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# Continuous long-term hydrolysis of wheat gluten using a principally food-grade enzyme membrane reactor system

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ABSTRACT

The potential of the enzyme membrane reactor technology has been shown in several studies. In our study, we designed a principally food-grade continuous long-term hydrolysis process of wheat gluten in an enzyme membrane reactor with the proteolytic enzyme preparation Flavourzyme®. Among others, ethanol was the most suitable food-grade processing aid for avoiding contamination and showed high potential for application. The Flavourzyme®/wheat gluten process was comprehensively characterized in the enzyme membrane reactor. Critical factors for the enzyme stability were the temperature, pump stress and enzyme leakage through the membrane. The hydrolysis and process conditions were optimized to increase the space–time yield. Respective process parameters were chosen to obtain sufficient microbial, enzyme and process stability for the whole process time. The long-term hydrolysis was carried out in the presence of 8% (v/v) ethanol with a substrate concentration of 100 g $L^{-1}$ at 37 °C and pH 7.5 for 96 h. The continuous process resulted in stable product quality (degree of hydrolysis) and space–time yield (6.33 g $h^{-1}$ $L^{-1}$) over time. However, a discontinuous removal of accumulated dry matter was inevitable for the present enzyme membrane reactor process and was performed for 30 min every 24 h. Due to the fact that the enzymes were reused, the enzyme productivity could be increased by 450% compared to a reference batch process.



## 1. Introduction

Cereal grain protein hydrolysates are versatile food commodities and play a major role in the food additive industry. Depending on the degree of hydrolysis (DH), these hydrolysates can be used for emulsification, gelatinization or as seasoning [1]. Wheat gluten powder contains up to 85% protein, is highly available [2] and its hydrolysates are already used in a wide range of culinary products [3–6]. For these applications, the desirable product should have a high DH [1]. The industrial biotransformation of proteins, nowadays, is mainly performed in discontinuous batch processes [7]. However, the use of an enzyme membrane reactor (EMR) for a continuous biotransformation is promising [8] and features various advantages. The enzymes are located in a reaction space, entrapped by a membrane and, thus, can be reused, which is a significant economical benefit [9]. Additionally, hydrolysates standardized with a molecular weight cut-off (MWCO) can be produced at high and constant space–time yields (STYs) [10]. The selective removal of products from the reaction site can also increase the conversion of product-inhibited or thermodynamically unfavorable reactions [8]. As a result, process efficiency and enzyme productivity can be increased compared to the classical batch processes in the case enzyme stability is sufficient and the lifecycle time satisfactory.

Membrane technology has been successfully used in the production of protein fractions with specific functional properties [11,2,12,13] and for the continuous production of protein hydrolysates in laboratory scale in an EMR [14–24]. However, microbial stability is not often mentioned or discussed in literature during long-term (>10 h) continuous hydrolysis [15,20] or non-food-grade additives, such as sodium azide, were used [14]. The microbial stability of a protein biotransformation is crucial,

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**Abbreviations**

| | |
|---|---|
| ACU | azocasein unit (endopeptidase activity) |
| DH | degree of hydrolysis [%] |
| DM | dry matter |
| EA | enzyme activity |
| EMR | enzyme membrane reactor |
| $g$ | constant of gravitation |
| $h$ | concentration of free amino groups [mol $L^{-1}$] |
| $h_{tot}$ | theoretical concentration of free amino groups at complete hydrolysis [mol $L^{-1}$] |
| $J$ | membrane flux [L $h^{-1}$] |
| $J_N$ | normalized flux [L $h^{-1}$ $m^{-2}$] |
| $k_T$ | temperature-dependent inactivation rate [$h^{-1}$] |
| $k_P$ | pump stress-dependent inactivation rate [$h^{-1}$] |
| $M$ | molecular weight [g $mol^{-1}$] |
| MWCO | molecular weight cut-off |
| $nkat_{glutenTNBS}$ | total proteolytic activity |
| $nkat_{H\text{-}Leu\text{-}pNA}$ | exopeptidase activity |
| *p*NA | *para*-nitroanilide |
| STY | space–time yield [g $h^{-1}$ $L^{-1}$] |
| TNBS | 2,4,6-trinitrobenzene sulfonic acid |
| $V$ | reactor volume [L] |

especially at moderate process conditions, and cannot be taken for granted.

In this study, an approach for a long-term hydrolysis with a principally food-grade EMR system was carried out to achieve a stable process with a high enzyme productivity and a constant STY over 96 h. The industrial peptidase preparation Flavourzyme®, which contains endo- and exopeptidase activities [18], was used for the wheat gluten hydrolysis. Factors affecting the operating stability of the wheat gluten enzymatic hydrolysis process, including microbial stability and enzyme stability inside the EMR, were identified and considered for the continuous process design.

## 2. Materials and methods

### 2.1. Materials and chemicals

Wheat gluten and Flavourzyme® 1000 L (Novozymes) were obtained from Nestlé Product Technology Centre (Singen, Germany). The 2,4,6-trinitrobenzene sulfonic acid (TNBS) and the azocasein were bought from Sigma (Taufkirchen, Germany). The exopeptidase substrate H-Leu-*p*NA was obtained from Bachem (Bubendorf, Switzerland). Ethanol was obtained from the department of Yeast Genetics and Fermentation Technology (150f) at the University of Hohenheim (Stuttgart, Germany).

### 2.2. Analytical methods

#### 2.2.1. Determination of amino groups with 2,4,6-trinitrobenzene sulfonic acid

Primary amino groups were determined after derivatization with TNBS according to the method of [25] with some modifications. A volume of 7.5 μL of the sample was transferred into a microtiter plate and sodium phosphate buffer (0.2125 M, 60 μL) and TNBS reagent [0.1% (w/v) in $H_2O$, 60 μL] were added. The plate was incubated at 60 °C for 10 min, HCl (0.1 M, 120 μL) was added afterwards and the absorbance was measured at 340 nm using a microtiter plate reader (Multiskan FC). The calibration was performed using L-leucine as a reference.

#### 2.2.2. Degree of hydrolysis

The DH was calculated according to [25] with modifications (Eq. (1)).

$$DH = \frac{h}{h_{tot}} \times 100 \quad [\%] \tag{1}$$

where $h$ is the concentration of free amino groups [mol $L^{-1}$; see Section 2.2.1] and $h_{tot}$ is the maximum concentration of free amino groups at complete hydrolysis [mol $L^{-1}$] calculated according to Eq. (2).

$$h_{tot} = \frac{c_{Protein}}{M^* - M_{H_2O}} \quad [mol L^{-1}] \tag{2}$$

where $c_{Protein}$ is the concentration of protein which is hydrolyzed (g $L^{-1}$) and $M^*$ is the average molecular mass of the amino acids in wheat gluten (133.5 g $mol^{-1}$). This gluten-specific average molecular mass was calculated by considering the wheat gluten amino acid composition [26]. The molecular mass of water ($M_{H_2O}$ = 18 g $mol^{-1}$) was subtracted due to the addition of water during the hydrolysis of a peptide bond.

#### 2.2.3. Total proteolytic activity of Flavourzyme® on wheat gluten

Wheat gluten was used as the substrate. For the substrate solution, 100 g $L^{-1}$ wheat gluten was suspended in the corresponding buffer and heated to 60 °C under stirring for at least 2 h and a sedimentation was carried out at 20 °C for 1 h. This supernatant was used for activity measurement and contained a soluble protein content of 2 g $L^{-1}$. The protein content was determined with the method of [27] and bovine serum albumin fraction V (BSA) as the reference. The standard assay contained 250 μL of substrate solution and was preincubated at 37 °C for 5 min. Then, the reaction was started with the addition of 50 μL appropriately diluted Flavourzyme® sample and was stopped by the addition of TCA (2 mol $L^{-1}$, 50 μL) and was centrifuged (20,000 × *g*, 4 °C, 5 min) afterwards. The supernatant (7.5 μL) was transferred into a microtiter plate and the TNBS assay was performed (see Section 2.2.1). One katal (kat) of total proteolytic activity was defined as the release of 1 mol L-leucine-equivalent amino groups per second at the defined assay conditions. Since, Flavourzyme® is known to contain exo- and endopeptidase activities both were also separately quantified (see below).

#### 2.2.4. Endopeptidase activity assay

The assay was performed according to the method of [28] with modifications. The reaction was carried out in 1.5 mL reaction tubes using a thermomixer (ThermoMixer comfort, Eppendorf, Hamburg, Germany). The substrate azocasein (2.5 g $L^{-1}$) was dissolved in Tris–HCl buffer (50 mmol $L^{-1}$, pH 7.5). The standard assay was performed as follows: the substrate solution (250 μL) was preincubated at 37 °C for 5 min. The reaction was started with the addition of an appropriately diluted Flavourzyme® sample (50 μL), stopped by the addition of trichloroacetic acid (TCA, 2 mol $L^{-1}$, 50 μL) and centrifuged (20,000 × *g*, 4 °C, 5 min, 5417 R, Eppendorf) afterwards. The supernatant (195 μL) was transferred into a microtiter plate, NaOH (1.5 mol $L^{-1}$, 50 μL) was added and the absorbance was measured at 450 nm using a microtiter plate reader (Multiskan FC, Thermo, Schwerte, Germany). One azocasein unit (ACU) was defined as the increase of one optical density unit per minute at 450 nm in 0.25 mol $L^{-1}$ NaOH.

#### 2.2.5. Exopeptidase activity assay

Exopeptidase activity was measured according to the method of [29] with modifications [30]. The standard assay contained 177 μL of Tris–HCl buffer (50 mmol $L^{-1}$, pH 7.5) and 50 μL appropriately diluted Flavourzyme® sample. Buffer and enzyme were preincubated at 37 °C for 5 min. The reaction was started with