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Full Length Research Paper

Malt factor induced flocculation in *Saccharomyces uvarum*

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Tests of fermentability of yeast were carried out with two species of malt; Sympa malt and Nymphea malt, and with their EP (Ethanol Precipitate). Flocculation of *Saccharomyces uvarum* was not observed with any of them. However, Flocculation can be induced by the EP from the bark of another specie of malt Trumpf malt. Analysis of the Trumpf malt EP was carried out using paper chromatography, fractionation on de Cellulose 23, IR (infrared) and UV (ultraviolet) Spectroscopy. Analysis of the effective (F1) fraction revealed the presence of ferrulic acid, arabinose and xylose. Effectiveness of the F1 osidic fraction and the results of IR spectra, which showed the presence of glucosidics bonds, plead for an active role of ferrulic acid and hemi-cellulose ester functions in yeast flocculation process.

Key words: Flocculation, *Saccharomyces uvarum*, malt, EP, osidic fraction, spectrographic analysis.

INTRODUCTION

Flocculation is a well known example of natural, active and reversible aggregation of cells into flocs (Calleja, 1987). The ability of yeast cells to flocculate is very important in industrial processes that are related to fermentation technology like brewing or bioconversion. Yeast flocculation is an important process for the production of beer that causes the yeast to sediment to the bottom of the fermenter at the end of the fermentation. Thus, the yeast can be harvested from the bottom of the fermenter and used for the next fermentation, while the beer may be matured without the need of centrifugation step (Stewart et al., 1975; Stewart and Russel, 1995). The aggregation of cells in flocs generally occurs during the late exponential or early stationary growth phase (Straver et al., 1993). As a result, the majority of cells are separated from the culture medium. However, an interesting yeast strain showed an inverse flocculation pattern (Strauss et al., 2003). The variability in the occurrence of flocculation is a problem (Straver et al., 1993); premature flocculation hampers complete fermentation of the growth medium, whereas, failure of the cells to flocculate at the end of fermentation requires the use of expensive techniques such as centrifugation or filtration to remove cells (Stratford, 1989, 1992; Sadosky and Schawrz, 2002).

Yeast flocculation is a complicated process that is currently only partly understood. It requires the presence of at least two types of molecules on the yeast cell surface. Mannans is one the molecule (carbohydrate chains), which are always present on the cell surface. The other type is flocculins (sugar binding proteins), which are the gene products of the *FLO* genes, that are activated only after depletion of nutrients. (Speers et al., 2006)

Classification of brewer's yeast strains

Brewing strains may be classified in four classes according to their ability to flocculate (Hussain et al., 1986): Non flocculent strains (powdery yeasts, completely dispersed), standard ale strains (Flocculating into small, loose lumps late in fermentation), highly flocculent yeasts (Flocculating into dense masses late in fermentation and producing beer under attenuated and sweet) and very highly flocculent yeasts (Flocculating very early in fermentation owing to non-separation of daughter cells).

Factors affecting flocculation

A number of factors influencing flocculation process have been reported (Jin and Speers, 1998). The genetic aspects

have been recognized since 1950s (Esser et al., 1987) and flocculation is under genetic control (Stratford, 1996; Teunissen and Steensma, 1995; Hidekatsu et al., 2001). The yeast cell wall, the cell age, and other factors of the growth medium can influence the process (Soares et al., 1994).

The flocculation depends on brewery conditions (Johnson et al., 1988) and on the nature of the strain used (Stratford, 1991; Versteepen et al., 2000). It is well established that Wort components influence the ability of the yeast cells to flocculate (Taylor and Orton, 1978). The kinetic approach shows that the calcium content, for example, has an influence on the flocculation process (Stan, 2002), and premature flocculation of yeast is induced by some Wort constituents (Fuji and Horie, 1975; Fujino and Yashida, 1976). The aim of this study was to determine the substance of Wort, which induces flocculation of S*accharomyces uvarum*.

MATERIALS AND METHODS

Organisms and media

The yeast strain used was a « local strain » of *S. uvarum* from the culture collection of « l'IFBM » of Nancy – France. The yeast was cultivated on 1 L of malt wort. 1 ml of the subnatent was added to 50 ml of Wort. The culture, with continuous stirring was incubated at 25°C for 3 days. Appropriate volumes were then transferred to 2 L of malt and the culture was left in the dark at 25°C for 5 - 6 days.

A day before its utilisation, culture was allowed to decant and appropriate volume was then transferred at equivalent volume of new Wort. Before experiments, yeast was dispersed in distilled water and centrifuged twice at 16000 g.

Conservation of yeast strain

Yeast strains were inoculated every 6 months on Y.M agar medium (malt extract 3 g/L, yeast extract 3 g/L, Biotripcase 5 g/L glucose 1 g/L, agar 2 g/L).

Growth experiments

S. uvarum was inoculated in 250 ml Wiame conical flasks using Stewart chemical liquid medium (Stewart et al., 1975) containing amino acids, sugars, vitamins, ions and other growth factors. When the OD (Optic Density) reached 0.85 - 1, appropriate volumes were then transferred to 500 ml conical flasks containing 100 ml of the same medium. The fermentations were carried out in glass vessels, cylinders containing 1000 ml of synthetic medium. Growth was observed at regular intervals by measuring changes in optical density using chemetrix –24- digital colorimeter (red filters) and the degree of flocculation was measured through growth by calculating the decrease in cell turbidity (800 nm) according to the method of Calleja and Johnson (Johnson et al., 1988).

Fermentability tests were performed using synthetic medium in presence of malt extracts of different species (malt Sympa and malt Nymphea), Ethanol Precipitate of each malt (300 ppm of Ethanol Precipitate of Sympa malt, Nymphea malt and Trumpf malt.), and with the different fractions of the Ethanolic precipitate of malt Trumpf.

Preparation of ethanolic precipitate (EP) of malt

The Ethanolic Precipitate was obtained according to Morimoto process (Morimoto et al., 1975). 55 g of malt were ground in MIAG Grinder and supplemented with 250 ml of water at 46°C, while shaking, the mixture was allowed to stand inside a large vat containing water at 45°C for 30 min. Temperature was then increased until it gets to 70°C (1°C by min) and the preparation was boiled during 30 min. After a decline in temperature, the mixture was homogenised and filtered, these were closely followed by the addition of 65 ml of Ethanol to 35 ml of the filtrate. The preparation was left at 0°C overnight. After centrifugation, the precipitate was dissolved in hot water and supplemented with cadmium acetate and mixture was filtered through "hyflo supercel". Ethanol (65%) was added to the filtrate, the precipitate obtained was dissolved in water and dialysed successively against tap water, demineralised water and in fine distilled water and finally lyophilised .

Analysis of malt trumpf barks EP contents

In order to analyse the EP (ethanolic precipitate) malt contents, its chemical degradation was necessary. This was carried out using saponification, acid hydrolysis and enzymatic degradation.

Saponification

Samples of 100 mg of ethanol precipitate were dissolved in 10 ml of NaOH 2 N and then shaken for 2 h. The solution was acidified by HCl 2 N up to pH 2. After extraction with ethylic ether, the organic phase was evaporated and the residue dissolved in 3 - 4 ml of sodium bicarbonate solution (5%) and another extraction was made by 5 - 6 ml of ethylic ether. Organic phase was dried over anhydride sodium sulphate containing phenolic acids salts. The aqueous phase was acidified (HCL 2 N) until pH 2 and added with ethylic ether containing phenolic acids esters. Identification of the acids was achieved by paper and thin layer chromatography.

Acid hydrolysis of the ethanol precipitate

Samples of 100 mg of Ethanol precipitate were hydrolysed by 5 ml of HCL 2 N at 100°C for 1 h. After a decline in temperature, the solution was filtered and extracted with ethylic ether. The acid and aqueous phases contain either sugars or osidic compounds. The acidity was neutralised using anionic resin (DOWEX 2) and the solution was then concentrated by evaporation. Sugars were identified by paper chromatography.

Enzymatic degradation of the ethanol precipitate

Samples of 100 mg of Ethanol precipitate were treated with 10 ml of enzymatic solution at 0.5 mg/L. Enzymes and processes used are shown on Table 1

Paper chromatography and thin layer chromatography

Paper chromatography was used to identify sugars, and phenolic acids were determined by paper and thin layer chromatography. The systems used are shown in Table 2 for sugar identification and Table 3 for phenolic acids identification.

In order to study the sugars, Watmann paper no. 1 was used, and Watmann paper no 4 and plates of cellulose MN 300 (20 × 20 × 0.03 cm) were used to study the phenolic acids.

Table 1. Enzymatic treatments applied to the ethanolic precipitate of trumpf malt.

Enzyme	Characteristic	Treatment
Celulysine TM	Grade B-Calbiochem (amylase+protease Hemicellulase)	At 37°C for 16 h with pH 6.5 and Centrifugation of 300 g for 15 min
Cellulase	Grade B-Calbiochem	At 40°C for 16 h
Hemicellulase	From rhizopus- Sigma chemical company	At 40°C for 16 h
β-Glucanase	Rapidase	At 25°C for 24 h
Esterase	From pig liver – Sigma chemical company	At 25°C for 24 h in phosphate buffer pH 8
α-amylase	Sigma chemical company	At 85°C for 1 h
Pronase	Grade B-Calbiochem	At 25°C for 12 h in water and At 40°C for 12 h in phosphate buffer pH8
Trypsine	Bovin pancreas–Sigma chemical company	At 40°C for 12 h in water and At 40°C for 12 h in phosphate buffer pH8
Papaine	Rapidase	Activation By 5 Mm Of Cystein-Chlorohydrate. At 25°C For 24 h

Table 2. Descendent chromatography of sugars in Trumpf malt EP (wattman paper N° 1). Migration is 24 h in solvent 1; and 24 h in solvent 2. Solvent 1: n-butanol : aceton : water(4:5:1:) ;solvent 2: n-butylacetat : ethanol : pyridine : water (8:2:2:1).

References (standards)	RGL	Revelation anisidine-phtalane
Rhamnose	3.0	Green-yellow
Glucose	1.0	Green- yellow
Arabinose	1.51	Purple
Mannose	1.33	Green-yellow
Xylose	1.82	Prune
Galacturonate	0.066	Orange
Galactose	0.83	Green-yellow

Table 3. Thin layer (cellulose MN300) and paper chromatography (Wathman no 4) of acid phenols of Trumpf malt EP.

Phenolic acids	Solvents: 1: isopropanol :ammoniac water(8:1: 2) 2 : benzene :acetic acid: water(6:7:3)			Fluorescence	Revelation	
	TLC 1	WP 2		UV	*PAN	Na_2CO_3 10%
Ferrulic acid	0.25	0.3	0.87	Blue	Red	Grey-blue
Vanillic acid	0.196	0.23	0.85	-	Yellow	Dark-blue
p-coumaric acid		0.38	0.47	-	Yellow	Black
p-OH benzoic acid	0.30	0.31	0.36	-	Yellow	Red
Salycilic acid	0.79	0.80	-	Violet	-	Yellow
Gentisic acid	0.67	-	0.12	Blue-green	Brown	Yellow
Syringic acid	0.17	0.19	0.93	-	Yellow	Yellow-green
p-OH-p-acetic acid	0.51	0.51	0.316	-	--	Purple
pOH-propionic acid	0.62	0.56	0.517	-	Yellow-blue	Purple
Sinapic acid	0.19	0.188	0.92	Blue	Red	Yellow-green

*PAN: Para Nitro Anilin.

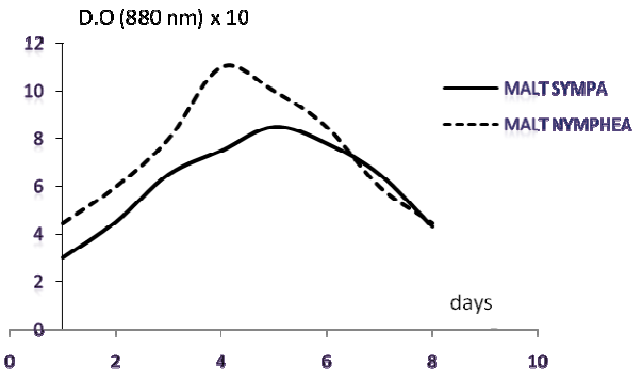

Figure 1. Static fermentation tests of flocculation of *S. uvarum* , comparing fermentation rate with Sympa or Nymphea malt wort, and with incubation time.

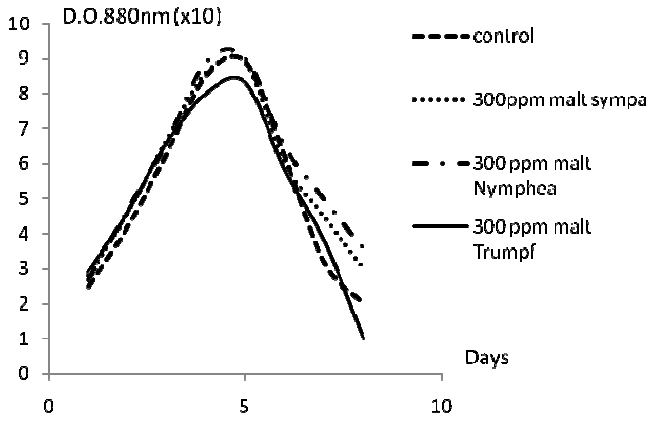

Figure 2. Static fermentation test for flocculation , comparing the effect of 300 ppm of different malt E.P.

The PAN (ParaNitrodiazotAnillin) specific revelatory of phenols and phenolic acids, was obtained by mixing 2 ml of paranitrilaniline (0.5%) with a solution of sodium acetate (20%) and $NaNO_2$(15%).

DE cellulose chromatography

DE –Cellulose-23 used was from W and R. Balston, LTD-n°catal.24231, England. The resin was treated with HCl (0.5 N) for 30 - 120 min, washed twice with NaOH (0.5 N) and H_2O until a pH of 4 - 8 was reached. After sedimentation in the dark, a solution of dissodic boric acid (0.2 M) was added and the mixture was filtered and washed with water. DE-cellulose was then dissolved in water and left for decantation and the columns were then filled.

350 mg of EP of malt Trumpf were fractionated using DE Cellulose 23 chromatography (3.0 × 50 cm). The column was eluted successively by deionised Water, Borax 0.01 M; Borax 0.1 M; NaOH 0.1 N and NaOH 0.3 N. At the same time, sugars were quantified by Dubois method, and proteins by measuring of Optic Density at 280 nm.

IR spectroscopy of bark's trumpf EP and deproteinated EP

200 mg of each sample (EP of malt Trumpf and deproteinated EP by cadmium acetate) were dispersed in 1 mg of K Br. The analysis was carried out following Flemming process (Flemming et al., 1974).

UV spectroscopy of bark's trumpf EP and deproteinated EP

Analysis of phenolic acids was carried out by Ethanol Precipitate, and Ethanol Precipitate deprotinated screening from 200 - 400 nm (spectrophotometer Uvikon 930-Kontron).

RESULTS

Static fermentations experiments

Results of static fermentation experiments, which compare the fermentation rate with yeast in suspension, in presence of wort of two species of malt (Sympa and Nymphea), showed that yeast behavior is the same for the two species (Figure 1). However, the curves obtained by adding Ethanol Precipitate (EP) of Sympha malt, Nymphea malt and Trumpf malt show the presence of flocculation using Trumpf EP (Figure 2).

Ethanol precipitates of trumpf malt contents

Sugars identified by paper chromatography were arabinose, glucose, xylose, galactose and a non identified type of sugar.

Phenolic acids identified by paper and thin layer chromatography are ferulic, isoferulic, paracoumaric, vanilic and syringic acids. The presence of ferulic acid is confirmed by UV (Ultra-Violet) spectrum (Figure 3) where pics at 280 and 300 nm are characteristic of these acids.

IR (Infra-Red) Spectrum (Figure 4) shows the presence of α glucosidics bonds (845 cm^{-1}) and ß glucosidics-bonds (898 cm^{-1}).

Fractionation by DE – cellulose of malt Trumpf EP had led to three fractions (Figure 5). Fraction F1 obtained with water constituted of arabo-xylanes, while arabinose residues are responsible for salvation in water of this fraction. The second fraction F2 (eluted by borax × 0.01 and 0.1 M) contains glycoproteins as a result of the overlapping of sugars and protein's picks. The third fraction F3 (Na OH elute), contains a heterogeneous protein mixture or a mixture of proteins and glycoproteins.

Then the effect of these different fractions of EP on the yeast flocculation was analysed.

Effect of the different fractions obtained by EP bark trumpf malt fractionation

The comparative studies of fermentations of the yeast with 70 ppm of each fraction and 70 ppm of the complete EP shows that the F1 fraction is more efficient than the two other fractions and the Ethanol Precipitate as well

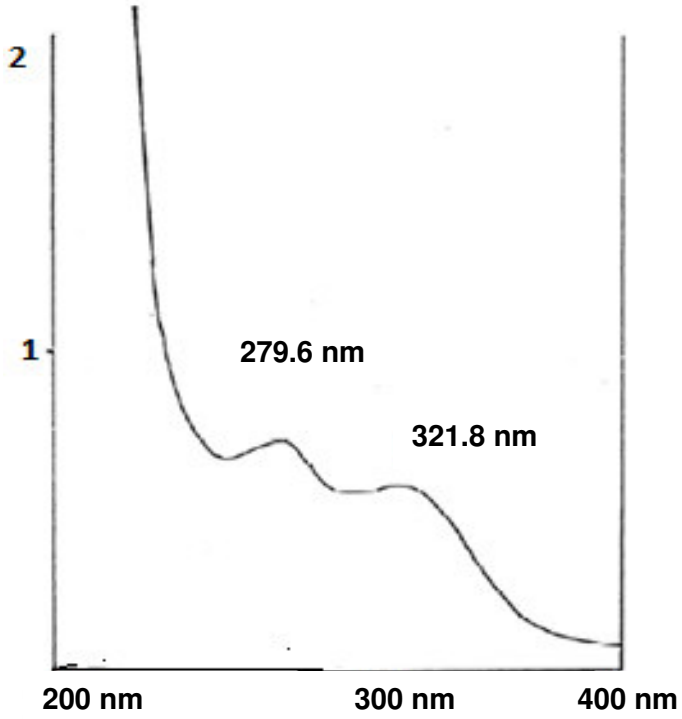

Figure 3. U V Spectrum of deproinated Ethanol Precipitate of Trumpf malt.

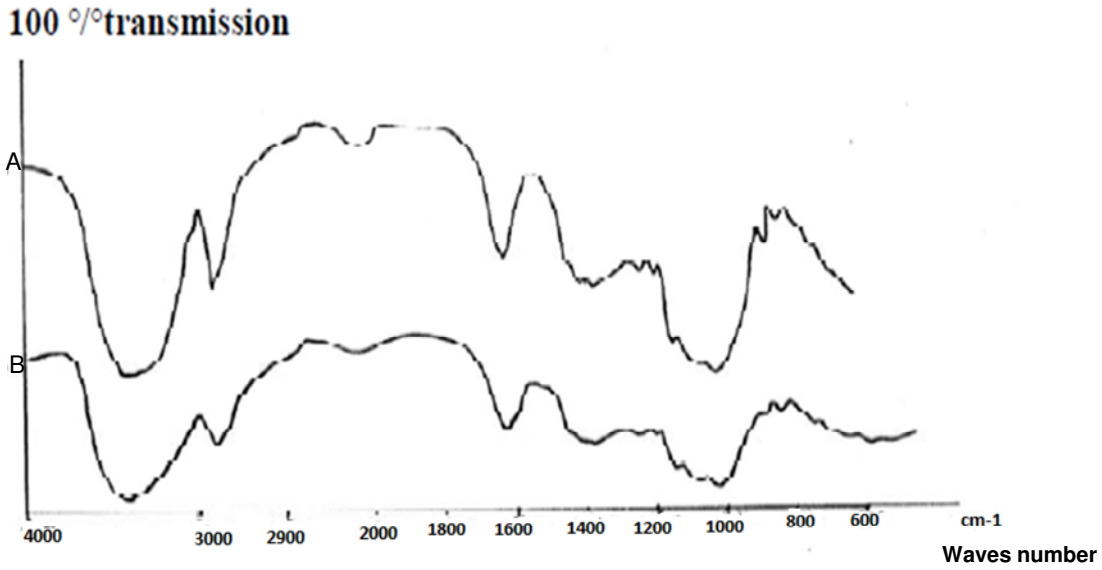

Figure 4. (A) I R spectrum of ethanol precipitate of Trumpf malt. (B) I R spectrum of deproinated Ethanol precipitate of trumpf malt.

(Figure 6). Treatment of the EP by saponification and by α amylase and ß glucanase did not have any effect on flocculation power, but treatment with trypsin or esterase led to the loss of flocculation power of the malt Trumpf. Indeed, saponified Ethanol Precipitate does not induce flocculation (Figure 7) and enzymatic treatments (α amylase, ß glucanase or pronase) did not significantly change the effect of EP.

Maraz and Gelata (2001) suggested that lectine-like cell surface proteins were involved in cell aggregation of

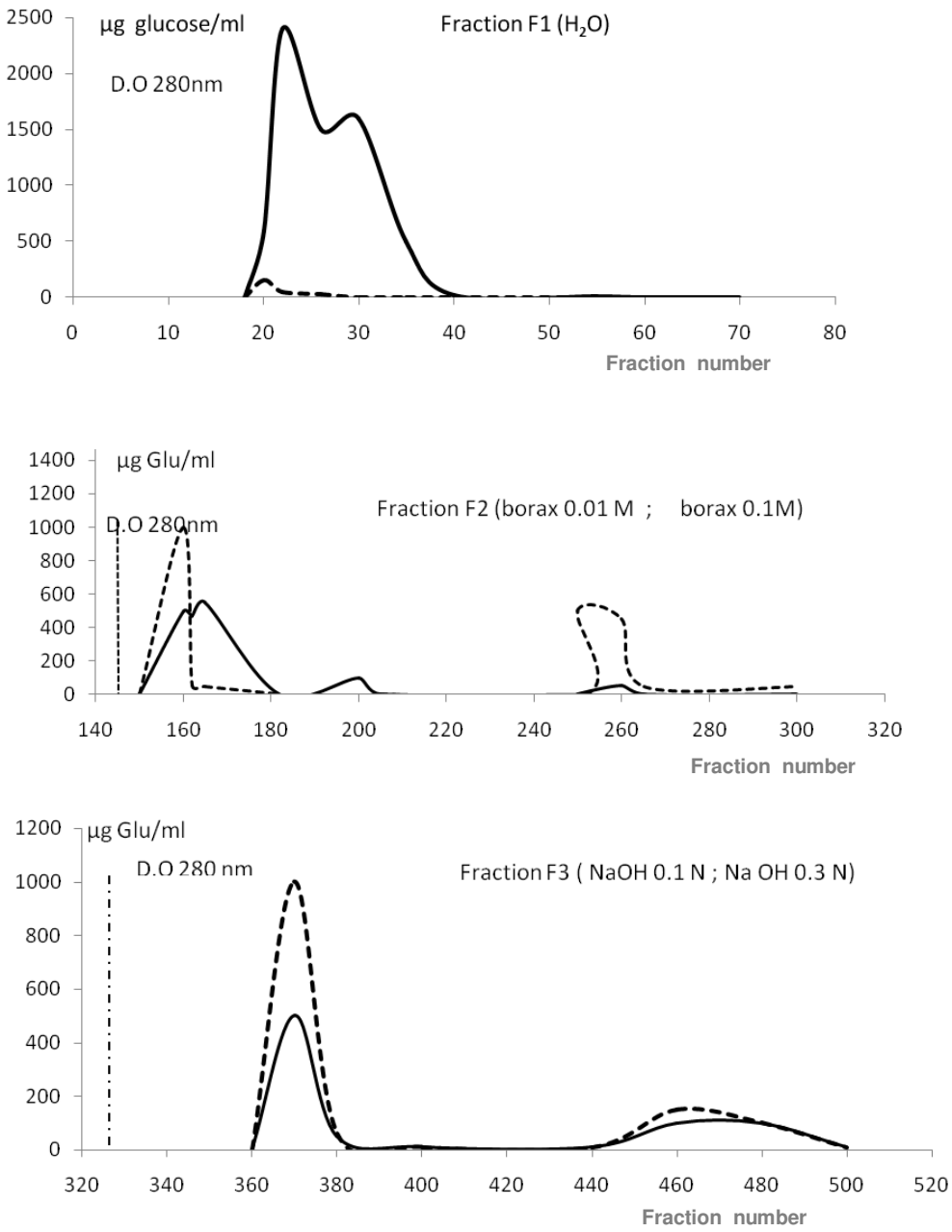

Figure 5. DE cellulose fractionations of the ethanol precipitate from the bark of trumpf malt.

the yeast. One possible role of the compounds of the fraction F1 (sugars, phenolic acids, glucosidic bounds) is that they can provide chemical modification in lectin itself by activating the molecules for binding to the carbohydrate moieties. Further work is needed to see if specific metabolic pathways are induced by those compounds, which directly or indirectly lead to cell aggregation.

The effect of the active compounds could have been caused also by the triggering of a set of events, which finally led to the excretion of bivalent cations (probably ca^{2+}) that are known to have a direct role in flocculation (Startford, 1989).

Conclusion

The efficiency of the osidic fraction F1 of the Ethanol Precipitate of Trumpf malt, which is the malt that appears to be the efficient on flocculation of *S. uvarum* is probably due to the ester-bonds between sugars and phenolic acids. This hypothesis is confirmed by the results obtained with addition of EP after saponification and enzymatic treatment. Indeed, trypsin is an enzyme which contains esterasic activity, and esterase leads to the loss of flocculation power, while the other enzymes do not show any effect on flocculation power.

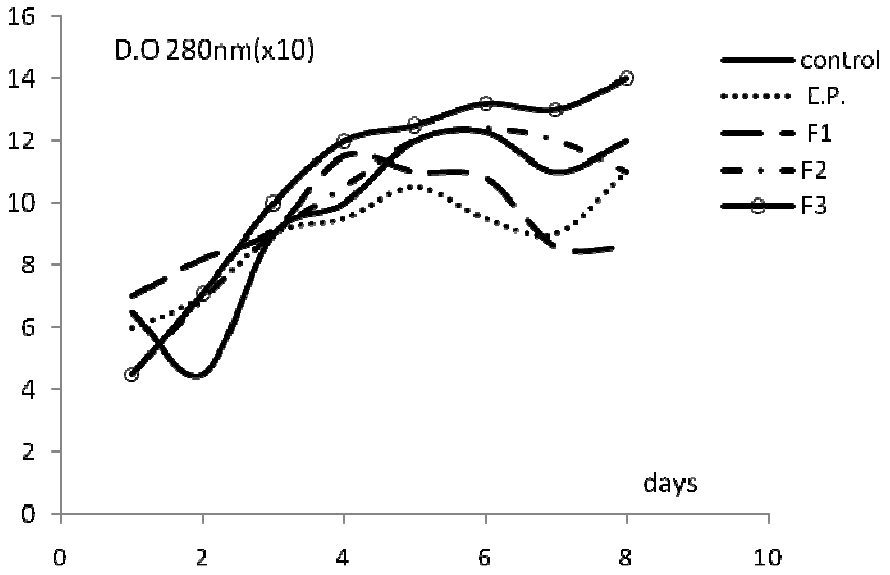

Figure 6. Effect of different fractions of Trumpf malt's EP on flocculation of S. *uvarum*.

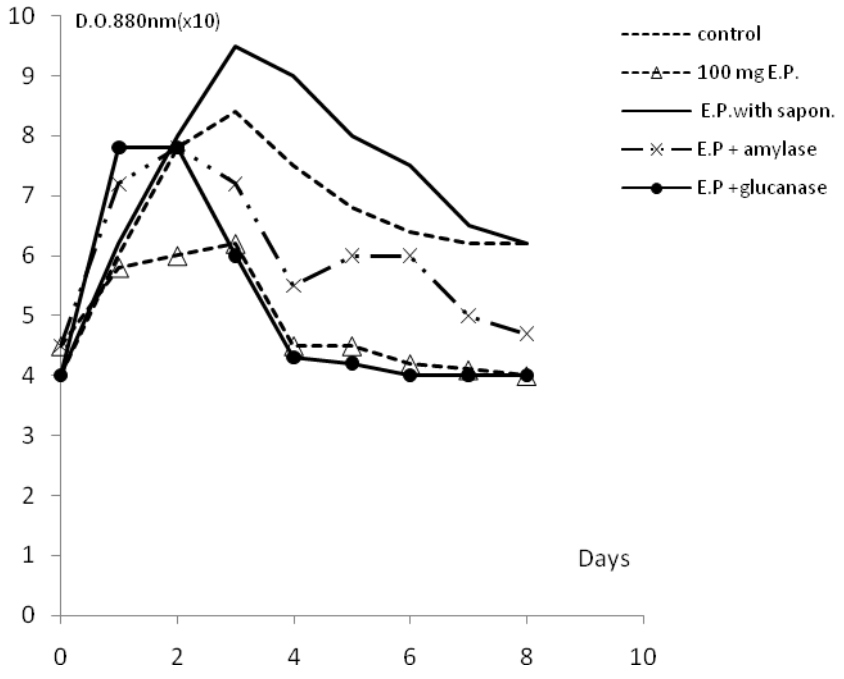

Figure 7. Effect of different treatments of trumpf malt's EP on flocculation induction of *S. uvarum.*

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