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# Effects of Medium Composition and Process Parameters on the Production of Extracellular Inulinase by *Thermomyces lanuginosus*

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**Summary**

The production of extracellular inulinase by eighteen *Thermomyces lanuginosus* strains was screened, and strain IMI 140524 was selected for further studies. The effects of various carbon and nitrogen sources on inulinase activity were investigated, and the best ones were found to be Jerusalem artichoke extract and peptone at the optimum fraction of 1.8 and 0.6 %, respectively. Effects of medium composition and fermentation conditions were also determined for the production of extracellular inulinase. The addition of 1 % (by volume) Tween 80 into fermentation medium enhanced the secretion of extracellular inulinase. In bench-scale fermentor, the age and amount of inoculum were also optimized and they were determined to be 40-hour-old culture and 5.0 % (by volume), respectively. The initial pH of the medium was adjusted to 6.5 and no further pH control was needed. Optimal aeration and agitation were 0.75 L/min and 150 rpm, respectively.

*Key words*: inulinase, optimisation, thermophilic fungus, *Thermomyces lanuginosus*, response surface methodology

## Introduction

Inulin is naturally accumulated polysaccharide in the underground organs of chicory, dahlia and Jerusalem artichoke (*1*) and is regarded as a prominent candidate for use as a renewable carbohydrate source (*2*). Thus, inulin has recently received interest since it is relatively cheap and abundant source for the biotechnological production of fructooligosaccharides, high fructose syrups (*3*), ethanol (*4*) and acetone/buthanol mixture. These products are important ingredients in food or pharmaceutical industry. Fructooligosaccharides (FOS) are recognized as prebiotics and their positive effects on human health have been widely acknowledged (*5*). FOS can be produced by the partial hydrolysis of inulin-type polymers (*6*) using endo-inulinase (EC 3.2.1.7). Fructose can be obtained by acid hydrolysis of inulin, but the degradation of fructose at low pH causes the discolouration of the hydrolysates and the formation of by-products difructose anhydrides (*1,7*). Fructose syrup can also be produced from starch by enzymatic methods involving α-amylase, glucoamylase and glucose isomerase resulting in a mixture consisting of 42 % of fructose, 50 % of glucose and 8 % of oligosaccharides (*8*). To produce high-fructose syrup (95 %) for application in pharmaceutical industry, HPLC technique is used, which makes this method uneconomical. Alternative procedure is one-step hydrolysis of inulin using exoinulinase (EC 3.2.1.26), which yields about 95 % fructose syrup (*1,9*). Industrial inulin hydrolysis is carried out at 60 °C in order to prevent microbial contamination and also because it permits the use of higher inulin substrate concentration due to increased solubility. Thus, a thermostable inulinolytic enzyme that may

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be produced by haemophilic organisms would be expected to play an important role in the hydrolysis of inulin for food and pharmaceutical purposes (*10*). The thermophilic fungus *Thermomyces lanuginosus* has been reported to produce high levels of extracellular thermostable cellulose-free xylanase with broad pH optimum, when grown on cheap carbon source such as corn cob (*11*). This fungus is also reported to produce extracellular thermostable amylolytic enzymes (α-amylase and glucoamylase) when cultured on starch-based medium (*12–14*). Furthermore, the production of mannanases, pectinases (*15,16*), lipase (*17,18*), phytase (*19*) and α-galactosidase (*20–22*) has been investigated worldwide by several groups. So far, very few studies are available in literature related to the inulinase from *T. lanuginosus*.

The development of proper fermentation media is a necessary and important step in efficient utilization of fermentation technology for the production of biocatalysts. The conventional 'one-factor-at-a-time' approach is laborious and time consuming (*23,24*). Moreover, it seldom guarantees the determination of optimal conditions. These limitations of a single-factor optimization process can be overcome by using statistics-based approach (*25, 26*). Statistical experimental design techniques are very useful tools for the selection of nutrient, as they can provide statistical models that establish the interactions among the process parameters at various levels and calculate the optimal level of each parameter for a given target (*27*). The application of statistical experimental design techniques in fermentation process development may result in improved product yields, reduced process variability, closer confirmation of the output response to nominal and target requirements and reduced development time and overall costs (*28*). Response surface methodology (RSM) is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors and searching for the optimum conditions (*9,23*). It is a statistically designed experimental protocol in which several factors are simultaneously varied. In RSM, the experimental responses to the design of experiments (DOEs) are fitted to quadratic function. The number of successful applications of RSM suggests that second-order relation can reasonably approximate many of the fermentation systems (*2*).

In this study, screening of thermophilic fungus *Thermomyces lanuginosus* strains is carried out to produce inulinase. Moreover, medium composition and fermentation conditions have been optimized for the production of inulinase using the selected strain. Scaling-up technology for the production of inulinase was also adapted in laboratory fermentor.

## Materials and Methods

### Chemicals

Dahlia inulin and some other analytical reagents as well as standards (glucose, fructose, maltotriose, *etc.*) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Yeast powder soluble starch (YPSS) agar and potato dextrose agar (PDA) were from Merck KGaA (Darmstadt, Germany). All other chemicals were purchased either from Reanal (Budapest, Hungary) or other companies.

### *Thermomyces lanuginosus* strains

*Thermomyces lanuginosus* strains originated from various culture collections and some strains were kindly supplied by Dr M. K. Bhat (Institute of Food Research, Norwich, UK). The investigated strains are the following: IMI 084400/ATCC 22070, IMI 110803, IMI 158749, ATCC 38905, ATCC 46882, IMI 140524, IMI 096218, CBS 218.34, CBS 224.63, CBS 288.54, CBS 395.62, ATCC 28083, ATCC 16455, IMI 131010, ATCC 34626, ATCC 36350, ATCC 44008 (RM-B) and DSM 5826. The strains were maintained in YPSS agar (*29*), and stored under refrigeration.

### Enzyme activity assay

Inulinase activity was assayed by determination of reducing sugars released by hydrolysis of inulin substrate. Briefly, 0.5 mL of adequately diluted enzyme solution were incubated with 1 mL of 2 % (by mass per volume) inulin from dahlia tubers and 0.5 mL of 0.05 M sodium acetate buffer (pH=5.5) at 55 °C for 30 min (*6*). The reaction was stopped by boiling for 15 min. The reducing sugars released were determined by Somogyi-Nelson method (*30,31*) using calibration curve prepared with fructose as a standard. One unit of enzyme activity was defined as the amount of enzyme that releases 1 μmol of fructose equivalent per minute under the assay conditions.

### Determination of soluble carbohydrates

Soluble carbohydrates in the fermentation broth were determined by HPLC. Briefly, samples taken at regular intervals were firstly centrifuged at 14 000×*g* for 15 min. Then the supernatants were filtered through 0.45-μm membrane (Waters, Milford, MA, USA). The analyses were carried out using the Waters HPLC system consisting of W610 pump, Waters HPLC controller, 717 plus autosampler, and W410 refractive index (RI) detector. A thermostatically controlled column compartment set at 45 °C containing Aminex® HPX-87H (BioRad Laboratories, Hercules, CA, USA) ion exclusion column was used at a flow rate of 0.6 mL/min using 5 mM $H_2SO_4$ as the mobile phase. The data acquisition and integration were performed using the Waters Millennium™ v. 4.0 software package. Each sample was injected three times. Standards (internal and external) for sugars (glucose, fructose, sucrose and maltotriose as $DP_3$, maltotetraose as $DP_4$, inulin as DP>4) were used to identify and quantify the components in the samples. Internal standards were also applied to check the analysis method.

### Shake flask studies

A three-stage cultivation technique was used for fermentation/enzyme production. In the first stage, the fungus was grown on PDA slant agar (Merck) for 8 to 10 days at 47 °C in humidified incubator. In the second stage a suspension of conidia was prepared using 0.1 % Triton X-100 solution. A volume of 5 mL of it was added to 100 mL of glucose-asparagine medium (glucose 4 g, L-asparagine 0.4 g, $KH_2PO_4$ 0.3 g, $K_2HPO_4$ 0.2 g, $MgSO_4$·

$7H_2O$ 0.05 g and 0.1 mL of Vogel's trace element solution (*32*) in 100 mL of distilled water; pH adjusted to 6.0) to initiate the cultivation. Fermentation was carried out at 47 °C and 220 rpm in an orbital shaker for 1 to 2 days to obtain a homogeneous mycelium growth, unless otherwise specified. In the third stage, 10 mL of the mycelial suspension were used as inoculum for initiating the production of inulinase in 150 mL of basal medium containing (in g/L): dahlia inulin 40, L-asparagine 4, $KH_2PO_4$ 3, $K_2HPO_4$ 2, $MgSO_4·7H_2O$ 0.5 and 1 mL of Vogel's trace element solution (*32*). The flasks were then incubated at 47 °C and 220 rpm in an orbital shaker. Samples (10 mL) were taken under laminar box from triplicate flasks at various times. The samples were filtered and the cell--free fermentation broth was analysed.

The effects of carbon sources were investigated by replacing the dahlia inulin in the basal medium with different carbon sources such as Jerusalem artichoke extract (JAE), glucose, fructose, *etc*. Various nitrogen sources (peptone, beef extract, meat extract, yeast extract, corn steep liquor, sodium nitrate, ammonium sulphate, urea and ammonium chloride) were substituted with L-asparagine at a fraction of 0.5 % (by mass per volume) in the medium to study their effects on the secretion of extracellular inulinase. Response surface methodology (RSM) was used to optimise carbon and nitrogen fraction for maximal production of inulinase by the selected *T. lanuginosus* strain. Full second-order polynomial model was applied for description of inulinase activity depending on the change of factors. Effects of various surfactants on the secretion of extracellular inulinase were also investigated by the addition of different fractions into fermentation medium.

### *Fermentor studies*

The first two stages of the production of inulinase on a lab scale were similar to those described for shake flask above. Fermentation system (BioStat® B, Sartorius BBI Systems GmbH, Melsungen, Germany) consisted of stirred tank reactor with working volume of 2.2 L, integrated gas-mixing and controlling module, controller and desktop computer running MFCS/win v. 2.1 software package. The tank reactor was fitted with two Ruston--type impellers with six blades per each. Production medium was prepared and filled into the bench-scale reactor then autoclaved at 121.1 °C for 45 min. Effects of various process parameters (initial pH of the medium, incubation time, agitation, aeration, inoculum age and inoculum size) on inulinase production were studied. Fermentations were carried out at 47 °C.

### *Statistical analysis*

Results are the mean of triplicate trials. Calculation of standard deviation, analysis of variance (ANOVA) and other statistical probe tests were performed using SPSS software package for MS Windows v. 12 (IBM SPSS, Armonk, NY, USA). Experimental design (design and analysis) was carried out based on the Design and Analysis of Experiments (DOE) module in STATISTICA v. 9 (StatSoft Inc., Tulsa, OK, USA).

## Results and Discussion

### *Studies in shake flasks*

#### Screening of strains

Eighteen *T. lanuginosus* strains were screened for inulinase activity in extracellular fraction using dahlia inulin as inducer. All tested strains seem to be able to synthesise extracellular inulinase and their activities varied from 0.09 U/mL in the case of IMI 084400 strain up to 0.61 U/mL in the case of IMI 140524 strain. Three strains (ATCC 16455, ATCC 28083 and IMI 140524) exhibited better results (0.52, 0.54 and 0.61 U/mL, respectively) compared with other strains. Based on the screening results, the strain IMI 140524 was selected for further studies.

#### Time course of inulinase production

The change of inulinase activity during fermentation is summarized in Fig. 1. Soluble carbohydrate content decreased from 3.2 % (by mass per volume) to zero after 120 h of fermentation with *T. lanuginosus* IMI 140524 strain, while the pH of the medium increased to 7.8. Intensive fermentation process was observed in the first three days of fermentation, whereas inulinase activity reached maximum value (0.61 U/mL) at 48 h, before the onset of stationary phase. Al-Dagal and Bazaraa (*33*) also reported that inulinase synthesis from *K. marxianus* was growth-associated and reached the optimum near the stationary phase.

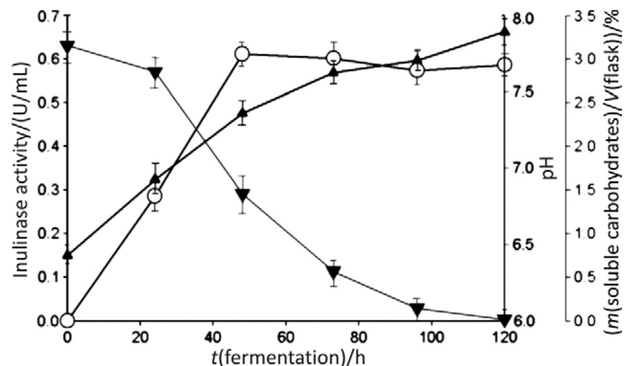


**Fig. 1.** Changes of pH (▲), inulinase activity (○) and the fraction of carbohydrates (▼) during fermentation with *Thermomyces lanuginosus* IMI 140524 strain. Data plotted are mean values of three parallel shake flask experiments

#### Effects of carbon sources

Effects of various carbon sources (dahlia inulin, Jerusalem artichoke extract, sucrose, glucose, starch, maltose, lactose and fructose) on the production of inulinase by *T. lanuginosus* IMI 140524 were studied. The order of inulinase activity was JAE>glucose>fructose>inulin>starch>lactose>maltose>sucrose (Fig. 2). About 1.6 times higher inulinase activity was assayed when JAE was used as substrate than in the case of inulin. JAE that contained fructose, glucose, sucrose, fructooligosaccharide and inulin seemed to be a good carbon source for both growth and inulinase production by *T. lanuginosus* IMI 140524 strain. Vandamme and Derycke (*1*) reported that microbial inulinases are usually inducible. Selvaku-

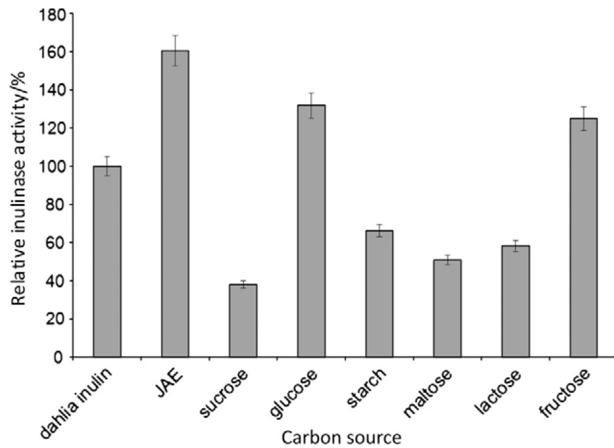


**Fig. 2.** Effects of carbon sources on inulinase production by *Thermomyces lanuginosus* IMI 140524 strain. Activity (0.6 U/mL) in the case of dahlia inulin was used as a reference (100 %). Data are mean values of triplicate shake flask experiments; JAE=Jerusalem artichoke extract

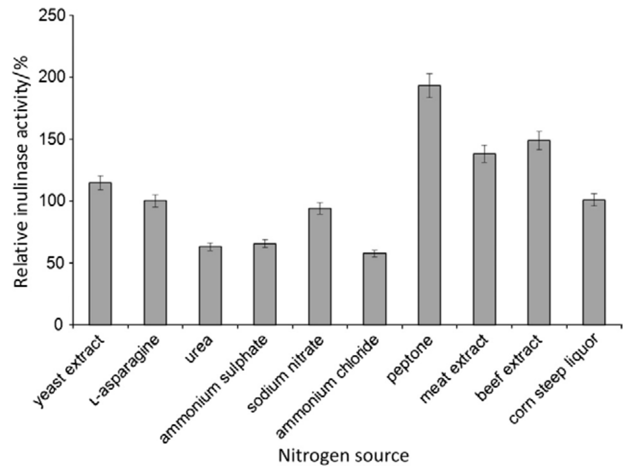


**Fig. 3.** Effects of nitrogen sources on the production of inulinase by *Thermomyces lanuginosus* IMI 140524 strain. Dahlia inulin was applied as the main carbon source. Activity (0.6 U/mL) in the case of L-asparagine was used as a reference (100 %). Data are mean values of triplicate shake flask experiments

mar and Pandey (*34*) found that the presence of inulin as a carbon source was essential for the synthesis of inulinase. Glucose and fructose are known as good growth substrates for *T. lanuginosus* (*23*), thus glucose was used as the main carbon source in the inoculum medium. Interestingly, *T. lanuginosus* IMI 140524 was able to secrete inulinase growing on glucose or fructose medium and better results were observed than when inducing it with dahlia inulin. This may indicate that *T. lanuginosus* IMI 140524 synthesises extracellular inulinase constitutively. Similar finding with α-galactosidase was also reported (*35*). When using sucrose as a carbon source, minimal inulinase activity (0.18 U/mL) was detected. Carbon compounds are the sources of carbon skeleton and they supply energy for microbial cells. Inulin is a relatively inexpensive and abundant substrate, thus, it can also be replaced in the industry by extracts of Jerusalem artichoke, chicory or dahlia.

## Effects of nitrogen sources

Based on the effects of nitrogen sources, inulinase activity can be ranked as follows: peptone>beef extract> meat extract>yeast extract>L-asparagine>corn steep liquor> sodium nitrate>ammonium sulphate>urea>ammonium chloride (Fig. 3). Generally, complex nitrogen sources were better than inorganic nitrogen sources. The best enzyme production (about 2 times higher than in the case of L-asparagine) was observed when using peptone. Meat extract has been reported as the best nitrogen source for the production of inulinase from *Chrysosporium pannorum* (*36*). Pork extract followed by beef extract were reported as good nitrogen sources for the synthesis of inulinase from *Kluyveromyces* sp. Y-85 (*37*). Gill *et al*. (*38*) found that yeast extract was the best nitrogen source for the production of inulinase by *Streptomyces* sp. Corn steep liquor, which is rich in amino acids, vitamins and other nutrients for the medium, is an important nitrogen source for inulinase production by yeasts such as *Kluyveromyces* S120 (*28*) or fungus such as *Aspergillus niger* AUP19 (*39*). In this study, it seems that corn steep liquor was not better than L-asparagine. Overall, peptone was selected as the main nitrogen source for further studies.

## Optimisation of the fraction of JAE and peptone by RSM

Based on the results of the effects of carbon and nitrogen sources on the production of inulinase by *T. lanuginosus* IMI 140524 strain, JAE and peptone were selected as the main medium components to be optimised. Central composite design (CCD) with full second-order polynomial model was selected. Numerous preliminary designs with different central points and steps were carried out and analyzed (data are not shown). The final design and the results of experiments are summarized in Table 1. Regression analysis was done by DOE in STATISTICA v. 9 and the following second-order regression model was obtained:

$$Y=3.10+1.71X_1-0.40X_1^2+4.78X_2-3.42X_2^2-0.39X_1\cdot X_2 \quad /1/$$

where $X_1$ is JAE fraction (% by mass per volume), $X_2$ is peptone fraction (% by mass per volume) and Y is inulinase activity (U/mL).

Table 1. The central composite design for the optimisation of Jerusalem artichoke extract (JAE) and peptone fractions

| Medium code | *m*(JAE)/*V*(medium) % | *m*(peptone)/*V*(medium) % | Inulinase activity U/mL |
|---|---|---|---|
| 1 | 2.5 | 0.9 | 5.48 |
| 2 | 2.5 | 0.1 | 5.24 |
| 3 | 0.5 | 0.1 | 4.70 |
| 4 | 0.5 | 0.9 | 5.57 |
| 5 | 2.9 | 0.5 | 5.76 |
| 6 | 1.5 | 0.0 | 4.49 |
| 7 | 0.1 | 0.5 | 4.28 |
| 8 | 1.5 | 1.1 | 5.08 |
| 9 | 1.5 | 0.5 | 6.05 |
| 10 | 1.5 | 0.5 | 6.09 |
| 11 | 1.5 | 0.5 | 6.02 |
| 12 | 1.5 | 0.5 | 5.89 |

Table 2. Analysis of the results of experimental design

| Factor | Effects | Standard error | Sum of squares (SS) | Degree of freedom | Mean square | *t* | F | p |
|---|---|---|---|---|---|---|---|---|
| $X_1$ | 0.63609 | 0.061294 | 0.8068 | 1 | 0.8068 | 10.3776 | 107.6956 | 0.001909 |
| $X_1^2$ | –0.79458 | 0.068528 | 1.0072 | 1 | 1.0072 | –11.5949 | 134.4418 | 0.001378 |
| $X_2$ | 0.61329 | 0.062961 | 0.7108 | 1 | 0.7108 | 9.7409 | 94.8843 | 0.002298 |
| $X_2^2$ | –1.09518 | 0.070621 | 1.8017 | 1 | 1.8017 | –15.5078 | 240.4922 | 0.000583 |
| $X_1 \cdot X_2$ | –0.31500 | 0.086554 | 0.0992 | 1 | 0.0992 | –3.6393 | 13.2447 | 0.035759 |
| lack-of-fit | | | 0.6586 | 3 | 0.2196 | | 29.3083 | 0.010073 |
| pure error | | | 0.0225 | 3 | 0.0075 | | | |
| total SS | | | 4.3866 | 11 | | | | |

$X_1$=Jerusalem artichoke extract (% by mass per volume), $X_2$=peptone (% by mass per volume)

The effect estimates, standard errors as well as the significant level of each medium variable were determined by *t*-test (Table 2). All effect estimates including interaction of both factors are statistically significant at 95 % or higher probability level. The ANOVA of the regression model demonstrates that Eq. 1 was a highly significant model (Table 2). The F-value (29.3083) of the lack-of-fit of the model with low p-value (0.010073) indicates the significance of the model at 95 % level model.

The model terms $X_1$, $X_2$, $X_1^2$ and $X_2^2$ were significant with a probability of 99 %; the interaction between two factors ($X_1 \cdot X_2$) was significant with a probability of 95 %. Three-dimensional graph was produced based on Eq. 1 by STATISTICA software to demonstrate the changes of inulinase activity depending on the fractions of JAE and peptone. Based on the regression model, the predicted maximum inulinase activity (6.09 U/mL) was obtained when JAE and peptone fractions were 1.8 and 0.6 %, respectively (Fig. 4).

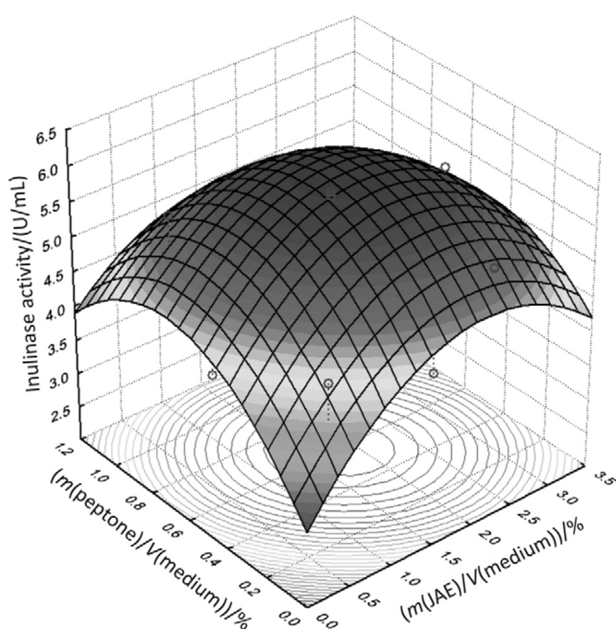


**Fig. 4.** Effect of Jerusalem artichoke extract (JAE) and peptone fractions on the production of inulinase by *Thermomyces lanuginosus* IMI 140524 strain

Validation of the experimental model

Inulinase production by *T. lanuginosus* IMI 140524 was examined during the submerged fermentation in shake flask applying the optimal medium (data not shown). The highest inulinase activity (obtained from five replications) was 6.15 U/mL at 48 h of fermentation, which was very close to the predicted value (6.09 U/mL with 5.96–6.22 U/mL at 95 % confidence interval). Thus, it can be concluded that the regression equation (Eq. 1) was proven to be adequate to describe the change of inulinase activity during fermentation.

Effects of surfactants on the secretion of extracellular inulinase

It is well known that surfactants dilate the pores by interaction with phospholipids located in the cell membrane, thus they affect its permeability (*40*). On the one hand, surfactants can increase the secretion of extracellular protein, while on the other hand, they may cause the death of living cells. Effects of supplementation of fermentation medium with individual surfactants at different fractions (0.01–1 % by mass per volume) on the secretion of extracellular inulinase by *T. lanuginosus* IMI 140524 strain were investigated. The addition of 1 % (by volume) Tween 80, 0.01 % (by mass per volume) sodium dodecyl sulphate or 0.01 % (by mass per volume) Triton X-100 to the fermentation medium increased inulinase activity from 6.12 (control sample) to 10.14, 8.60 and 7.25

Table 3. Effects of surfactants on the secretion of extracellular inulinase by *Thermomyces lanuginosus* IMI 140524 strain

| Surfactant | Inulinase activity |
|---|---|
| | U/mL |
| Tween 20 (1 % by volume) | 4.79±0.72 |
| Tween 40 (1 % by volume) | 5.58±0.45 |
| Tween 60 (1 % by volume) | 5.07±0.63 |
| Tween 65 (1 % by volume) | 5.46±0.46 |
| Tween 80 (1 % by volume) | 10.14±1.53 |
| Tween 85 (1 % by volume) | 5.19±0.81 |
| SDS (0.01 % by mass per volume) | 8.60±1.17 |
| Triton X-100 (0.01 % by mass per volume) | 7.25±0.96 |
| control | 6.12±0.31 |

U/mL, respectively (Table 3). Tween 80 is polyoxyethylene (20) sorbitan monooleate, where oleate is unsaturated fatty acid residue with one double linkage located at position 9–10 of the carbon chain. This linkage is supposed to play an important role in the formation of interaction with phospholipids that dilate the pores in the cell membrane. Interestingly, while the addition of Tween 80 enhanced the secretion of extracellular inulinase, no significant effects were observed in the case of Tween 85, which contains three unsaturated oleic acid residues. This means that the effects of polysorbates on the secretion of extracellular proteins depend on the nature of fatty acid as well as on the number of fatty acid residues. It was confirmed that there were not any significant effects on enzyme secretion detected when 0.01–1 % (by mass per volume) of Tween 20, Tween 40, Tween 60, Tween 65 and Tween 85 were supplemented into the fermentation medium. Our results with Tween 80 are in agreement with those reported by Arnesen *et al*. (*41*), who studied the production of α-amylase by *T. lanuginosus*. Interestingly, in the same study they found that the addition of Triton X-100 had an inhibitory effect on the growth of *T. lanuginosus* at all fractions (0.1–1.3 % by mass per volume). In the case of yeast *Kluyveromyces maxianus* YS-1, supplementation of 0.002 % sodium dodecyl sulphate (SDS) affected the secretion of inulinase, but no effect of the addition of 0.002 % Tween 80 or Triton X-100 was detected (*7*). Generally, interaction with SDS disrupts all non-covalent protein bonds causing the macromolecules to unfold. This mechanism is valid for both membrane and extracellular proteins including inulinase. High SDS fraction causes denaturation of inulinase, but at low fraction (0.01–0.001 %) in the fermentation broth it may cause only reversible unfolding of proteins or dilate pores in the cell membrane, enhancing the secretion of extracellular enzymes including inulinase. Another effect of Tween 80 on glucosyltransferase (GTase) synthesis was also reported by Wittenberger *et al.* (*42*). Basically, no extracellular GTase was detected in the medium, but the addition of Tween 80 stimulates the production of extracellular GTase by acting either directly or indirectly at the level of enzyme synthesis.

### *Studies in bench-scale fermentor*

#### Effect of initial pH of the medium

Due to the increase of pH of the medium during fermentation process (Fig. 1), its effect on the production of inulinase by *T. lanuginosus* was investigated. In the first experimental series, the pH of the fermentor was kept in a range from 4.5 up to 7.5 increasing stepwise by automatic controller of BioStat® B laboratory fermentor system. Unfortunately, no increase in enzyme production was detected and, moreover, lower enzyme activity was assayed when the pH was adjusted to 4.5, 5.0, 5.5 and 6.0 (data not shown). Hence, controlling pH did not give any good results; the effect of initial pH of the medium was investigated by preparing a fermentation medium with 50 mM phosphate buffer with different pH values (from 5.0 to 8.0). The best inulinase activity (12.92 U/mL) was assayed at 48 h when medium was prepared with phosphate buffer at pH=6.5. At pH=5.0 and 8.0, lower inulinase activity (8.33 and 3.27 U/mL, respectively) was observed. Interestingly, similar effect was also found in our previous report (*23*) related to the production of amylolytic enzyme by *T. lanuginosus* ATCC 34626 strain. This means that strictly controlled pH did not result in higher inulinase production. Other authors reported that optimal pH of the medium for the production of inulinase by *K. marxianus* should be 6.4–6.6 (*7, 43,44*). Selvakumar and Pandey (*34*) also found that initial pH of the medium for the production of inulinase by *Staphylococcus* sp. was pH=6.5.

#### Effects of inoculum size and age

Generally, spores of *T. lanuginosus* take about at least 24 h for growing, thus the effects of the age of inoculum on the production of inulinase were investigated by inoculating fermentation media with 24- to 62-hour-old cultures. Inoculum level was kept at 5.0 % (by volume). Maximum inulinase production (about 14.0 U/mL) was observed when 40- to 48-hour-old inoculum was applied. To investigate the effect of inoculum size, inulinase production was carried out by culturing media with different inoculum levels (2.5, 5.0, 7.5 and 10.0 % by volume). The obtained results showed that the best inulinase production was obtained by using 5.0 % (by volume) 40- to 48-hour-old inoculum. In this case after 48 h of fermentation, about 14.5 U/mL of enzyme activity was assayed (Table 4). Wei *et al*. (*45*) reported that 5.0 % (by volume) inoculum should be good for initiation of fermentation in 15-litre fermentor. Our results are in agreement with other published literature (*7,46*).

Table 4. Effects of inoculum age and size on the production of inulinase by *Thermomyces lanuginosus* IMI 140524

| Inoculum age<br>h | Inulinase activity<br>U/mL |
|---|---|
| 24 | 9.59±1.05 |
| 32 | 11.16±1.61 |
| 40 | 14.29±1.43 |
| 48 | 14.08±0.97 |
| 54 | 12.69±1.22 |
| 62 | 9.46±1.01 |
| **Inoculum size<br>% by volume** | |
| 2.5 | 10.52±0.93 |
| 5.0 | 14.45±1.38 |
| 7.5 | 13.52±1.02 |
| 10.0 | 12.42±1.17 |
| 12.5 | 11.86±1.18 |

#### Effects of agitation speed and aeration

Effects of shaking speed on inulinase production were preliminary studied in shake flasks (data not shown), but due to the difference in oxygen transfer mode as well as shearing, the effects of agitation (50, 100, 150, 200 and 250 rpm) and aeration (0.5, 0.75, 1.0, 1.25 and 1.5 L/min) were investigated in a BioStat® B laboratory fermentor. Changes in dissolved oxygen (DO) fraction in the fermentor were also recorded. Generally the DO values dropped drastically in the first 8 h (from about 60 to

19–20 % of saturation), then remained nearly constant until the end of fermentation process (data not shown). This result is in agreement with the one reported by Ilias and Hoq (*47*), who studied the effect of agitation rate on the growth and production of cellulase-free xylanase by *T. lanuginosus* MH4. Agitation at low speed (less than 150 rpm) always causes inhomogeneity of the reaction mixture in the fermentor and additionally, growth of the strain on the walls was also detected. The maximum inulinase production was 15.83 U/mL at 150 rpm agitation and 0.75 L/min aeration rate. Increase in the agitation speed (higher than 150 rpm) caused the decrease of inulinase activity in the fermentation broth. Papagianni *et al.* (*48*) reported the effects of agitation and medium viscosity on phytase production by *Aspergillus niger* and they found that best phytase production was achieved at 300 rpm after 125 h of fermentation. The difference may be due to the distinction in morphology and physiology of filamentous fungi. Agitation at high speed resulted in shear stress on the fungal filaments, thus affecting their growth and physiology. Sensitivity to shear stress is an intrinsic characteristic of microorganisms and varies from strain to strain (*47*). Similar effect was also detected when aeration was increased from 0.75 to 1.5 L/min. In this case, inulinase production decreased from 15.83 to 6.22 U/mL. Growth of the strain on the fermentor wall was observed when aeration rate was 0.5 L/min. Generally oxygen supply and shear stress act in antagonism. The former increases the biomass growth and the latter decreases the enzyme yield (*48,49*).

The optimisation of medium composition and process parameters was successfully carried out and the medium for the production of extracellular inulinase by *T. lanuginosus* IMI 140524 strain should be 50 mM phosphate buffer, pH=6.5, containing (in g/L): JAE 18, peptone 6, $KH_2PO_4$ 3, $K_2HPO_4$ 2, $MgSO_4·7H_2O$ 0.5, with 10 mL of Tween 80 and 1 mL of Vogel's trace element solution. In the bench-scale fermentor, 5.0 % (by volume) of inoculum (40- to 48-hour-old) is needed to start the fermentation at 47 °C, 150 rpm agitation and 0.75 L/min aeration rate. Applying the suggested medium and fermentation conditions, about 16 U/mL of enzyme titre (Table 5) should be produced, which is similar to the one from *Xanthomonas campestris* pv. *phaseoli* (*50*), but definitely lower than the one from *A. niger* (*51–54*). The production of inulinase by *Kluyveromyces marxianus* varied from 0.5 up to 47 U/mL depending on the strain applied (*34,55,56*).

Table 5. Production of extracellular inulinase by different microorganisms

| Microorganism | Enzyme activity<br>U/mL | Ref. |
|---|---|---|
| *Thermomyces lanuginosus* | 15.8 | this study |
| *Xanthomonas campestris* pv. *phaseoli* | 17.4 | (*50*) |
| *Aspergillus niger* NK-126 | 52.3 | (*51*) |
| *Aspergillus niger* 13/36 | 79.8 | (*52*) |
| *Aspergillus niger* van Teighem UV11 | 302 | (*53*) |
| *Aspergillus niger* van Teighem UV11 | 290 | (*54*) |
| *Kluyveromyces marxianus* DSM 70106 | 26 | (*55*) |
| *Kluyveromyces marxianus* ATCC 52466 | 0.5 | (*34*) |
| *Kluyveromyces marxianus* YS-1 | 47.3 | (*56*) |

## Conclusions

Thermophilic fungus *Thermomyces lanuginosus* is able to synthesise extracellular inulinase. The enzyme synthesis can be induced by inulin, but constitutive production was also detected when grown on glucose substrate. The addition of Tween 80 to the fermentation medium increased the secretion of extracellular inulinase enzyme. Initial pH of the medium, inoculum size and the age as well as agitation and aeration played important roles in the growth and enzyme production by *T. lanuginosus* IMI 140524. Agitation at high speed or aeration at higher rate may cause fragmentation of filaments, and thus affect the physiology of a fungus.

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