1	0,99	1,3	0,6	0,3	3,4	2,4	0,2	0,8	4,8	2	3,4	2,7	0,9	7,2	0,001	2	2,7
CD	2,4	11,4	9,9	0,2	4,6	1,8	4,3	6,7	0,5	0,8	4,3	3,2	0,1	2,6	0,2	0,9	0,01	8,1	1,1	0,1	1,3
CE	0,01	1,3	1,6	0,8	0	0,3	0,7	0,2	4	1,5	0,1	1,2	0,1	1,2	1,1	7,2	4	3,5	0,8	1,8	3,1
DE	7.4	1,3	2,3	5,2	3,4	18,9	6,2	3,3	1,5	3,1	0,9	6,9	7,4	3,9	4,5	6,7	5,8	2,5	2	14	6
R^2 ADJUSTED	**0,87**	**0,90**	**0,88**	**0,97**	**0,97**	**0,85**	**0,92**	**0,89**	**0,83**	**0,73**	**0,91**	**0,86**	**0,74**	**0,83**	**0,82**	**0,74**	**0,86**	**0,72**	**0,91**	**0,75**	**0,8**
R^2 PREDICTED	**0,77**	**0,85**	**0,80**	**0,96**	**0,94**	**0,71**	**0,85**	**0,76**	**0,57**	**0,35**	**0,76**	**0,73**	**0,51**	**0,61**	**0,66**	**0,45**	**0,69**	**0,33**	**0,84**	**0,43**	**0,6**
SIGNAL / NOISE	**15,2**	**17,7**	**15,1**	**27,1**	**29,6**	**13,9**	**20,4**	**12,3**	**10,6**	**9,7**	**14,7**	**13,8**	**9,8**	**11,8**	**12,9**	**9**	**13**	**8,4**	**18,9**	**9,9**	**12**

(*): underlined numbers show the factors or interactions found, for $\alpha = 0,05$, statistically significant.

Table 3 also demonstrates that the relative importance of the effect of temperature decreases between day 2 and 8, from 27% to 13%, with an intermediate value of 6% for day 5. This decrease in the effect of temperature on the formation of the halo can be explained by its initial effect on the synthesis processes and the secretion of lipases, which once activated do not depend significantly on its effect. In a study with *Candida* species in a temperature range between 30-35°C after 24 hours of incubation, Slifkin (2000) did not observe a significant difference in the occurrence and intensity of the halos at these temperatures.

In contrast to the factors mentioned, the effect of the relative importance of Tween 80® increases for the study period from 14% to 36%, with an intermediate value for day 5 of 25,8%. The increase of the effect on the time of this carbon source is explained by its oily nature, which induces the production and excretion of lipases. Studying the growth of the filamentous fungus *Rhizopus oryzae*, Shukla and Gupta (2007) observed that the secretion of these enzymes was dependent on the nature of the oily carbon source used. Moreover, the effect of the carbon source on the size of the halo in the species O. floccosum has a more relevant effect on the entire evaluation period, with respect to the strains of the species *O. piceae* and *O. piliferum*, exhibiting a notable difference at 48 hours of growth with respect to these same strains (Table 3).

The relative importance of peptone (the source of nitrogen) on the size of the halo, although significant in the ANOVA of the strains analyzed, is the least relevant factor and only explains approximately 2,8% of the total variability observed for the days of growth analyzed. The values suggest that this factor is the least important, in general and in the study period, for the species *O. floccosum*. Nevertheless, Gao and Breuil (1995), Elibol and Ozer (2001) and Sharma *et al.* (2001) suggest that the nitrogen source has a significant effect on the production of microbial lipases. In particular, for a strain of the species *O. piceae* grown in liquid culture medium, these researchers observed that the addition of peptone as well as ammonium sulfate induced lipase production (Gao and Breuil 1995).

The effect of the presence of antibiotics used to prevent the growth of unwanted microorganisms had no statistically significant effect on the development of the halo for any species.

Relative importance of the interactions between variables

In this study, it was observed that only some of the second-order interactions were significant, of which the AC interactions (temperature/Tween 80), AD (temperature/calcium chloride), and DE (calcium chloride/peptone) were notable for their percentage contribution in the explanation of the total observed variability. The effect on the response variable of the AC and DE interactions was maintained during the study period, reaching values as high as 6,2% (Table 3). Nevertheless, for the AD interaction, the value increased from 1,3 to 7,5%. The effect of the AC and AD interactions is explained by the effect that the temperature, which is involved in both interactions, has on the enzymatic hydrolysis reaction of Tween 80® as on the calcium ion, which simultaneously acts as an inducer of enzymatic activity and as a precipitating agent of the free acids produced by the reaction (Hoshino *et al.* 1991). The effect of the DE interaction, however, can also be explained by the induction effect that calcium has on the synthesis of lipases (Hoshino *et al.* 1991), while peptone is the source of amino acids for the synthesis of these enzymes (Gao and Breuil 1995).

Moreover, the second-order interactions, AB, AE, BC, BE, CD, and CE, do not obey the pattern observed in the AC, AD, and DE interactions. In the first case, these interactions only contribute significantly to the explanation of the total variability observed in at most one biotreatment period. However, for the second set of interactions, their effect is observed in at least two periods of biotreatment, for the AB and BE interactions and for the growth period of 5 days, with percentages of 3 and 2%, respectively. Similarly, the same finding occurs for the CE interaction, which only contributes 3,1% for the period of 8 days of growth. An explanation for the diverse contributions of these interactions to the explanation of the total variability observed is found in the inherent variability of each strain and species studied. The interactions of the third and fourth order, which are not presented in Table 3, were not statistically significant.

Correlation coefficients, signal/noise ratio and cube diagrams

The average adjusted and predicted correlation coefficients and those from the signal/noise ratio indicate that, from the incubation periods studied, the shorter incubation period, with a predicted correlation coefficient of 85%, is the most appropriate for rating the precipitant area (Table 3). The average predicted correlation coefficients for periods of 5 and 8 days, of 61% and 60%, demonstrate that the mathematical models associated with these ANOVAs explain the lower percentage of the variability observed. Moreover, the greatest value of the signal/noise ratio of approximately 20 for 2 days of incubation, compared with the value of 12 for at 5 and 8 days, demonstrates that in this period, there is a higher probability of detecting statistically significant differences (Table 3).

Figure 1 displays the combined effect of the significant factors, temperature, Tween 80, and calcium chloride, for 2 days of incubation, on the halo area size. The cube plots also show, for both, low and high level of peptone, the predicted responses at each point of the design and the conditions that favor the formation of a halo of maximum area. From their analysis it is concluded that the solid growth medium of the Tween 80 assay, to maximize the halo area, must be prepared without antibiotics, with peptone (8 g/L), Tween 80 (4 g/L), $CaCl_2$ (1 g/L), and the test must be performed at a temperature of 25°C.

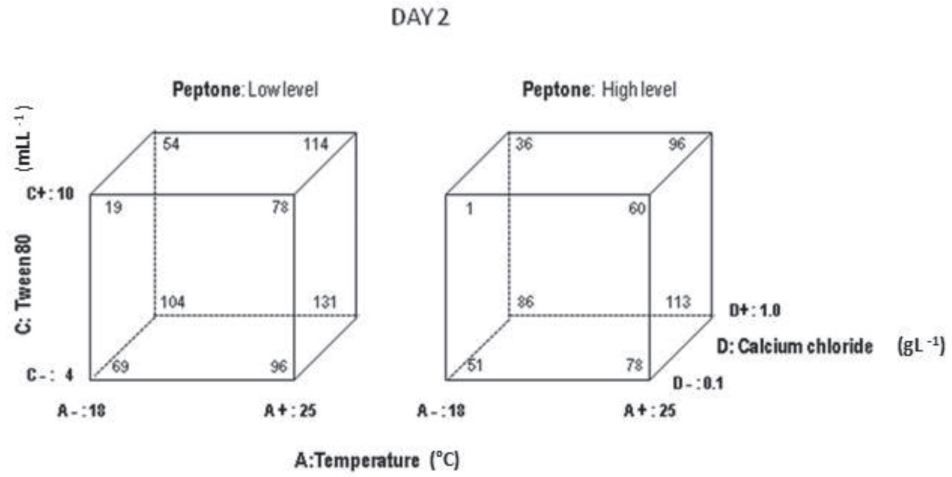

Figure 1. Cube diagram of predicted response halo area size by the model derived from the analysis of variance for the strain *O. floccosum* FLF_1A18 measured at day 2 as a function of temperature, calcium chloride and Tween 80, at the lowest (8g/L) and highest level (20g/L) of Peptone.

These results indicate that the Tween 80® test is a suitable methodology for identifying albino strains of the species *O. piceae, O. piliferum* and *O. floccosum* with better lipase activity and that the optimum levels of the variables for its application are concentrations of 1 g/L of $CaCl_2$, 4 g/L of Tween80®, and 8 g/L of peptone, with an incubation time of 2 days at 25°C.

Evaluation of the effect of the albino fungus species on lipase activity

The extracellular lipolytic activity of 30 fungi of the *O. floccosum, O. piceae* and *O. piliferum* species, 10 from each species, was evaluated using the Tween 80® test adapted to this genre. All of the strains of the species under study, unlike other species of fungi and yeasts, exhibited statistically significant lipolytic activity (Slifkin 2000). For the studied strains, it was observed that the species factor significantly affected the time of formation and the visibility of the halo of the calcium precipitant. The albino strains of *O. floccosum* were the first to form a visible halo at 12 hours of growth, while the strains of *O. piceae* formed a halo at 48 hours (Figure 2). Halo growth for albino strains of *O. piliferum* was observed after approximately 24 hours.

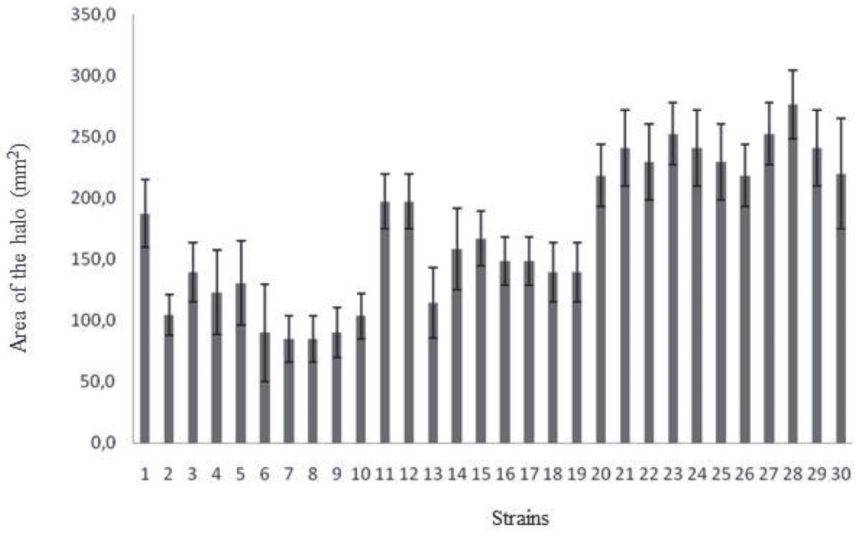

Figure 2. *Ophiostoma* albino strains halos´s area in Tween 80® media amended with calcium chloride after 48 h of incubation at 25ºC.

Note: The numbers in the x-axis correspond to the identification of the strains informed in Table 1

The ANOVA of the factorial design for studying the effect of the species on the halo size demonstrated that the effect of this factor on the size of the halo of the precipitated calcium salts was statistically significant with a *p value* of less than 0,0001 ($F_0 = 51$). Likewise, the values of the predicted and adjusted coefficients of correlation, 0,741 and 0,775, respectively, indicate that the model explains nearly 75% of the observed variability. The appropriate statistical prediction indicates that the signal/noise ratio was 14,2, a large enough value to ensure an adequate prediction for the model. The results of the test method for the least significant difference of Fisher's LSD demonstrated that all the possible comparisons of the means were significant (Figure 3).

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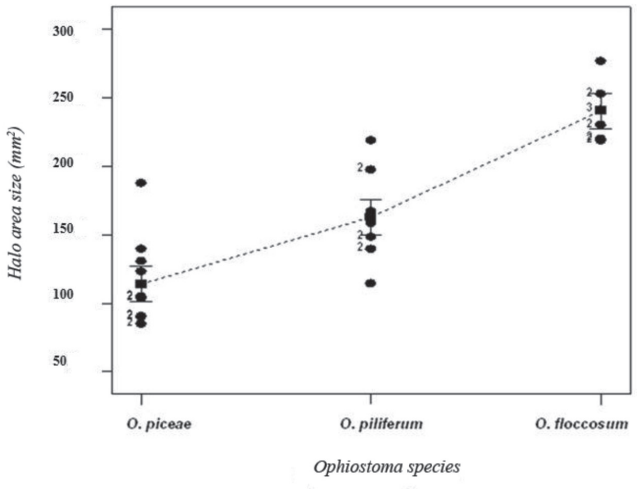

Figure 3. Scatter diagram of halo area size versus *Ophiostoma* species.
Note: Numbers in the figure represent the overlap of data with the same value.

Figure 2 demonstrates that the species *O. floccosum* provides the highest levels of precipitation of the calcium salts of the fatty acids, evaluated at 25°C after 48 hours of incubation, which is an indirect measure of the enzymatic activity of this species. This figure also reveals that the evaluated strains of each species have different levels of lipolytic activity. The areas of the halos of the species *O. piceae, O. piliferum* and *O. floccosum* ranged from 84,4–187,4; 114,3–218,5 and 176,4-218,5 mm^2, respectively. The major differences between the minimum and maximum values of the halo areas of each population of individuals were observed in the *O. piceae* and *O. piliferum* species (Figure 2).

Other authors have corroborated the variability of the enzymatic activity in strains of the same species and in different species. Cihangir and Sarikaya (2004) isolated fungi samples of the genera *Aspergillus, Penicillium, Neurospora* and *Cladosporium*, among others, from soil and observed that of the strains analyzed, a strain of the species Aspergillus exhibited the greatest lipase activity. In another study, Colen *et al.* (2006), also working with soil samples, discovered a strain of the species *Colletotrichum gloeosporioides* with high lipolytic activity among 59 isolated strains.

No reports have considered the search for or the development of strains with high lipolytic capacity as objectives in the study of albino fungi of the genus *Ophiostoma*. The screening studies in this area have intended to discover, through stress tests, fungi with a permanent albino character for use as biological control agents. Held *et al.* (2003) reported obtaining thousands of spores and subsequently evaluating their albino character. The authors selected a large number of albino strains that maintained their albino character and were tested as potential agents for the biological control of blue stain exhibited by fungi such as *Leptographium procerum, O. piliferum* and *Sphaeropsis sapinea*. Meanwhile Gao *et al.* (1994) and Gao and Breuil (1995) directed their work to the characterization of lipases from a strain of the staining species *O. piceae* for its possible application as a reducing agent of lodgepole pine wood extractives. In another study, Martínez et al. (2000) reported the characterization of the lipolytic activity of 90 strains of Basidiomycetes and Ascomycetes fungi that were collected in plantations of *Eucalyptus globulus* by inoculating sterile splinters from that wood. In addition, Calero-Rueda *et al.* (2004) conducted a search for fungi capable of degrading extractives from *Eucalyptus* wood and discovered a strain of the species *O. piceae* that exhibited a great production capacity for lipases and esterases.

This study, in the context of the search for microorganisms with lipolytic enzymes with exceptional features, provides a simple and robust methodology that minimizes the use of resources while simultaneously reducing the time required to obtain results. The Tween 80® assay will be especially useful in the development of microorganisms with this potential, particularly when an early classification of this ability among hundreds of potential microorganisms is desired, as for studies performed with fungi of the genus *Ophiostoma.*

CONCLUSIONS

The results of this study demonstrate that the Tween 80® test is a suitable methodology for identifying albino strains of the species *O. piceae, O. piliferum* and *O. floccosum* with better lipase activity and that the optimum levels of the variables for its application are 1 g/L of $CaCl_2$, 4 g/L of Tween80®, and 8 g/L of peptone, with an incubation time of 2 days at 25°C.

In addition, by applying this methodology, it was possible to determine that the species *O. floccosum* has greater lipase activity compared with the species *O. piliferum* and *O. piceae* and that the species *O. piliferum* has greater lipolytic capacity compared with the species *O. piceae*.

Future studies will aim to relate the genetic variability of the genus *Ophiostoma*, its enzymatic activity, and the reduction of pitch in extractives of *Pinus radiata* wood.

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