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MODELING ISOLATION OF AMARANTH PROTEIN BY ENZYMATIC BREAKDOWN OF POLYSACCHARIDES

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ABSTRACT

This article deals with technology of biochemically separating proteins from amaranth. Technology we examined was enzymatic degradation of polysaccharides from amaranth flour in order to liquefy starch by hydrolysing it into soluble glucose, and to enrich the solid phase with vegetable protein. Three specific enzymes (amylase, glucoamylase and cellulase) were chosen to this purpose. Measured data were mathematically processed applying the mechanism of first-order kinetics in relation to non-decomposed starch. From processed experimental data it followed that the level of activation energy of starch hydrolysis is 9.7 x 10^4 J/mol and frequency factor is 29 min^{-1}. Separated amaranth components valuable for health (proteins, liquid proteins and sugars, oil, fibre) may serve to prepare functional foodstuffs and quality supplements, cosmetics, as an ingredient in animal feed or as a source of biological fertilisers.

Key words: Amaranth, Biochemical isolation, Enzyme; Protein, Starch.

INTRODUCTION

Amaranth grain, depending on strain, has very small dimensions (0.6 to 2.1 mm), low mass (approx. 1,000 seeds / g), and whitish to brown colour. Shape of grain is lenticular, under a tough husk is a sprout curled along the fringe about the plain of greatest circumference, and that takes up a third of grain volume and encircles the perisperm[1]. Reserve nutrients (lipids, proteins, polysaccharides, organic phosphates as well as a number of inorganic components) are not evenly stored in the grain. Proteins, bound to cells of embryo and endosperm, are stored in a membrane. The lipids are also found there. On the contrary, polysaccharides appear as starch in perisperm (seed) and are not found in endosperm[2]. Amaranth grain may yield as much as 18 % high-quality protein possessing a very well balanced composition of essential amino acids. Protein content in amaranth grains is higher, as opposed to proteins of current cereals. The higher content of lysine and tryptophane is comparable to that in animal proteins. As much as 65 % protein in amaranth is concentrated in the sprout. Protein content varies depending on amaranth species and cultivation conditions[3].

Starch content in amaranth ranges from 48 % (Amaranthus cruentus) to approx. 62 % (Amaranthus hypochondriacus). It was microscopically determined that minute grains of starch from amaranth grain are very small, of 1–3 μm diameter, angular polygonal shape. Starch bonds very strongly but is highly sensitive to action by amylases. Compared to wheat starch, it displays lower amylose content, lower swelling, higher solubility, greater water reception, lower and higher gelation temperature range. The very small size of minute starch grains and residual activity of amylases is probably responsible for recorded differences in swelling strength and solubility[4].

Amaranth grain contains about 3–6 % coarse fibre and as much as 15 % dietetic fibre. Thus, its fibre content is markedly greater as compared to other cereals. Dietetic fibre reduces cholesterol level, reduces the hazard of cancer of the colon and rectum. Coarse fibre is important for preventing and curing constipation. The recommended daily reception of fibre for adults is 30–40 g while the ratio of insoluble and soluble component should be 3:1[5].

Total fat content of cereal amaranth is 5.4–17.0 % containing almost 50 % linolenic acid. Fat contains 6–8 % squalene, which is a natural compound of isoprenoid type, a precursor in the synthesis of

steroids and important antioxidant compounds (coenzyme Q10). Squalene inhibits formation of oxygen radicals very efficiently and is a significant means of preventing tumorous diseases, brain damage or atherosclerosis[6, 7].

Kinetics of enzymatic reactions

Usual enzymatic reaction involves a case in which component *S* (substrate) reacts with component E_n (enzyme) in a homogeneous environment in such manner that component E_n is reclaimed, so that on termination of the reaction its concentration $e = e_i$ [8]:

$$S + E_n \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [E_nS] \xrightarrow{k_2} P + E_n \quad (1)$$

The rate at which final product (*P*) originates is expressed by relationship:

$$r = \frac{dp}{dt} = k_2 c \quad (2)$$

The rate of enzymatic reactions is governed by kinetics of saturation type, that is, rate of action first increases with growing concentration of substrate in almost linear manner, but at higher substrate concentrations the increase in rate is lower and reaction rate slowly approaches a maximum level (V_{max}). Such behaviour is brought about by the active centre of enzyme being able to process only a certain maximum quantity of substrate molecules in a unit of time. The rate of forming the product is directly proportional to concentration of complex E_nS:

$$V = K[E_nS] \quad (3)$$

Concentration of complex E_nS is a result of equilibrium, where dissociation constant is:

$$K_m = \frac{[E_n][S]}{[E_nS]} \quad (4)$$

Dissociation constant K_m depends on physico-mechanical parameters of the system, particularly on temperature and pH. At constant concentration of enzyme (as of catalyst that does not "participate" in the reaction nor is consumed therein), the resultant rate of product *P* yield is described by means of the well-known Michaelis-Menten equation[9]:

$$V = \frac{V_{max}[S]}{K_m + [S]} \quad (5)$$

Constant K_m in this relationship is designated the Michaelis-Menten constant; it is physically equal to substrate concentration at which enzymatic reaction rate *V* equals just half the maximum rate V_{max} which is the rate when enzyme is saturated with substrate. The numerical value of constant K_m expresses affinity of enzyme to given substrate. Low values of this constant signify that enzyme is highly specific to the given substrate and is already active at low substrate concentrations. In the opposite case, enzyme exhibits merely very low activity to the substrate. Values of constants V_{max} and K_m are always specific for a particular enzyme substrate couple and cannot be generalised and applied to other enzymes or substrates without experimental verification.

The first function of enzyme is reaction initiation. Molecules of reacting substances must be present in a certain minimum quantity in the medium for the reaction to proceed. When these molecules meet, start of the reaction requires a certain quantity of energy which is called activation energy; its magnitude depends on external conditions, particularly on temperature. The course of biochemical reactions is mostly much limited regarding high temperature because elevated temperatures could cause damage to reacting components. Around 20 °C, activation energy of many reactions is so high that the reaction might not proceed at all. Thanks to the catalytic effect of enzymes, activation energy may be much reduced; enzymes thus enable the reaction to carry on. Their further action, besides reducing activation energy, may be summarised as follows: Enzymes, similar to catalysts, act at minimum concentrations on the limits of threshold concentrations. They come from the reaction unchanged and unconsumed. Which, of course, does not mean enzymes remain in the medium at

unchanged concentration, because owing to their protein basis they undergo natural breakdown through the activity of bacteria. Enzymes exert no influence on the equilibrium position the chemical reaction arrives at (change in thermodynamic energy *G* is the same with a non-catalysed as well as catalysed reaction). Hence, enzymes make possible and accelerate a reaction, but do not shift equilibrium condition either to the side of substrate or product. Many enzymes start acting only under the effect of another agent, the so-called activator. Apart from chemical compounds, the role of activator may be played by such factors as temperature, pH or redox potential, that is, physical and chemical parameters of the environment[8].

Reactions leading to formation of an enzyme-substrate complex may be interfered with various chemical and physico-chemical influences generally contained under the term of inhibition. Prior to potential artificial application of enzymes in a system, a check thus has to be carried out on whether inhibitory influences will act against these enzymatic reactions[10].

Objective of work

Fractionation of amaranth flour has been performed so far by water extraction during which starch and protein are washed out together. Protein may be separated from starch only with great difficulty. However, amaranth protein contains a high proportion of essential amino acids and can be a significant component in functional foodstuffs (as special nutritive dietetic component); it is thus suitable to be concentrated. The aim of tests was to examine possibilities for separating proteins and starch of amaranth flour. The procedure we selected was enzymatic breakdown of polysaccharides to the purpose of liquefying starch by hydrolysing it into soluble glucose, and enriching the solid phase with vegetable protein. Three enzymes were selected to this aim (amylase, glucoamylase and cellulase), and dosed in a conveniently chosen ratio. The reason for applying several enzymes during the reaction was their high specificity, given by the typical three-dimensional structure of enzyme in which the active centre is found.

EXPERIMENTAL

Materials

Polarimeter Kruss P1000 (Germany) with polarimetric tube 200 mm, drier WTB Binder E/B 28 (Germany), shaft stirrer Heidolph RZR1 (Germany), electronic balance KERN 770/GS/GJ (Germany), water bath HGL W 16 (Germany), centrifuge WERK EBA 20 (Germany), mineralisation apparatus HACH Digesdahl (USA), muffle furnace Nabertherm L 9/S 27 (Germany), Parnas-Wagner distillation apparatus, Soxhlet extractor.

Amaranth flour was supplied by the AMR Amaranth Company (Hradec Kralove, The Czech Republic); its composition is presented in Tab. 1.

Analytical methods

Dry matter was determined by drying a weighed quantity of sample in glass weighing bottle at 103±2 ^{o}C for 12 hours and weighing after cooling. Inorganic solid was determined by carefully incinerating a sample of flour in a ceramic crucible over a gas burner and then by annealing at 600 ^{o}C in a muffle furnace and weighing after cooling. Total Kjeldahl nitrogen was determined by mineralising a sample of flour by boiling for 30 min (at approx. 440 ^{o}C) in sulphuric acid with added catalyst. Nitrogenous substances were thus transformed into ammonium sulphate from which ammonia was released in an alkaline environment, then steam distilled and determined by titration. Coarse proteins were determined by multiplying nitrogen content by conversion factor 5.70[11]. Fat was extracted from the flour sample with n-hexane in a Soxhlet extraction apparatus for 4 hours. After distilling off the solvent and drying the flask containing fat for 1 hour and cooling, fat content was determined by gravimetry. Starch content was determined according to ISO 10520:1997[12]. This determination is based on transforming starch into soluble starch by action of diluted HCl while warm. After clarification, soluble starch is determined by polarimetry. The method for determining fibre consists in eliminating accompanying substances from the sample by hydrolysis in an acid and alkaline medium; after a 90 min hydrolysis in 1.25 % H_2SO_4, the undissolved solid fraction was washed with water and hydrolysed for another 90 min in 1.25 % KOH. Non-hydrolysed residue (fibre), after washing with water and drying at 103±2 ^{o}C for 6 hours, was weighed[13].

Enzymatic breakdown of polysaccharides

Liquefying starch into soluble glucose used a combination of 3 specific enzymes (from Novozymes A/S, Bagsvaerd, Denmark): BAN 480 L (α-amylase), AMG 300 L (glucoamylase), CELLUCLAST 1.51 FG (cellulase). These were mixed in ratio 4:3:3 in such manner that an enzyme stock solution

was prepared from concentrated solutions of enzymes: 2 ml BAN + 1.5 ml AMG + 1.5 ml CELLUCLAST and filled to the mark with water in a 500 ml volumetric flask. For enzymatic breakdown of polysaccharides from amaranth flour, the stock solution of enzymes was dosed in a quantity of 5 l / 1,000 kg of dry matter of flour. Amylases break down starch chains and glucoamylases break cross-linked starch segments. Cellulase exerts specific hydrolytic action on cellulose which is present in the shell of amaranth grain. This reaction is a kind of biochemical modification, as such it can lead to many functionalities depending on the extent of enzymatic hydrolysis; thereby various chain lengths that correspond to glucose (dextrose), maltose, oligosaccharides or polysaccharides may be obtained.

Kinetics of enzymatic breakdown of polysaccharides from amaranth flour was investigated at temperatures of 60, 70 and 80 °C under conditions that had been proposed already earlier and optimised to this purpose. Amaranth flour was mixed with water (at 22±2 °C) in a ratio of 1:20. Under laboratory conditions, 5 g flour dry matter was dosed into a 250 ml boiling flask and 100 ml distilled water was added. The flask containing mixture was put into a water bath and shaft stirrer set into motion (600 rpm), with simultaneous heating at a rate of 1.5 °C min^{-1}. On attaining the desired temperature, 2.5 ml stock solution of enzyme was added (corresponding to a dose of 5 l per 1,000 kg flour dry matter, or 25 µl enzyme / 5 g flour dry matter). When enzymatic breakdown was over, the mixture was centrifuged (10 min) at a rate of 6,000 rpm. The liquid fraction and solid phase were separated and dried at 103±2 °C to constant mass. Starch content was determined from the dry matter of liquid and solid fraction. A scheme indicating complex processing of amaranth grain and separation of its components is shown in Fig. 1.

RESULTS AND DISCUSSION

Enzymatic breakdown of polysaccharides from amaranth flour

Measured experimental data were mathematically processed according to mechanics of first-order kinetics related to non-decomposed starch. Assuming the rate of conversion degree *(y)* is directly proportional to fraction of non-decomposed starch, we may write the differential equation as follows:

$$\frac{dy}{d\tau} = k(1-y) \tag{6}$$

Integration of equation (6) gives:

$$-\ln(1-y) = k\tau \tag{7}$$

Results of experimental data on enzymatic breakdown of polysaccharides from amaranth flour at temperatures of 60, 70 and 80 °C are presented in Tab. 2.

Plotting the natural logarithm of unreacted fraction: $-\ln(1-y) = k\,\tau$ against time (τ) gives a straight line whose gradient is equal to rate constant of starch hydrolysis (k). Fig. 2 shows experimental data processed in this manner at temperatures of 60, 70 and 80 °C.

Evaluation of activation energy (E) and frequency factor (A) assumes validity of the Arrhenius equation:

$$k = Ae^{-\frac{E}{RT}} \tag{8}$$

Integrating relationship (8) gives:

$$\ln k = \ln A - \frac{E}{R}\frac{1}{T} \tag{9}$$

An estimate of activation energy and frequency factor was made from evaluated rate constants (k) at three different temperatures (60, 70 and 80 °C). Rate constants were determined from line gradients. Tab. 3 summarises results of kinetic measurements.

Plotting the natural logarithm of rate constant ($ln\ k$) against inverse value of absolute temperature ($1/T$) produces a straight line whose gradient enables to determine activation energy, and section on axis of ordinates to determine the value of frequency factor (see Fig. 3).

$$-\frac{E}{R} = k \tag{10}$$

From processed experimental data it follows that level of activation energy for hydrolysis of starch E = 9.714 x 10^4 J/mol, and frequency factor A = 28.967 min^{-1}.

Proposal of measuring system

Enzymatic breakdown of starches may be monitored using a measuring system of fermentors. Studying the process has to focus on determining its optimum conditions for projecting a technological procedure isolating amaranth protein. We propose a measuring system consisting of two identical fermentors with identical system of probes and other auxiliary equipment. Fermentor A serves for reactions of a reference medium, and fermentor B for reactions of the medium under evaluation. Fermentors have to be equipped with stirrers incorporated in a vessel bottom. We propose that heating bodies should be placed along the perimeter in the bottom part of fermentor bodies. An outlet valve is likewise to be situated in the bottom part. Fermentor lids have to be designed so that the vessels can be hermetically closed, with three gas-tight apertures for probes, with a gas exhaust aperture and filling device. A computer-controlled measuring system with further complementary sensors. This system comprises a unit capable of measuring principal physical and chemical quantities. The instrumental part should be equipped with galvanically separated measuring inputs, logical inputs and a continuous control output. A sensor can be attached through every measuring input, and measuring input should enable conversion into analogue inputs. An analogue measuring input may then be set as a voltage input (for example, 0–10 V) or current intensity input (for example, 0–20 mA). Proposed connections of measuring instruments are described in Fig. 4.

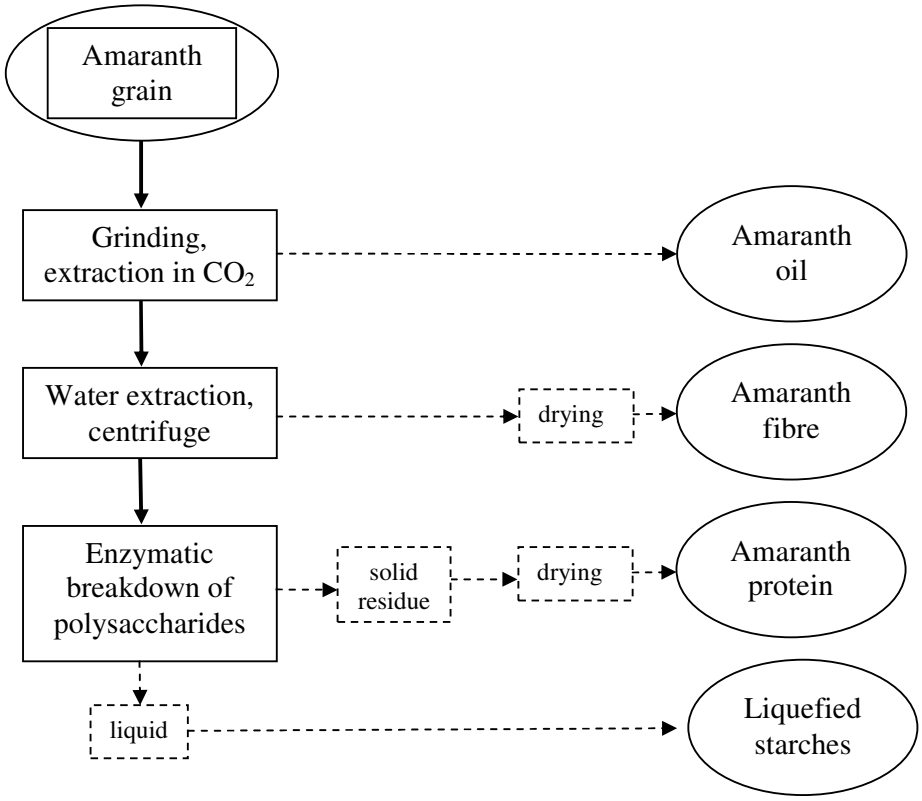

Fig.-1: Technology for processing amaranth grain.

CONCLUSIONS

This study verified a procedure for separating amaranth protein. The selected procedure was biochemical, consisting in employment of enzymes acting specifically on breakdown of polysaccharides into simple water-soluble sugars (glucose). Non-decomposed protein was then separated. Enzymatic hydrolysis of starch was investigated at temperatures of 60, 70 and 80 ^{o}C, assuming a first-order mechanism of hydrolysis which is satisfactory up to approx. 50 % conversion. Rate constants were determined by evaluating experimental data. Their numerical values were as follows: $k = 1.7 \times 10^{-3}$ min^{-1} for 60 ^{o}C, $k = 1.0 \times 10^{-2}$ min^{-1} for 70 ^{o}C, and $k = 1.3 \times 10^{-2}$ min^{-1} for 80

^{o}C. Activation energy E = 9.714 x 10^4 J/mol was evaluated from the dependency of natural logarithm of rate constant (*ln k*) on inverse value of absolute temperature (*1/T*). Corresponding frequency factor was A = 28.967 min^{-1}. The proposed separation procedure, or concentration of proteins through enzymatic breakdown of starch into glucose, sets this technology onto a no-waste level. Separated amaranth protein may be effectively applied in the production of functional foodstuffs and quality supplements. Another field of application is animal feed because it helps, after forbidden use of meat-and-bone flour, to overcome the deficit in proteins. In addition, amaranth protein may also be combined with proteins from other sources (for example, collagen) and employed to prepare mixed products for other than food applications. A sugar glucose solution may be utilised for producing yeast biomass. The amaranth plant, due to its composition and qualities, has substantial food potential.

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Table-1: Composition of amaranth flour [a. in dry matter]

Parameter	Value (%)
Dry matter	86.91
Ash [a]	3.57
Total Kjeldahl nitrogen [a]	2.82
Coarse proteins (nitrogen x 5.70) [a]	16.07
Fat [a]	9.81
Starch [a]	65.79
Fibre [a]	4.85

Table-2: Enzymatic breakdown of polysaccharides from amaranth flour.

τ (min)	z (%)	y	1–y	–ln (1–y)
Enzymatic breakdown of polysaccharides at temperature of 60 ^{o}C				
10	2.5	0.025	0.975	0.025317808
20	4.1	0.041	0.959	0.041864204
30	5.8	0.058	0.942	0.059750004
40	7.0	0.070	0.930	0.072570693
50	8.1	0.081	0.919	0.084469157
60	13.6	0.136	0.864	0.146182510
100	15.5	0.155	0.845	0.168418652
Enzymatic breakdown of polysaccharides at temperature of 70 ^{o}C				
1	21.9	0.219	0.781	0.247180
3	25.6	0.256	0.744	0.295714
5	26.2	0.262	0.738	0.303811
10	26.6	0.266	0.734	0.309246
15	39.6	0.396	0.604	0.504181
35	49.0	0.490	0.510	0.673345
60	56.3	0.563	0.437	0.827822
Enzymatic breakdown of polysaccharides at temperature of 80 ^{o}C				
5	15.0	0.150	0.850	0.162518929
10	30.0	0.300	0.700	0.356674944
20	42.0	0.420	0.580	0.544727175
25	46.0	0.460	0.540	0.616186139
30	48.0	0.480	0.520	0.653926467
40	55.0	0.550	0.450	0.798507696
50	60.0	0.600	0.400	0.916290732
65	71.0	0.710	0.290	1.237874356
100	75.0	0.750	0.250	1.386294361

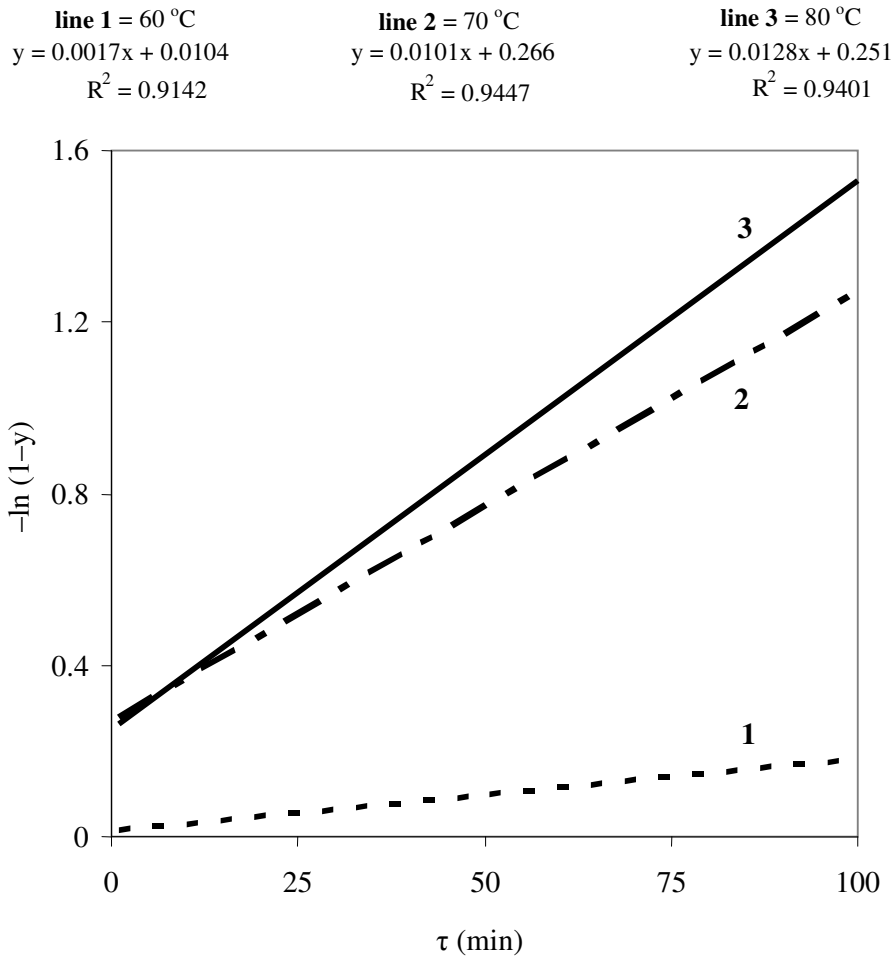


Fig.-2: Rate of enzymatic liquefying of starch to soluble glucose at temperatures of 60, 70 and 80 °C.

Table-3: Determining activation energy

t (°C)	T (K)	k (min^{-1})	ln k	1/T
60	333	0.0017	–6.37713	0.00300
70	343	0.0101	–4.59522	0.00292
80	353	0.0128	–4.35831	0.00283

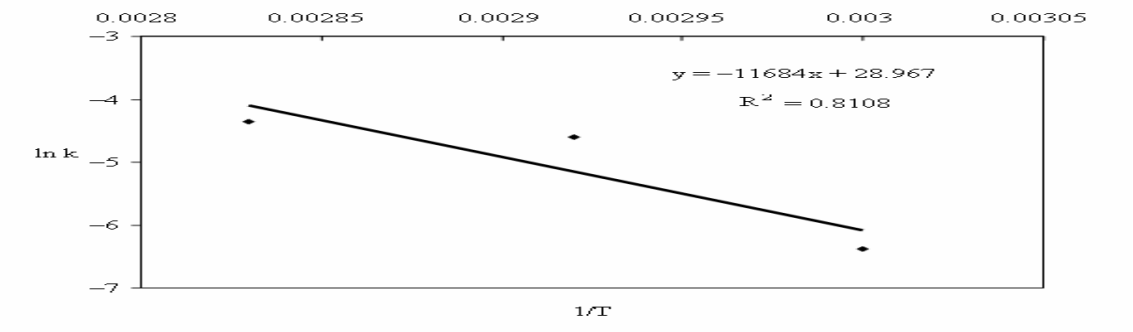
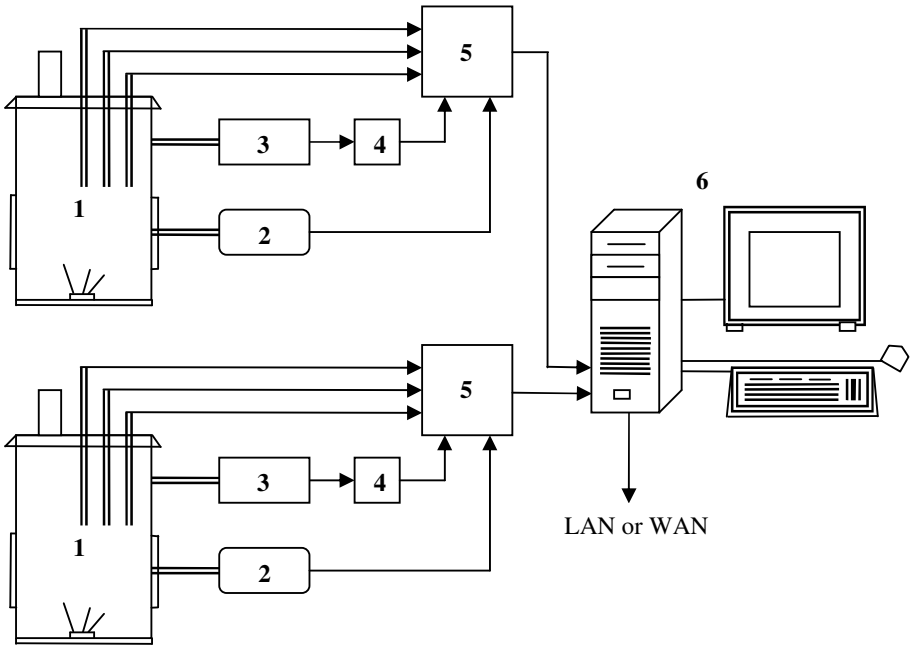


Fig.-3: Determining activation energy.



Legend: 1–fermenting tub, 2–heating control unit, 3–flow recorder, 4–analogue-digital convertor, 5–interface, 6–personal computer

Fig.-4: Scheme of measuring system.

Nomenclature

A	frequency factor
c	intermediate concentration $[E_nS]$
E	activation energy
E_n	enzyme
$[E_nS]$	reaction intermediate (enzyme-substrate complex)
k	rate constant (min^{-1})
k_1, k_{-1}, k_2	reaction rate constants
K_m	dissociation constant
p	product concentration
P	product
r	reaction rate of final product
R	molar gas constant (8.314 J/K.mol)
S	substrate
T	absolute temperature (K)
t	reaction temperature (^oC)
τ	time of enzymatic breakdown (min)
V	reaction rate
y	conversion degree
1–y	unreacted fraction
z	conversion of starch into glucose (%)

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