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Evaluation of Effective Parameters on Enzymatic Hydrolysis at Low Starch Concentrations

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Abstract. Enzymatic hydrolysis of starch is one of the most important enzymatic technologies nowadays, leading to important amounts of sweeteners in food industry. It is accomplished at industrial scale using high starch concentrations; the present study deals with a less explored pathway, the one of low starch concentrations, originating from the residual starch in waste waters. The effective parameters of enzymatic starch hydrolysis were studied at laboratory scale, using a commercial enzymatic product (Dextrozyme® GA); experiments were carried out using low concentration starch systems, which were hydrolyzed monitoring the influence of pH, temperature, starch concentration and mixing rate on the reaction rate. Enzymatic reactions were monitored using high performance liquid chromatography, with a system enabling the quantification of glucose, fructose, saccharose and maltose. A complete factorial design at two levels was applied, the measured system's response being the reaction rate. The obtained data demonstrated that the most important effect on saccharification is due to the pH, this being followed by starch concentration, temperature and mixing rate. Temperature, starch concentration and mixing rate are positively related with the reaction rate, while pH is inversely related with this. Analysis on the parameters' interactions revealed that the most important interaction is the one between temperature and pH, followed by concentration-temperature and concentration-pH, while the combined interaction concentration-temperature-pH-mixing rate can be considered negligible.

Keywords: starch, hydrolysis, enzyme, HPLC.

INTRODUCTION

Starch is one of the most important raw materials in food industry, originating from sources like potatoes, maize, wheat or tapioca. Its' industrial applications are diverse: cosmetics, adhesives, food products, paper industry, pharmaceuticals, textile industry, detergents, plastics, etc (Ellis et el., 1998). Starch can be modified by thermal, chemical or enzymatic treatment; from these, enzymatic treatment is by far the most important one, hydrolases being used to break glucosidic bonds (Aehle, 2007; Guzman-Maldonado and Pardes-Lopez, 1995; Othmer et al., 2005; Tharanathan, 2005).

Enzymatic hydrolysis leads to a wide range of products, depending of the enzymes and the reaction conditions (Bravo-Rodriguez, 2006; Fullbrook, 1984; Kennedy et al., 1988): maltodextrins (used as texture provider in food products, ingredients for added nutritional value, carrier or bulk agents, etc.), high maltose syrup (used in brewing industry, in confectionery industry for production hard sweets, frozen deserts, etc.), high dextrose

equivalent syrups (used in brewing and fermentation industries and in food products such soft drinks, jams, sauces, etc.) and high glucose syrups (used in brewing and fermentation industries, in beverages, as a raw material in producing D-glucose or fructose syrup, etc.).

Enzymatic hydrolysis of starch involves usually three main steps: gelatinisation, liquefaction and saccharification. Gelatinisation increases the substrate accessibility, enhancing thus the hydrolysis rate, while liquefaction is the first stage of enzymatic starch hydrolysis, being in fact a partial hydrolysis which leads to a decrease in viscosity of the gelatinised starch. During the saccharification stage, the partially hydrolysed starch chains are further broken to dextrins, higher oligomers, maltotriose, maltose and finally glucose, the properties of the end product being determined by the enzyme type used (Van der Maarel et al., 2002). Starch hydrolysates are usually characterized by their dextrose equivalent (DE), which can be considered a measure of the hydrolysed glucosidic bonds' amount (Chaplin and Bucke, 1990); thus, after liquefaction, the formed maltodextrins have between 10 and 30 DE, while after saccharification the products varies between 40 and 98 DE.

Industrial starch enzymatic hydrolysis is usually carried out at high starch concentrations, being an intensive-studied process due to its importance. The present paper deals with a less studied pathway, the one of low starch concentrations, specific for residual starch fractions which can be found in residual waters resulted from some processes in food industry; it presents some of the data obtained in a larger project's framework, dedicated to recovery and conversion of residual starch from waste waters originating from food industry.

MATERIALS AND METHODS

Lab-scale starch hydrolysis was achieved using a 1000 ml glass round – bottom flask and a Heidolph MR Hei-Stand thermostated magnetic stirrer; the process includes preparation of starch gell, liquefaction, pH adjustment, enzyme addition and heating under stirring. Starch gells of 2% and 6% were prepared by homogenizing the necessary weighed starch amounts (Merck) in 100 mL cold water, then adding 800 mL hot water, while stirring; the obtained system was brought to a final volume of 1000 mL in a graduated flask with distiiled water. Liquefaction was accomplished in 2000 mL round bottom glass flasks, in which starch gells were introduced, together with a phosphate buffer, up to a final pH of 6; after the addition of 0.2 g $CaCl_2$ (Merck), the glass flask was introduced in the magnetic stirrer thermostated at 105^0C. 5 ml Thermamyl® (Novozymes - Austria) were then added and the liquefaction was carried out for two hours. As two levels of pH were tested (4.5 and 6.5), the pH adjustement was achieved using 250 mL Erlenmeyer flasks, with 100 mL liqueffied starch aliquotes, using each time 100 mL appropriate phosphate buffers (Merck). For saccharification, Erlenmeyer flasks containing the working starch sollutions were heated up to the tested temperatures (56, 60 and 64^0C), then 500 μL Dextrozyme® GA (Novozymes) dilluted 1/ 250 were added and the chronometer was started; after 10, 30, 60, 90 and 120 minutes, 5 mL samples were removed from the reaction system, these being immediately subjected to HPLC analysis. The studied stirring rates were 100 to 500 rotations per minute.

High performance liquid chromatographic analysis (HPLC) was accomplished on a Shimadzu system, consisting from a Prominence LC-20AP solvent delivery module, a Prominence DGU 20As online degasser, an automatic sample injector SIL-10AF, a RID-10A differential refractive index detector, a Prominence CTO-20A column oven and a Prominence CBM-20A system controller. Instrument control, data acquisition and data analysis were accomplished by „LCsolution" ver.1.2. software. Isocratic separations were conducted at 40^0C,

with an EC 250/ 4 Nucleodur 100 – 5 NH_2 RP column (250 x 4.6 mm), using as mobile phase 77 : 23 v/ v % acetonitrile in water at 1 mL min^{-1} (Muntean, 2009; Muntean et al., 2009). Injection sample volumes were of 10 µL; the external standard method was used for quantifications. HPLC mobile phase was prepared from HPLC grade acetonitrile (Merck) and ultrapure water with a specific resistance of 18.2 MΩ□cm^{-1}, this bein also utilised for sample dilution; ultrapure water was obtained from a Direct Q 3UV Smart (Millipore). Mobile phase was filtered through a 0.45 µm membrane (Millipore), then degassed using an Elmasonic S30 H ultrasonic bath. Standard carbohydrate working calibration solutions were prepared from reagent-grades glucose, fructose, saccharose and maltose (Merck). Sample pre-treatment for HPLC analysis included enzyme inactivation with 1 mL HCl 1N, then a dillution with ultrapure water in a 10 ml flask, homogenisation and filtration through a 0.47 µm membrane filter (Millipore) in 2 mL vials, which were then introduced in the HPLC's autoinjector, being immediately analysed.

Data processing was accomplished using MatLab (The Mathworks Inc., USA).

RESULTS AND DISCUSSIONS

A two level factorial design focussed on the saccharification step was applied, the considered levels for effective parameters being mentioned in "Materials and methods' section"; a supplementary central point was assigned for temperature. The monitored response was the reaction rate, this being calculated from the slopes of the regression lines corresponding to glucose concentrations' evolutions during hydrolytic processes (Tab. 1). The linearity of these regressions was very good in all cases, with correlations coefficients greater than 0.977.

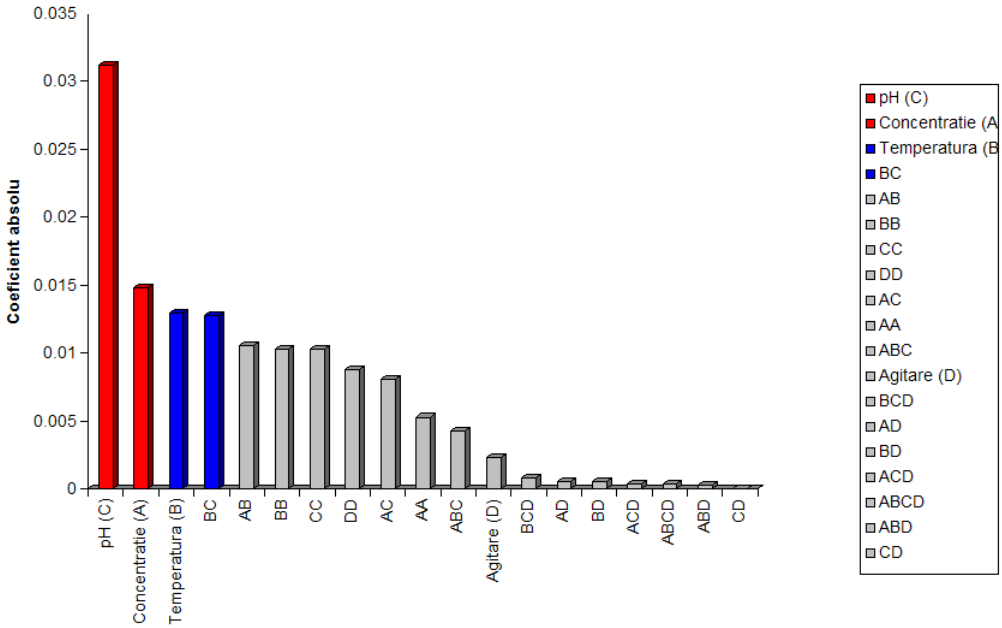

Fig 1. Paretto histogram corresponding the regression analysis of experimental data

Analysing the recorded responses, one can conclude the maximum influence on data variability is due to pH, this being twice higher than those corresponding to temperature and concentration, while the smallest effect is due to mixing. The Paretto histogram from figure 1 describes best the complex effects of the studied parameters on the enzymatic reaction rate,

revealing significative interactions for temperature – pH (the most important one), concentration-pH, concentration-temperature, even for the third order interaction concentration-temperature-pH; the quadratic effects for temperature, pH, concentration and mixing are also significative.

Tab.1.

Experimental matrix with the recorded responses for the two levels factorial experiments

Experiment no.	Starch concentration [%]	Temperature [^{0}C]	pH	Stirring [rpm]	Reaction rate [mol/ min]
1	2	56	4.5	100	0.227
2	2	56	4.5	500	0.231
3	2	56	5	100	0.172
4	2	56	5	500	0.178
5	2	56	5.5	100	0.173
6	2	56	5.5	500	0.180
7	2	60	4.5	100	0.205
8	2	60	4.5	500	0.220
9	2	60	5	100	0.219
10	2	60	5	500	0.232
11	2	60	5.5	100	0.163
12	2	60	5.5	500	0.169
13	2	64	4.5	100	0.266
14	2	64	4.5	500	0.268
15	2	64	5	100	0.193
16	2	64	5	500	0.110
17	2	64	5.5	100	0.145
18	2	64	5.5	500	0.147
19	6	56	4.5	100	0.229
20	6	56	4.5	500	0.234
21	6	56	5	100	0.227
22	6	56	5	500	0.228
23	6	56	5.5	100	0.190
24	6	56	5.5	500	0.198
25	6	60	4.5	100	0.279
26	6	60	4.5	500	0.288
27	6	60	5	100	0.257
28	6	60	5	500	0.268
29	6	60	5.5	100	0.305
30	6	60	5.5	500	0.316
31	6	64	4.5	100	0.290
32	6	64	4.5	500	0.299
33	6	64	5	100	0.257
34	6	64	5	500	0.261
35	6	64	5.5	100	0.222
36	6	64	5.5	500	0.224

pH has a maximum influence on enzymatic activity, acting both alone and in interaction with temperature, starch concentration and mixing; its' contribution is also important as a

quadratic term. pH is negatively related with the reaction rate, while the interactions in which pH is involved are positively related with this.

In a smaller extent, starch concentration and temperature influences the reaction rate, the enzymatic activity being positively correlated with these parameters. The reaction rate increases with the increase in the substrate concentration, this dependence being illustrated by the response surface from figure 2. The residual starch concentration in waste water is never as high as the one from dedicated industrial processes; in fact, there is an unexploited potential of the enzyme at the experimented concentrations, this being far from saturation. Interactions concentration-temperature, concentration-pH and even concentration-temperature-pH and concentration-concentration are significative, so the concentration effect is a complex one.

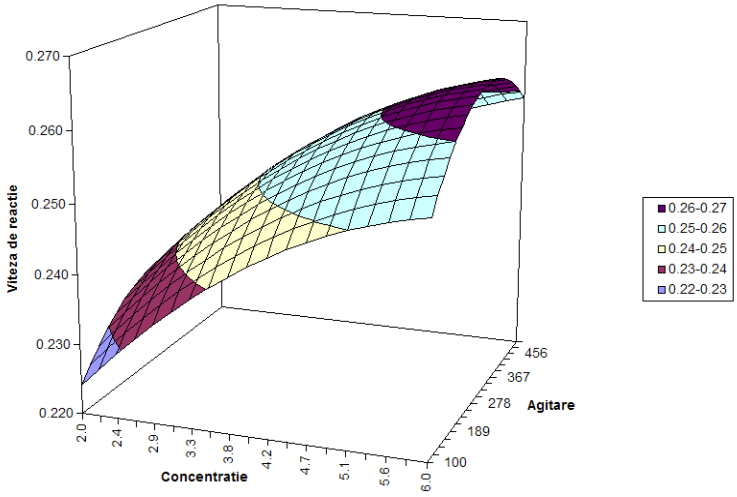

Fig.2: Response surface reaction rate – concentration - mixing

Temperature has a similar effect with concentration; increasing temperature causes increasing of the reaction rate on the experimental domain; however, a higher temperature means a higher energy consumption, while an exaggerated increase in temperature can lead to the enzyme degradation.

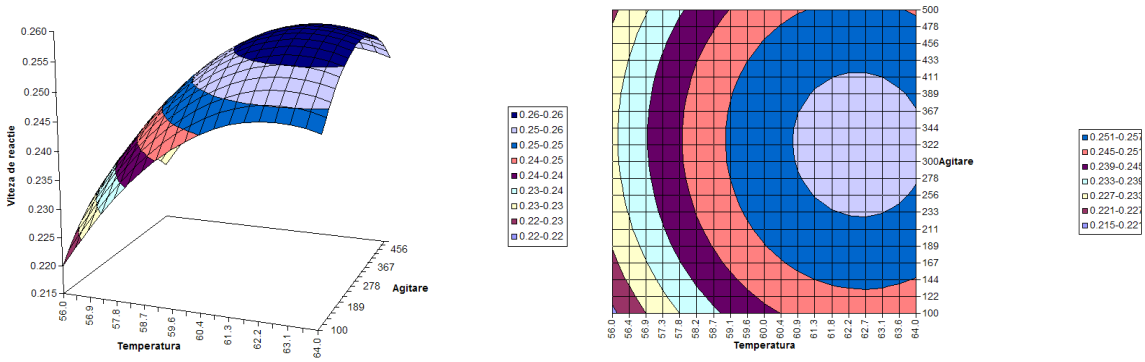

Fig.3: Response surface reaction rate – temperature – mixing and the corresponding contour plot

Mixing rate alone has a small influence on reaction rate, while the interactions in which this is involved are not significative; the importance of the quadratic term is however bigger.

The response surface and the contour plot from figure 3 shows clearly the domain in which the hydrolytic process has a maximum efficiency, this being between 61 – 63^{0}C and 300 – 340 rotations per minute.

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