High-level Production of Bovine Enterokinase Light Chain Using Fed-batches by Recombinant *Pichia pastoris*

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A recombinant *Pichia pastoris* containing enterokinase light chain gene from bovine was cultured in flasks firstly at different pH, methanol addition, and cell mass concentration for enterokinase production. Activity of enterokinase increased as cell mass concentration increased at more than φ = 6 % methanol added. No significant change of activity of enterokinase was observed when methanol added was less than φ = 4 %. No activity of enterokinase lost after 120 h conservation at different pH. Secondly, high-level enterokinase production was achieved in 3.7 L bioreactor at pH 4.0, φ = 0.6 % methanol, p = 52.5 kPa. Activity of enterokinase was not cell mass concentration dependent at φ = 0.6 % methanol in bioreactor. Yield of enterokinase was 479.99 mg L^{-1} after 97.5 h induction. Maintenance coefficient and methanol consumed were calculated to analyse the enterokinase production.

Key words:
High cell mass concentration fermentation, *Pichia pastoris,* enterokinase, amount of methanol consumption

Introduction

Enterokinase (EK, EC 3.4.21.9), discovered in 1984,[1] was a protease of the intestinal brush border that specifically cleaved the acidic hexapeptide (Val-$(Asp)_4$-Lys) from trypsinagen to yield active trypsin. This cleavage initiated a cascade of proteolytic reactions leading to the activation of many pancreatic zymogens.[2] Enterokinase had been found from porcine,[3,4] bovine,[5] human,[6] ostrich intestine.[2] Enterokinase was reported as a disulfide-linked heterdimer with a heavy chain of 82–140 kDa and a light chain of 35–63 kDa. The heavy chain was postulated to mediate association with the intestinal brush border membrane, the light chain contained catalytic center with high specificity and tolerance to wide range of cleaves condition.[7] The broad application of enterokinase to cleave hexapeptide regardless of amino acid sequence of down-stream is known worldwide. Many heterologous hosts were used including *E. coli,*[8,9] filamentous fungus,[10] *Pichia pastoris,*[11,12] and COS cells[13] to produce enterokinase. However, much work focused on high expression of enterokinase at molecules manipulation and recombinant host cultivation in flasks. Therefore, yield of enterokinase was low and price of enterokinase was high although enterokinase was used widely. In our study, high-level production of bovine enterokinase light chain (EK_L) was achieved in recombinant *Pichia pastoris* by fed-batches.

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Materials and methods

Strain

Pichia pastoris GS115-PAO815-EK_L

Medium

YPD (L): yeast extract 10 g, peptone 20 g, glucose 20 g, agar 15 g (if necessary).

BMGY (L): yeast extract 10 g, peptone 20 g, potassium phosphate buffer 0.1 mol, pH 7.0, YNB 13.4 g, biotin $4 \cdot 10^{-4}$ g, glycerol 20 g.

Basal salts medium (BSM) (L): H_3PO_4 26.7 mL, $CaSO_4$ 0.93 g, K_2SO_4 18.2 g, $MgSO_4 \cdot 7H_2O$ 14.9 g, KOH 4.13 g, glycerol 40 g, PTM1 4.35 mL, pH was adjusted by w = 28–30 % NH_4OH to 5.0.

PTM1 (L): $CuSO_4 \cdot 5H_2O$ 6.0 g, KI 0.08 g, $MnSO_4 \cdot H_2O$ 3.0 g, $Na_2MoO_4 \cdot 2H_2O$ 0.2 g, H_3BO_3 0.02 g, $ZnSO_4 \cdot 7H_2O$ 20 g, $FeSO_4 \cdot 7H_20$ 65.0 g, $CoCl_2 \cdot 6H_2O$ 0.5 g, biotin 0.2 g, H_2SO_4 5.0 mL.

Flask cultivation

Cells were transferred from YPD agar plates into the 250 mL flasks, which contained 25 mL BMGY and grew at 30 °C 250 rpm for 18–20 h. Cell was used as inoculum for flask experiments.

Flask experiments were performed in the 250 mL flask containing 25 mL BMGY medium at 30 °C, 250 rpm with φ = 4 % YPD inoculums. A φ = 2 % methanol was added into BMGY after 24 h cultivation to induce EK_L every 24 h.

Bioreactor cultivation

A V = 3.7 L bioreactor (KLF2000 3.7 L, Bioengineering AG, Switzerland) equipped with a pH electrode, a dissolved oxygen electrode and two peristaltic pumps were used to scale up. Each fed-batch began with φ = 4 % inoculums in 2.0 L BSM at 30 °C, 500 rpm, 100 L h^{-1} air. The pH value was adjusted by ammonium hydroxide and maintained automatically through fed-batches. The oxygen was supplied by air flow (Q = 100–300 L h^{-1}) and agitation (500 to 1000 rpm). After depletion of the γ = 40 g L^{-1} glycerol, φ = 50 % glycerol containing 12 mL L^{-1} PTM1 was fed for cell growth. In the second phase, methanol feed rate was 1.0 mL L^{-1} h^{-1} after 0.5–2 h of glycerol depleted. The methanol feed rate was increased by 1.0 mL L^{-1} h^{-1} until settled methanol volume fraction was reached. Antifoam was injected manually throughout the fermentation to avoid excess foam.

Analytical procedures

Biomass

Cells were harvested by centrifugation at 6000 rpm for 3 min, and then washed twice with deionized water, and dried to a constant mass at 90 °C. The cell optical density was positively related to the dry cell mass (DCM) with an experimentally determined calibration curve. The cell optical density of the sample (properly diluted) was monitored by a spectrophotometer (U-1100, Hitachi Ltd., Tokyo, Japan) at λ = 600 nm.

Methanol fraction

Cell supernatant liquid was mixed with isopyknic mixture of 1-butanol and 1-propanol (Ψ = 4:1) to extract methanol. Sodium sulfite was used to pull out water from the mixture. Methanol fraction was determined by gas chromatography (GC 6890N, Agilent, USA) with a DB-WAX column. Temperatures of injection and detector were θ = 200, and 250 °C, respectively. Column temperature profile was 100 °C for 1 min, and increased at a rate of 20 °C min^{-1} until the temperature reached 200 °C.

Yield and activity of EK_L

In flask experiments, activity of EK_L was determined by percentage of cleaving a fusing protein (Trx-PTH) using supernatant after centrifugation. Reaction system (40 μL) was following: γ = 40 g L^{-1} Trx-PTH, pH 8.0, c = 50 mmol L^{-1} Tris-Cl, 1 mmol L^{-1} $CaCl_2$, 1 μL supertant. SDS-PAGE was done according to protein protocol after cleaving reaction, which was preformed at 37 °C for 20 min. The protein brands were analyzed by Smartview (FR200A Shanghai Furi Science & Technology Co. Ltd., Shanghai). The activity of EK_L was quantified according to the method of Invitrogen Ltd (CA, USA). In bioreactor experiments, yield of EK_L was determined by SDS-PAGE after centrifugation of broth. The protein brand was analyzed as stated previously. EK_L activity was also determined by using the fluorogenic enterokinase substrate Gly-Asp-Asp-Asp-Asp--Lys-naphthylamide.[14]

Maintenance coefficient analysis

Maintenance coefficient was analyzed according to the mass balance. As EK_L was secreted from the cell and yield of EK_L was too low compared with biomass, mass balance equation can be described as eq. (1). Specific methanol consumption rate can be depicted as eq. (2). Eq. (3) can be expressed by combining eq. (1) with eq. (2). $Y_{X/S}$ and "μ" were calculated from data of bioprocess. "k_m" were obtained by curve fitting.

$$\frac{ds}{dt} = \frac{\mu \gamma_X}{Y_G} + k_m \gamma_X \tag{1}$$

$$q_s = -\frac{1}{\gamma_X} \cdot \frac{ds}{dt} = \frac{\mu \gamma_X}{Y_{X/S}} \tag{2}$$

$$\frac{1}{Y_{X/S}} = \frac{1}{Y_G} + \frac{1}{\mu} k_m \tag{3}$$

Results and discussion

Effect of air pressure on yield of EK_L in bioreactor

Known from Henry formula, oxygen solution was improved when air pressure increased. High air pressure was used as a treatment to avoid oxygen limitation during high cell mass concentration fermentation.[15] On the other hand, too much air pressure accelerated cell lysis.[16] For avoiding the influence of methanol fraction on yield of EK_L. No methanol was detected during induction phase using Do-stat strategy fermentation. Air pressure was increased to 52.5 kPa in this study for EK_L expression. Fig. 1a showed the curves of cell growth with the DCM of γ = 37.96 g L^{-1} and γ = 68.60 g L^{-1} after 90 h cultivation (63 h induction) under 10.5 kPa and 96 h cultivation (62.5 h induction) under 52.5 kPa respectively. No significant difference of biomass was observed between the two

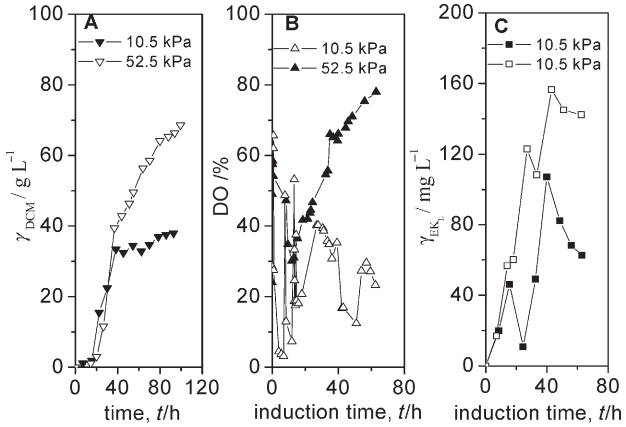

Fig. 1 – *Effects of different air pressure on yield of EK_L, DO and cell growth*

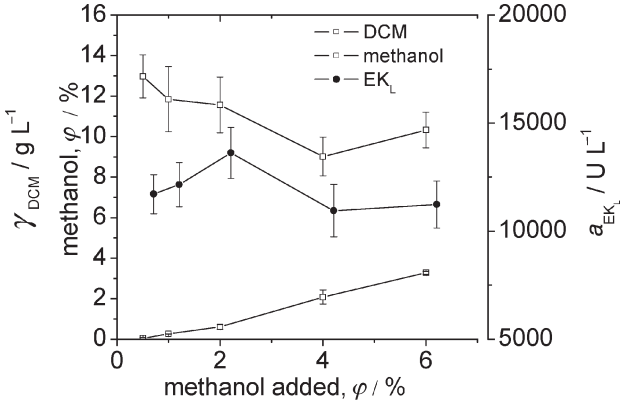

Fig. 2 – *Effect of different amounts of methanol added on DCM, activity of EK_L, and methanol fraction after 144 h cultivation*

curves during cell growth at cost of glycerol. During the induction phase, the yield coefficients of biomass per methanol were 0.07233 g mL^{-1} and 0.09458 g mL^{-1} under 10.5 kPa and 52.5 kPa respectively. The methanol consumption rates were 4.4293 mL L^{-1} h^{-1} and 4.8699 mL L^{-1} h^{-1}, therefore, the yield coefficient of biomass per methanol and methanol consumption rate were improved under 52.5 kPa compared with that under 10.5 kPa. DO was enhanced under 52.5 kPa as the results of higher oxygen solution than that under 10.5 kPa (Fig. 1b). The final yield of EK_L in the culture under 10.5 kPa was 62.63 g L^{-1}, whereas that in the culture under 52.5 kPa was 142.24 g L^{-1} (Fig. 1c). Another effect of air pressure on cell metabolism was described by maintenance coefficient. Maintenance coefficient during induction phase under 52.5 kPa (0.0724 mL g^{-1} h^{-1}) was lower than that under 10.5 kPa (0.1312 mL g^{-1} h^{-1}), which benefited cell metabolism for EK_L expression. The yield coefficients of EK_L per methanol were 0.4350 and 0.4673 mg mL^{-1}.

Effect of methanol added on activity of EK_L in flask experiments

In flask experiments, different fraction of methanol (φ = 0.5, 1.0, 2.0, 4.0, and 6.0 %) was added every 24 h to induce EK_L after 24 h cultivation. Fig. 2 showed the biomass, activity of EK_L and methanol volume fraction after 144 h cultivation (120 h induction). Little inhibition was observed on cell growth as methanol added increased. The highest activity of EK_L (13619.14 U L^{-1}) was obtained as φ = 2 % methanol was added. Methanol scarcity might be the reason why activity of EK_L was low at φ = 0.5 % and φ = 1.0 % methanol added. Activity of EK_L decreased when φ = 4.0 % or φ = 6.0 % methanol was added, this may be due to the influence of methanol toxicity.[17] Therefore, φ = 2 % methanol was added to induce EK_L in following flask experiments. Methanol volume fraction should be investigated in bioreactor for EK_L expression.

Effect of pH on the activity of EK_L in flask experiments

Recombinant EK_L was secreted to broth during induction phase by φ = 2 % methanol added per 24 h at different pH. Therefore, different pH in medium was prepared by potassium phosphate buffer, phosphate acid or potassium hydroxide. The pH value during the cultivation was maintained by addition of potassium hydroxide every 12 h. The bell shape of the biomass suggested that it was beneficial to cell growth at the range of pH 5.0–7.0. At the point of activity of EK_L, pH 4.0–6.0 gave high relative activity of EK_L. Specific activity of EK_L at pH 4.0 was the highest among the different pH. Fermentation broth containing EK_L at different pH was lay at 37 °C for 120 h to investigate the stability of EK_L. The activity of EK_L did not decrease during the period of conservation (Fig. 3). There-

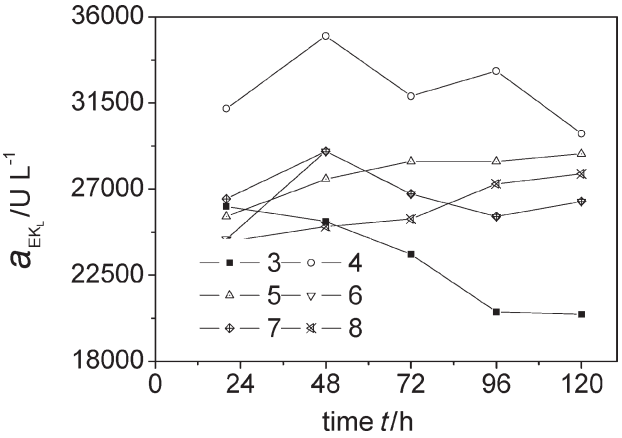

Fig. 3 – *Curves of activity of EK_L during conservation at different pH*

Table 1 – *Effect of pH on the cell growth and yield of EK_L in fed-batches*

pH	Biomass γ_x/g L^{-1}	Induction time t/h	EK_L γ/mg L^{-1}	EK_L fraction w/mg g^{-1}	Methanol consumption rate/mL L^{-1} h^{-1}	Maintenance coefficient k_m/mL g^{-1} h^{-1}
4.0	84.52	90	277.15	3.28	5.52	0.0724
5.0	73.09	105	174.68	2.39	5.18	0.0899
6.0	76.36	117	156.09	2.04	4.04	0.0964

fore, it was considered that activity of EK_L was not lost during fed-batches.

Effect of pH on yield of EK_L in 3.7 L bioreactor

As EK_L was stable in broth at different pH, three fed-batches with different pH (4.0, 5.0, and 6.0) were performed to clarify the influence of pH on EK_L expression at the condition of 52.5 kPa. Methanol was added after 10 h glycerol fed almost by DO-stat strategy. Thirty percent DO was maintained during induction phases without methanol detected. Table 1 showed the summary of parameters of three fed-batches. No distinguished difference of biomass was observed during fermentation, however, the yield and specific yield of EK_L increased as pH decreased from 6.0 to 4.0. The methanol consumption rate increased when pH decrease from 6.0 to 4.0. This may be because of metabolism condition improved at pH 4.0, as maintenance coefficient was decreased from 0.0964 to 0.0724 mL g^{-1} h^{-1}. The maximal yield and specific yield of EK_L were 277.15 mg L^{-1} and 3.28 mg g^{-1} at pH 4.0 after 136.5 h cultivation (90 h induction).

Effect of biomass and amount of methanol added on the yield of EK_L in flask experiments

Methanol volume fraction was known as an important factor during *Pichia pastoris* fermentation for heterogeneous protein expression. Yield of EK_L was reported as a cell concentration dependent by Tan *et al.*[18] Effect of different amount of methanol added and cell concentration on activity of EK_L was investigated in flasks to optimize the yield of EK_L (Fig. 4). Activity of EK_L decreased significantly

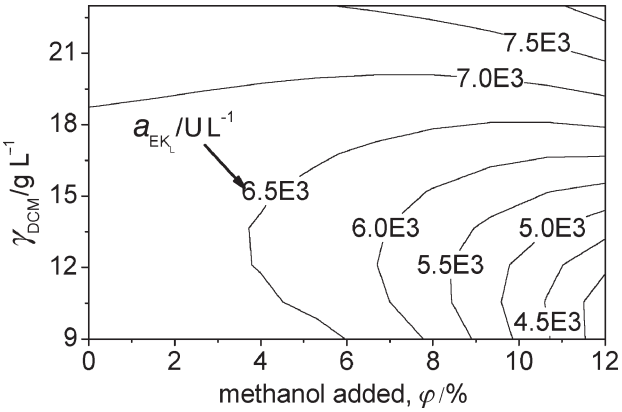

Fig. 4 – *Contour plots of activity of EK_L at different amounts of methanol added and cell mass concentration*

when amount of methanol added increased, especially higher than φ = 6.0 %. Activity of EK_L did not change much when the amount of methanol was lower than φ = 4.0 % regardless of cell concentration. Activity of EK_L increased as cell concentration increased as the amount of methanol added increased, especially higher than φ = 6.0 %. This result was confirmed by Tan's work. However, methanol fraction was maintained between φ = 1.0–3.0 % in Tan's report which was too high for EK_L expression.

Effect of methanol fraction on yield of EK_L in 3.7 L bioreactor

Three grades of methanol fractions (φ = 0, 0.6, and 1.0 %) were preformed during induction phase to improve the yield of EK_L. Table 2 is the summary of parameters of three fed-batches. Biomass (86.36 g L^{-1}) at φ = 0 % methanol was higher than that at φ = 0.6 and 1.0 % methanol. This might be

Table 2 – *Effect of methanol volume fractions on biomass and yield of enterokinase in fed-batches*

Methanol volume fraction φ/%	Biomass concentration γ_x/g L^{-1}	Induction time t/h	EK_L γ/mg L^{-1}	EK_L mass fraction w/mg g^{-1}	Methanol consumption rate /mL L^{-1} h^{-1}	Maintenance coefficient k_m/mL g^{-1} h^{-1}
0	86.36	90	277.15	3.21	5.52	0.0724
0.6	68.19	95.5	323.98	4.75	5.63	0.1219
1.0	37.97	60	124.46	3.28	4.27	0.1629

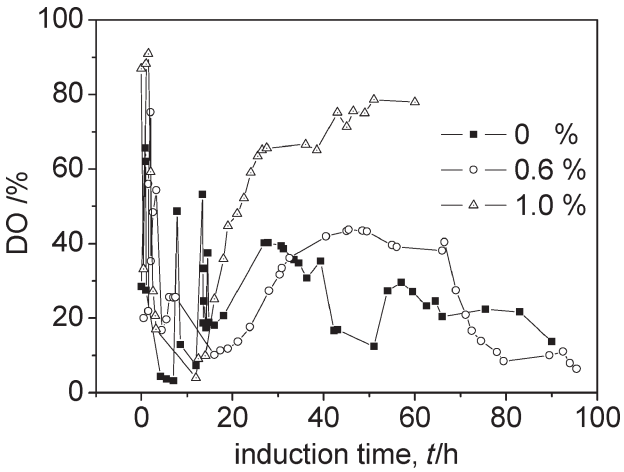

Fig. 5 – *Curves of DO at different methanol fractions during induction phase*

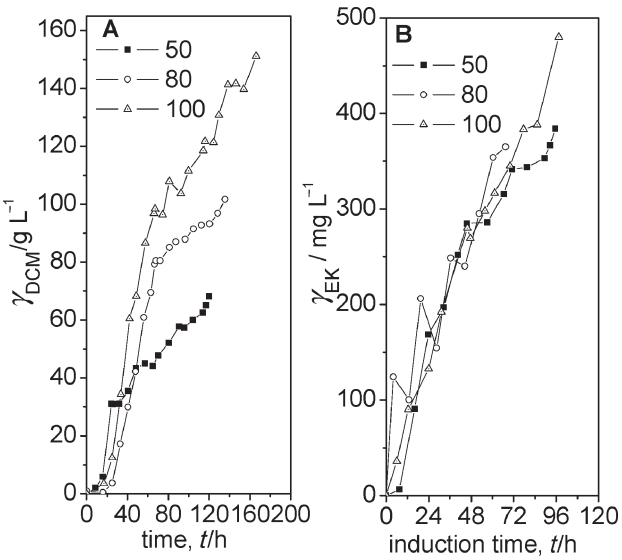

Fig. 6 – *Curves of cell growth and yields of EK_L during the cultivation when methanol was added at different cell mass concentration*

that methanol was oxidized efficiently when methanol fraction was φ = 0 % because DO at φ = 0 % methanol was higher than that at φ = 0.6 % methanol (Fig. 5). At the condition of φ = 1.0 % methanol, DO was higher than that of other grades because of methanol toxicity. Yield of EK_L was 323.98 mg L^{-1} at φ = 0.6 % methanol, which was the results of higher intensity of methanol induction than φ = 0 % methanol. Disadvantageous effect of methanol toxicity on yield of EK_L was the lowest methanol consumption rate and maintenance coefficient increasing.

Effect of cell mass concentration on yield of EK_L from *Pichia pastoris* in 3.7 L bioreactor

Scale up of effect of cell concentration on yield of EK_L was performed in 3.7 L bioreactor by three cell concentration grades (50, 80, 100 g L^{-1}). Fermentation conditions were pH 4.0, φ = 0.6 % methanol fraction and 52.5 kPa. Fig. 6a shows the curves of cell growth during cultivation. Fig. 6b shows the curves of EK_L yield during induction phase. Yields of EK_L increased without changes significantly regardless of cell concentration which was confirmed by flask experiments. The methanol fraction was too high for EK_L expression in Tan's work which gave lower activity of EK_L than that of φ = 0.6 % methanol in this work. Activity of EK_L was not the cell concentration dependent at φ = 0.6 % methanol. The yield of EK_L was 479.99 mg L^{-1} at the condition of 100 g L^{-1} after 97.5 h induction.

Before this work, a few works have been done done for production of EK_L because it was a useful tool for cleavage of fusion protein. Recombinant bovine EK_L exhibited a high level of activity in Lavallie's work.[13] Vazza *et al.*[11] reported that the yield of pure bovine EK_L from recombinant *Pichia pastoris* was 6.3 mg L^{-1}. And yield of pure EK_L from recombinant *E. coli*[9] was 4.3 mg L^{-1}. Mix-carbon source was fed during cultivation of recombinant *Pichia pastoris* for EK_L production, with the resultant of 350 mg L^{-1} EK_L without purification after 120 h induction.[12] Improved yield of EK_L and short induction time were found after our work for EK_L production compared to previous work. Methanol fed controlled in this work was easier than that of mix-carbon source fed. Therefore, EK_L production through this work was advantaged industrially.

Conclusion

Yield of recombinant EK_L in *Pichia pastoris* was optimized by fed-batches. DO was improved for yield of EK_L enhancement (46.19 %) at 52.5 kPa air pressure. In flask experiments, good stability of EK_L was known at different pH. The amount of methanol added, not the cell concentration at low amount of methanol added, played an important role in high EK_L expression by *Pichia pastoris*. Scaled up in 3.7 L bioreactor, pH 4.0, φ = 0.6 % methanol, and 100 g L^{-1} cell concentration were the final conditions for EK_L expression by *Pichia pastoris*. The yield of EK_L was 479.99 mg L^{-1} after optimization. Maintenance coefficient had the low value when high yield of EK_L was obtained.

List of symbols

a – EK_L activity, U L^{-1}
c – concentration, mol L^{-1}
k_m – maintenance coefficient, mL g^{-1} h^{-1}
Y_G – yield biomass per substrate, g g^{-1}

$Y_{X/S}$ – observed yield biomass per substrate, g g^{-1}
p – pressure, kPa
Q – volume flow rate, L h^{-1}
q_s – substrate consumption rate, h^{-1}
s – substrate, g
t – time, h
V – volume, mL, L
w – mass fraction, %
γ – mass concentration, g L^{-1}
γ_x – biomass concentration, g L^{-1}
θ – temperature, °C
λ – wave length, nm
μ – specific cell growth rate, h^{-1}
φ – volume fraction, %
Ψ – volume ratio

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