A T-flask based screening platform for evaluating and identifying plant hydrolysates for a fed-batch cell culture process

Canghai Lu · Carlos Gonzalez · Joseph Gleason ·
Jennifer Gangi · Jeng-Dar Yang

Received: 10 April 2007 / Accepted: 25 July 2007 / Published online: 18 August 2007
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Abstract This paper presents a T-flask based screening platform for evaluating and identifying plant hydrolysates for cell culture processes. The development of this platform was driven by an urgent need of replacing a soy hydrolysate that was no longer available for the fed-batch process of recombinant Sp2/0 cell culture expressing a humanized antibody. Series of small-scale experiments in T-flasks and 3-l bioreactors were designed to gain an insight on how this soy hydrolysate benefits the culture. A comprehensive, function-oriented screening platform then was developed, consisting of three T-flask tests, namely the protection test, the growth promotion test, and the growth inhibition test. The cell growth in these three T-flask tests enabled a good prediction of the cell growth in the fed-batch bioreactor process. Fourteen plant hydrolysate candidates were quickly evaluated by this platform for their ability to exert strong protection, high cell growth promotion, and low cell growth inhibition to the culture. One soy hydrolysate was successfully identified to support the comparable cell growth as the discontinued soy hydrolysate. Because of the advantage of using small-scale batch culture to guide bioreactor fed-batch culture, this proposed platform

approach has the potential for other applications, such as the medium and feeding optimization, and the mechanism study of plant hydrolysates, in a high throughput format.

Keywords Hydrolysate · Screening platform · Fed-batch · Bioreactor process · Mammalian cell culture · Sp2/0

Introduction

In a mammalian cell culture process, an optimal high product yield can be achieved by the combination of high viable cell density and high cell specific productivity. To achieve the best cell growth, an optimized physical environment and a balanced nutrient supplement should be supplied to the culture. The former includes constant temperature, pH, gentle but sufficient mixing, etc. The latter should be tailored to each transfected cell line to fulfill its own unique nutrient requirements.

Driven by the regulatory concerns of potential viral or prion contamination, more and more processes have moved to serum-free, animal-derived component free medium. The ultimate goal is to cultivate the culture in a chemically defined medium. However, it is time and resource consuming for the development of a fully defined medium. The process may not be cost-efficient and the product yield may be unsatisfactory.

C. Lu (✉) · C. Gonzalez · J. Gleason ·
J. Gangi · J.-D. Yang
Immunomedics, Inc., 300 American Road, Morris Plains,
NJ 07950, USA
e-mail: clu@immunomedics.com

Supplementation of plant hydrolysates into the medium and/or feeding could be an alternative strategy (Jayme and Smith 2000; Kuchibhatla et al. 2004). The plant hydrolysate is the plant protein digests hydrolyzed by microbial proteases (produced in animal-free medium), so it is animal-component free. It contains low-molecular weight components, including peptides, amino acids, vitamins and trace elements (Sung et al. 2004; Chun et al. 2005). Its comprehensive component matrix may cover the cells' needs so the medium/feeding optimization may take less time and could meet the production goal in the tight process development timeframe.

Plant hydrolysate has been used in cell culture medium to boost cell growth and productivity in various applications and processes (Heidemann et al. 2000; Franek et al. 2000; Burtreau et al. 2003; Sung et al. 2004; Chun et al. 2005). The drawbacks of using plant hydrolysates include lot-to-lot quality variation (Schwartz et al. 2002), undesired components coupling with the useful ones, and the difficulty in identifying a second source as a back-up. The last issue will be the main subject explored in this paper, while the second issue will be discussed as well.

In our fed-batch process for the production of a humanized monoclonal antibody, a soy hydrolysate, namely TC soy, was supplemented to enhance the cell proliferation and antibody expression. The antibody is expressed in the Sp2/0 mouse myeloma cell line and produced in 2,500 l bioreactors using a serum-free medium. In the clinical production phase, we encountered a supply issue of TC soy. The manufacturer discontinued its production and was unable to transfer the proprietary manufacturing process to other contractors. Therefore, a substitute of the soy hydrolysate needed to be identified. In the race to identify the suitable replacement before the depletion of the existing inventory, a strategy of replacing the current soy hydrolysate with another plant hydrolysate instead of a chemically defined feeding was adopted.

A good hydrolysate replacement should satisfy the following requirements. First, it is a non animal-derived product, i.e., a plant hydrolysate. To minimize the potential impact on the product quality, it is preferred that the replacement is also a soy-based product. Second, it should support comparable cell growth and antibody production with the similar feeding schedule/concentration. Third, it should not

impact downstream purification. Fourth, the product from the manufacturing process using the new plant hydrolysate should possess comparable biochemical attributes and potency.

It was our goal to identify a plant hydrolysate which meets the above four requirements. It was achieved by screening through a hydrolysate candidate pool comprising of various soy hydrolysates and one yeastolate. Ideally, bench-scale bioreactor fed-batch process is the most direct approach to do the screening. However, it is time and labor consuming. It is also often limited by the instrument availability. As such, we conducted the screening in the small-scale T-flask batch culture. Its smaller volume, faster turnover, and simpler experimental requirements allowed us to do a high throughput screening from a large candidate pool.

There are challenges using T-flasks. First, the static culture in T-flasks may not represent the suspension culture in bioreactors for the cultures are subjected to different hydrodynamic forces. The hydrodynamic impact has been found minimal for our cell line. Under the current experimental settings, the results of batch growth and productivity from static culture (i.e., T-flask) and suspension culture (i.e., spinner flask or bioreactor) are comparable. Therefore, this challenge did not exist in our case. Second, the experimental results of the *batch* culture may not be directly translatable to the *fed-batch* culture. This was the challenge we encountered in this study. Only through the resourceful experimental design and careful data interpretation to avoid the potential misleading, the results from T-flask cultures can reveal valuable information for fed-batch process. Then, its higher throughput screening capability can facilitate the cell culture process development and medium optimization.

The conventional method of evaluating hydrolysates is to culture the cells in the hydrolysate-supplemented growth medium (Franek et al. 2000; Franek and Katinger 2002; Burtreau et al. 2003; Sung et al. 2004). As we demonstrated in this study, this method failed to translate T-flask results to the bioreactor. Not until a dramatic modification was made on the conventional method, the advantages of T-flask screening will not be realized.

This paper reports our efforts in modifying the above conventional method. Specifically, a T-flask based screening platform was developed through

which various plant hydrolysates were evaluated for the fed-batch process. The platform was built based on the varied functions of the plant hydrolysates to the cell culture. The plant hydrolysate that passed a series of T-flask testing as outlined in the platform would have a high probability of bringing good performance in the fed-batch process.

Materials and methods

Plant hydrolysates

TC soy, Phytone, Soy 01 to Soy 10, and TC yeastolate were provided by BD Biosciences (Sparks, MD). Hypep 1510 and Hypep 1511 were provided by Kerry Bio-Science (Rochester, MN). For convenience, a yeastolate (yeast cell hydrolysis) is defined here as a plant hydrolysate. The stock solutions of the hydrolysates were prepared at a concentration of 120 g l^{-1} by dissolving them in 40–50°C WFI (water for injection) and passing them through $0.22 \text{ }\mu\text{M}$ sterile filters.

Fatty acid

An unsaturated omega-6 fatty acid was used in the fed-batch process and T-flask experiments. It is one of the essential fatty acids to mammalian cells. This fatty acid was critical to the growth of the cell line in this study and the feeding amount was optimized for the process. Its identity is not for disclosure. The “fatty acid” described in the experiments means this specific fatty acid.

Fatty acids are poorly soluble in water, so they need a carrier to be delivered to the culture. Bovine serum albumin (BSA) is an often-used carrier, as it has the binding sites for fatty acids (Greenberg-Ofrath et al. 1993; Kobayashi et al. 1994). To avoid the animal derived component, our process used methyl- β -cyclodextrin, instead of BSA, as the carrier to present fatty acids to the culture. Methyl- β -cyclodextrin is a cyclic oligosaccharide consisting of seven glucopyranose units. Hydrophobic molecules, such as fatty acids, can be incorporated into its cavity by displacing water and become water-soluble.

The fatty acid solution carried by methyl- β -cyclodextrin was supplied by Invitrogen (Carlsbad, CA).

The fatty acid solution carried by BSA was prepared by mixing 4 moles fatty acid (Sigma, St. Louis, MO) with 1 mole BSA (Sigma). Unless specified, the fatty acid used in the experiments was carried by the cyclodextrin.

Cell line and medium

The recombinant Sp2/0 mouse myeloma cell line expressing a humanized antibody was generated by transfection of the host cell line with the DHFR-based amplifiable expression plasmid carrying the product producing genes. The transfected cells were amplified by increasing the methotrexate (MTX) concentration followed by sub-cloning and gradual weaning off of serum.

Complete medium, a customer-modified Hybridoma Serum Free Medium (HSFM, Invitrogen), was used to maintain and expand the cell culture in T-flasks. Basal medium (Invitrogen), a customized formulation based on the complete medium with much lower glucose and glutamine concentrations, was used in the 3-l fed-batch bioreactor process.

Cell culture

The cultures in T-flasks were maintained and expanded at 37°C in a humidified atmosphere with 5% CO_2 in the CO_2 incubator. The fed-batch experiments were conducted in 3 l Bellco spinner-flask bioreactor systems (Bellco glasses, Vineland, NJ) with a 2 l working volume. The bioreactor temperature, pH, and dissolved oxygen (DO) were monitored and controlled by single loop controllers. The concentrated feeding cocktail containing the hydrolysate, fatty acid, amino acids, glucose, vitamins and trace metals was fed on the daily base. When evaluating different plant hydrolysates, the amino acid contents of the feeding cocktail will be adjusted to avoid amino acid deficiency based on the free amino acid profiles of the hydrolysates.

Complete medium hydrolysate supplementation experiments

The experiments were conducted in 25 cm^2 T-flasks with the culture volume of 10 ml using the complete

medium. The cultures were inoculated at a viable cell density (VCD) of 1×10^5 cells ml^{-1} with the supplementation of 1 or 5 g l^{-1} of plant hydrolysates. The cultures were incubated until the decline phase. The experiments were conducted in duplicate.

Fatty acid add-on experiments

The experiments were conducted in 25 cm^2 T-flasks with the culture volume of 10 ml using the complete medium. The cultures were inoculated at a viable cell density (VCD) of 1×10^5 cells ml^{-1} . Cyclodextrin-carried fatty acid or BSA-carried fatty acid was added into the culture at various concentrations. The cultures were incubated for 2 days. The experiments were conducted in duplicate.

Fatty acid and hydrolysate add-on experiments

The experiments were conducted in 25 cm^2 T-flasks with the culture volume of 10 ml using the complete medium. The cultures were inoculated at a VCD of 5×10^5 cells ml^{-1} with the supplementation of 1 g l^{-1} of plant hydrolysate and 8 $\mu\text{g ml}^{-1}$ of cyclodextrin-carried fatty acid. The cultures were incubated for 3 days. Cell growth was compared with the control (the culture without additions of hydrolysates and fatty acid) and the negative control (the culture with the addition of fatty acid but no hydrolysates). The experiments were conducted in duplicate.

Spent medium hydrolysate supplementation experiments

Spent medium is defined herein as the cell-free in-process culture medium. Cultures were collected on days 2, 3, 4, and 5 from the 2,500 l bioreactor. The cells were removed by centrifugation and the supernatant was filtered by 0.22 μm filters. The spent medium was supplemented with glucose and glutamine to the same concentrations of the complete medium.

The experiments were conducted in 25 cm^2 T-flasks with the culture volume of 10 ml using the glucose and glutamine supplemented spent medium. The cultures were inoculated at a VCD of 1×10^5 cells ml^{-1} with the supplementation of 1 g l^{-1} of plant

hydrolysate. The cultures were incubated until the decline phase. The control was cultured in the spent medium without hydrolysate supplementation. The experiments were conducted in duplicate.

Assays

The VCD and the cell viability were measured by microscopic counting using a hemocytometer after staining with 0.4% trypan blue dye (Invitrogen). The Integral Viable Cells (IVC) is the time integral of the total viable cells in the culture and is approximated using a trapezoidal point-to-point summation method. The antibody concentration was determined by HPLC (Alliance Separations Module, Model 2695, Waters, Milford, MA) using a Protein A affinity chromatography column (P/N 2-1001-00, Applied Biosystems, Foster City, CA).

Results

Limitation of conventional screening method

The conventional method was first conducted to test two soy hydrolysates, named Soy 06 and Soy 08. They were both animal-free, enzymatically-hydrolyzed soy protein, and ultrafiltered for cell culture use. They both exhibited similar free and total amino acid profiles, carbohydrate profiles, and peptide profiles to those of TC soy (data not shown).

The cells were cultured in the complete medium with the supplementation of 1 or 5 g l^{-1} of TC soy, Soy 06 or Soy 08. The growth curves and the antibody concentration of the final culture are shown in Fig. 1. In general, the culture supplemented with Soy 06 or Soy 08 showed a very similar growth and productivity as its TC soy counterpart at both supplementation levels. At 5 g l^{-1} , some growth inhibition effects were observed across the board and they were comparable. Based on these results, Soy 06 and Soy 08 seemed to be promising candidates to replace TC soy.

The 3-l bioreactor experiments were further conducted that used the same amounts of TC soy, Soy 06 or Soy 08. On the contrary of the T-flask results, both runs showed poor cell growth as compared to the run using TC soy (Fig. 2A). This discrepancy clearly

Fig. 1 The growth curves (viable cell density (A1, B1) and viability (A2, B2)) and the final antibody concentrations (bars, from left to right, represent the control, TC soy, Soy 06, and Soy 08, respectively (A1, B1)) of the T-flask experiments using the complete medium supplemented with hydrolysates. Panel

A: with 1 g l⁻¹ of hydrolysates; Panel **B:** with 5 g l⁻¹ of hydrolysates. Data shown include the control (△), TC soy (●), Soy 06 (◆), and Soy 08 (■). The control was cultivated without hydrolysate supplementation

demonstrated the limitation of this T-flask screening method.

The resultant dissolved oxygen (DO) profiles of the above 3-l runs were very different among TC soy and the tested hydrolysates as shown in Fig. 2B. An initial drop on DO was observed across all hydrolysates after day 2 feeding. Thereafter, the DO of the TC soy run continued to decrease until it triggered oxygenation. By contrast, the DO profiles of the Soy 06 and 08 runs rebound rapidly to the level prior to feeding. The rapid DO rebound indicated the slowdown in culture respiration activity, which was consistent with slowdown in the corresponding cell growth shown in Fig. 2A. It appeared that the feeding of the Soy 06 or 08 run either introduced toxicity to the culture or it failed to replenish the depleted nutrients.

The above observations indicated that TC soy helped the culture in a complex way. Before a replacement can be identified, we should first

characterize its functions in the process. The conventional T-flask screening method using complete medium was inadequate in providing such information and it did not yield satisfactory screening outcome. The method required modification before it can be used to produce useful information to bioreactor fed-batch process.

Protection effect of TC soy

The first hypothesis for the failure of above Soy 06 and Soy 08 runs was that the cell growth was halted by the toxicity from the day 2 feeding. In the process, the day 2 feeding comprised of a soy hydrolysate and a fatty acid. Figure 1 previously showed that in the absence of the fatty acid, Soy 06 and 08 exhibited similar growth and antibody production profiles as TC soy. This implied that the hydrolysate portion of the feed is not inhibitory. As such, the toxicity

Fig. 2 Viable cell density (graph **A**) and dissolved oxygen (graph **B**) profiles of the 3-l bioreactor fed-batch runs using TC soy, Soy 06 and Soy 08. The 3-l bioreactor runs used the same process except feeding the equal amount of the different hydrolysates

introduced by day 2 feeding was likely resulted from the fatty acid portion.

Fatty acids are prone to oxidation which will cause cell damage and death (Gutteridge and Halliwell 1990; Spitz et al. 1992). The cytotoxicity introduced by cyclodextrin-carried fatty acid to our cell line was confirmed by a T-flask experiment (Fig. 3). In the T-flask culture, the fatty acid carried by cyclodextrin or by BSA was supplemented to the culture at different concentrations. After 2-day incubation, the results showed that the increase in the cyclodextrin-carried fatty acid concentration resulted in a decrease in the viable cell density. The culture containing 4 $\mu\text{g mL}^{-1}$ of cyclodextrin-carried fatty acid had no survived cells. If the fatty acid was coupled with BSA, the

inhibition was reduced significantly. In that case, cells grew well in the presence of 26 $\mu\text{g mL}^{-1}$ of fatty acid. They even survived at higher concentration of 52 $\mu\text{g mL}^{-1}$. The results agreed with the literature that serum albumin not only acts as a carrier of fatty acids, but also inhibits the oxidation and protects the cells from damage (Stocks et al. 1974; Kobayashi et al. 1994).

Based on the above finding, it was hypothesized that TC soy may possess the similar protection effect as BSA. As such, the good cell growth seen in the 3-l run using TC soy in Fig. 2A might result from its protection effect on reducing the toxicity introduced by the fatty acid to the culture. This might also explain why Soy 06 and Soy 08 failed. These two soy

Fig. 3 Viable cell density results on the second day post inoculation of the fatty acid add-on experiments. The experiments were conducted using the complete medium. The fatty acid carried by cyclodextrin or by BSA was supplemented to the culture at inoculation with different concentrations

hydrolysates may simply not exert the same level of protection as TC soy.

Based on the above analysis, the fatty acid and hydrolysate add-on experiment was designed. A hydrolysate was supplemented into the culture together with the fatty acid. The cell growth was compared with the control and the negative control. The control was the culture added neither the hydrolysate nor the fatty acid. The negative control was the culture with the addition of the fatty acid but no hydrolysates. The concentration of hydrolysate in the culture was set at 1 g l^{-1} to avoid any inhibition effects caused by hydrolysates. The starting VCD of $5 \times 10^5 \text{ cells ml}^{-1}$ with $8 \mu\text{g ml}^{-1}$ fatty acid were experimentally determined for our cell line that would best differentiate the protection potentials of various hydrolysates.

The results are shown in Fig. 4. The T-flasks supplemented only with the fatty acid (the negative control) had few survived cells. The T-flask cultures containing fatty acid supplemented with TC soy had a comparable cell growth with the control. Soy 06 and Soy 08 also showed their protection but they were not as good as TC soy. The results revealed the protection exerted by soy hydrolysates.

The protection test and its limitation

The lower protection potential of Soy 06 and Soy 08 shown in the fatty acid and hydrolysate add-on

Fig. 4 Viable cell density results of the fatty acid and hydrolysate add-on experiments with TC soy, Soy 06 and Soy 08. The experiments were conducted using the complete medium. One gram per litre of plant hydrolysate and $8 \mu\text{g ml}^{-1}$ of fatty acid were supplemented into the culture at inoculation. The control was the culture without additions of hydrolysates and fatty acid. The negative control was the culture supplemented with the fatty acid only

experiment corresponded to their worse cell growth in the 3-l fed-batch runs (Fig. 2A). This correlation suggested that this T-flask experiment could be a good screening method of hydrolysates. The method used the protection potential of hydrolysates as the indicator of their bioreactor performance, so it was named protection test.

Three other soy hydrolysates (Phytone, Hypep1510 and Hypep1511) and one yeastolate were tested using this method, and the results are shown in graph A of Fig. 5. Hypep1511 showed comparable protection potential to TC soy. Phytone and Hypep1510 did not provide any protection to the cells. Yeastolate was in the middle, showing less protective efficacy than that of TC soy. The 3 l experiments were further conducted, with the results shown in graph B of Fig. 5. The results demonstrated that only yeastolate yielded the performance that came close to that of TC soy. The rest of the hydrolysates all resulted in poor cell growth.

The results from the T-flask protection test and the corresponding 3 l bioreactor evaluation for the above hydrolysates are compiled into Table 1. TC soy, Phytone and Hypep 1510 showed good correlation between the T-flask protection test results and the 3-l performance. TC soy had the highest protection potential in T-flask and it yielded the best cell growth in the 3-l process. On the other end, Phytone and Hypep1510 lacked of the protection potential and also yielded the poorest cell growth in the 3-l process.

Fig. 5 Viable cell density results of the T-flask protection test (graph **A**) and 3-l fed batch evaluation (graph **B**) for TC soy, yeastolate, Phytone, Hypep1510 and Hypep1511. (**A**) The experiments were conducted using the complete medium. One gram per litre of plant hydrolysate and $8 \mu\text{g mL}^{-1}$ of fatty acid were supplemented into the culture at inoculation. The control was the culture without additions of either hydrolysates or fatty acid. The negative control was the culture supplemented with the fatty acid only. (**B**) The 3-l bioreactor runs used the same process except feeding the equal amount of the different hydrolysates

The 3-l performances of Soy 06 and Soy 08 were in the middle, which may be attributed to their sub-optimal protection potential.

Controversial results were found for Hypep 1511 and yeastolate. Hypep 1511 scored high in the protection test but yielded a very poor cell growth in the 3-l bioreactor. Yeastolate had a lower score than Soy 06 in the protection test, but it exhibited a far superior performance in the 3-l evaluation.

The above results showed both value and limitation of the protection test. The non-correlative results of Hypep 1511 and yeastolate indicated that TC soy should provide other benefits besides the protection effect. As a result, additional tests were developed to characterize these effects.

The growth promotion test

Another hypothesis for the poor cell growth with the Soy 06 and Soy 08 feeding in Fig. 2 was related to the nutrient deficiency. The potential of hydrolysates to promote cell growth by replenishing the depleted nutrients in the culture was the second subject to study.

Experiments were designed to differentiate the growth promotion strength of different plant hydrolysates. As previously demonstrated in Fig. 1, complete medium was not suitable for screening the hydrolysates with the respect to their growth promotion potentials. This was because of the fact that the complete medium already contained all the necessary nutrients and growth factors to support the cell growth in a batch culture. The growth promotion benefit provided by hydrolysates became excessive and cannot be revealed in the experiment. Therefore, a spent medium, in which cell growth could be largely promoted by supplemented key nutrients or growth factors from hydrolysates, was the appropriate medium to screen the growth promotion.

In this study, the spent medium was obtained from the 2,500 l fed-batch bioreactor. Since it was taken from the real environment of the cell growth, it would best represent the conditions of nutrient consumption and metabolite formation of the culture.

The spent media collected from the different days of a 2,500 l fed-batch run were tested with the hydrolysates tested in the protection test. Glucose and glutamine were replenished into the spent media to the same level of the complete medium. Hydrolysates were, respectively, added at the final concentration of 1 g l^{-1} . It was found that Day 3 spent medium best differentiated the cell growth of the cultures when supplemented with different hydrolysates (Fig. 6). Spent media collected from other days were not effective in differentiating the difference (data not shown).

The results in Fig. 6 showed that the control, cultured with the spent medium, grew only to a VCD of $\sim 1 \times 10^6 \text{ cells mL}^{-1}$ and started to decline afterwards. TC soy and yeastolate supported a very good cell growth to a VCD of $\sim 2 \times 10^6 \text{ cells mL}^{-1}$, which was close to the cell growth in the complete medium (the control in Fig. 1). Soy 06, Soy 08, Hypep 1510 and Phytone yielded mediocre cell growth. Spent medium with Hypep 1511 exhibited a similar poor

Table 1 T-flask-bioreactor correlation table

	TF protection test result	TF growth promotion test result	3 l fed-batch results (IVC) (percentage to control)
Control (TC soy)	+++	+++	100%
Hypep1511	+++	—	8.76%
Soy 06	++	++	16.72%
Soy 08	++	++	12.62%
Yeastolate	+	+++	78.60%
Phytone	—	++	9.41%
Hypep1510	—	++	11.64%

Summary of T-flask (TF) protection test, T-flask growth promotion test, and 3 l fed-batch run results of TC soy, Hypep1511, Soy 06, Soy 08, yeastolate, Phytone, and Hypep1510. The 3-l evaluation results were presented as the percentage of the IVC value to the TC soy run. The T flask test results for each hydrolysate were scored qualitatively based on the cell growth in Figs. 4–6, by comparison to TC soy and the negative control in terms of the IVC values. The negative control was the culture without hydrolysates. Three plus (+++) is given to the one comparable to TC soy. Negative (—) is given to the one comparable to the negative control. Two plus (++) and one plus (+) are given based on how close they were to TC soy

Fig. 6 Viable cell densities of the T-flask cultures using the spent medium collected on day 3 of a bioreactor fed-batch run. The spent medium was supplemented with glucose, glutamine, and 1 g l^{-1} of hydrolysates: TC soy, yeastolate, Soy 06, Soy 08, Hypep 1510, Phytone or Hypep 1511. The control was cultivated in the spent medium without hydrolysate supplementation

cell growth as the control. This spent medium hydrolysate supplementation experiment was named the growth promotion test.

The above results were inserted into Table 1 to expand this TF-BR (T-flask-Bioreactor) correlation table. As the table indicated, the growth promotion test fits nicely into the gap left in the table. The poor performance of Hypep 1511 in the 3-l bioreactor evaluation was caused by its poor capability in growth promotion although it showed a high protection potential. Phytone had similar growth promotion capacity as Soy 06. Its poor performance in the bioreactor was primarily due to the combination of its

low protection potential and sub-optimal growth promotion capability. The results suggested that both protection and growth promotion properties of a hydrolysate were the integral parts of its overall capacity to support superior growth and antibody yield in the fed-batch process.

The growth inhibition test

As shown in Table 1, Yeastolate was the hydrolysate that had the closest performance to TC soy among the six plant hydrolysates under evaluation. One may ask whether the yeastolate can perform better if the culture is also supplemented with Hypep 1511? After all, Hypep 1511 showed a high protection potential. A 3-l run was conducted with the addition of yeastolate and Hypep 1511. To our disappointment, no improvement on cell growth was seen as compared to the one using yeastolate only (data not shown). That implied that it was not the lack of protection potential that made the yeastolate-supplemented culture decline earlier at the late stage of the growth (Fig. 5B). This additional factor was the subject of the subsequent study that led to the growth inhibition study. The above results also suggested that the protection potential of a hydrolysate equivalent to that of yeastolate (score of one plus) should be the minimal requirement for our process.

Previously, the experimental data showed the growth inhibition at 5 g l^{-1} level for Soy 06, Soy 08, and TC soy (Fig. 1-B1). In the late stage of the

fed-batch process, the accumulation of the hydrolysate from feeding could reach the inhibition threshold that harmed the culture. This might explain why the late-stage cell growth declined so rapidly in the yeastolate-supplemented culture in Fig. 5B. To verify the hypothesis, the same T-flask experiment design as that in Fig. 1 was conducted. The experiment used the complete medium supplemented with 5 g l^{-1} of yeastolate. The results are shown in Fig. 7 and confirmed the hypothesis. Yeastolate showed stronger growth inhibition on the culture than TC soy. This T-flask experimental setting was named the growth inhibition test.

Based upon the above growth inhibition theory, one may postulate that if the feeding amount of yeastolate was further reduced, the culture should grow better since the associated growth inhibition effects were reduced. This idea was tested in a 3-l bioreactor. The results showed a worsen cell growth (data not shown). It was obvious that the decrease in the feeding amount, although helping alleviate the inhibition effects, may also reduce the protection and the growth promotion effects to the culture.

The screening platform and its application

The above studies helped us understand the roles of hydrolysates in our process. A good hydrolysate was expected to possess high protection potential and growth promotion and to also show low/none growth inhibition effect. The three tests (protection, growth

Fig. 7 Viable cell densities of the T-flask cultures using the complete medium supplemented with 5 g l^{-1} of hydrolysates. Data shown include the control, TC soy, and yeastolate. The control was cultivated without hydrolysate supplementation

promotion, and growth inhibition) can compose a screening platform, as shown in Fig. 8, to evaluate hydrolysates and identify a good replacement for TC soy. Based on all the results on hand, we have defined the protection test and the growth promotion test as the primary screening criteria. If a hydrolysate can obtain a full score in the growth promotion test (comparable to TC soy) and a score equal to or higher than one plus (comparable to yeastolate) in the protection test, it can proceed to be evaluated in the 3-l fed-batch process. Meanwhile, the growth inhibition test will be conducted, serving as a secondary screening criterion. The results from the growth inhibition test can also be used as a reference for feeding dosage optimization in the bioreactor process.

Eight additional soy hydrolysates were evaluated using this platform. The qualitative scores of both growth promotion and protection tests were summarized in Table 2. In the growth promotion test, Soy 09 showed the closest growth to TC soy (full score) while Soy 04, Soy 05 and Soy 10 had mediocre growth (score of two plus). The rest hydrolysates, namely Soy 01, Soy 02, Soy 03 and Soy 07, exhibited poor growth but still better than the control (score of one plus). In the protection test, Soy 03, Soy 09 and Soy 10 showed a strong protection potential. Soy 05 scored lower with two plus but it was higher than Soy 04 and Soy 07 (both with one plus). For comparison, Soy 01 and Soy 02 did not show any protection at all (negative).

Extrapolated from the existing TF-BR correlations (Table 1), the prediction of cell growth in the 3-l bioreactor for all eight soy hydrolysates was included in Table 2. The table predicted that Soy 09 would

Fig. 8 The flow chart of the hydrolysate screening platform, which is composed of the protection test, the growth promotion test, and the growth inhibition test

Table 2 T-flask-bioreactor prediction table

	TF growth promotion test result	TF protection test result	3 l fed-batch (predicted)
Soy 01	+	—	Poor
Soy 02	+	—	Poor
Soy 03	+	+++	Poor
Soy 04	++	+	Medium
Soy 05	++	++	Medium
Soy 07	+	+	Poor
Soy 09	+++	+++	Good
Soy 10	++	+++	Medium

Summary of T-flask protection test and T-flask growth promotion test results and the predicted results in 3-l fed-batch runs of Soy 01, Soy 02, Soy 03, Soy 04, Soy 05, Soy 07, Soy 09, and Soy 10. The scoring method for the T-flask tests was the same as in Table 1

yield the best outcome. Therefore it was selected for further 3-l bioreactor evaluation. Soy 04 was also included in the evaluation for the purpose of theory validation. The same amount of each soy hydrolysate was supplemented in the runs. Figure 9 compares their VCD, viability and antibody yield profiles. As predicted, Soy 09 had a cell growth close to TC soy while Soy 04 supported cell growth only to a peak VCD of $\sim 4 \times 10^6$ cells mL^{-1} , which was approximately one-third of the TC soy counterpart. In addition, the culture declined much earlier, which also fell in line with our prediction. Besides cell growth, the Soy 09 yielded similar antibody productivity as the TC soy.

It is noticed in Fig. 9 that the cell growth for the Soy 09 run was slightly worse than the TC soy run. The peak VCD reached $\sim 1.1 \times 10^7$ cells mL^{-1} , which was a little lower than $\sim 1.3 \times 10^7$ cells mL^{-1} achieved by TC soy supplemented culture. Furthermore, the late stage viability of the Soy 09 culture was a little lower than its TC soy counterpart. This slightly lower cell growth might be enhanced by increasing the feeding of Soy 09 as long as the feeding amount did not exceed the inhibition threshold as per our theory. The supplementation of 1, 1.5 and 2 g l^{-1} of Soy 09 to the culture with the spent medium confirmed the dosage-dependent response of cell growth to Soy 09 (Fig. 10). The results suggested that the feeding of Soy 09 could be optimized by increasing its dosage within the range of 1× to 1.5×.

Fig. 9 Viable cell density, viability and antibody yield results of 3-l fed-batch runs using the same feeding amount of TC soy, Soy 09 and Soy 04. Graph A shows the viable cell density (closed symbols) and concentration (open symbols) for TC soy (● and ○), Soy 09 (■ and □), and Soy 04 (◆ and ◇). Graph B shows the viability for TC soy (●), Soy 09 (■), and Soy 04 (◆)

The growth inhibition test showed that Soy 09 had a similar level of inhibition to cell growth as TC soy and it was much lower than that of yeastolate (data not shown). Based upon the information gained from the above T-flask experiments, 1.25× Soy 09 was ultimately used in the process. This dosage was the results of balancing several scale-up concerns such as the resultant volume increase, excessive foaming, etc. The optimized 3-l run with 1.25× Soy 09 achieved comparable growth and antibody yield with TC soy run as shown in Fig. 11. This was an example of this platform's application on process optimization.

Downstream impact of Soy 09

Once Soy 09 was identified, its impact on downstream purification and product properties was evaluated. If non-favorable impacts were observed,

Fig. 10 Viable cell density results of the Soy 09 dosage study (1, 1.5 and 2 g l⁻¹) in the growth promotion test. The cells were cultivated in the spent medium supplemented with 1, 1.5 or 2 g l⁻¹ Soy 09 or 1 g l⁻¹ TC soy. The control was cultivated in the spent medium without hydrolysate supplementation

Fig. 11 Viable cell density, viability and antibody yield results of Soy 09 dosage study (1× Soy 09 and 1.25× Soy 09) in 3-l fed-batch runs. Graph A shows the viable cell density (closed symbols) and antibody concentration (open symbols) for 1× TC soy (● and ○), 1× Soy 09 (■ and □), and 1.25× Soy 09 (▲ and △). Graph B shows the viability for 1× TC soy (●), 1× Soy 09 (■), and 1.25× Soy 09 (▲)

another round of screening with more candidates had to be conducted. In this study, the 3-l bioreactor harvest using Soy 09 was purified using a scaled-down model of large-scale purification process. A comparability study was conducted on the purified antibodies from the new (Soy 09) and the current (TC soy) processes. The panel of analytical tests consisted of physicochemical assays, such as SEC-HPLC, SDS-PAGE, and IEF, functional assays, such as a binding assay and a potency assay, and structural characterization assays, such as oligosaccharide profiling, C-terminal lysine distribution, and tryptic peptide mapping. The results demonstrated that the products from the new and current processes were comparable (data not shown).

Discussion

In this paper, we reported a T-flask based platform to evaluate and screen plant hydrolysates for the fed-batch cell culture process. It was developed during a study of replacing an existing soy hydrolysate in a fed-batch cell culture process. Cell growth, antibody productivity, and downstream impact need to be evaluated for a potential candidate. In this study, achieving the same cell growth in the process was identified as the most difficult part. The screening platform reported here used cell growth as an initial indicator to pre-screen the plant hydrolysate candidates. This comprehensive, function-oriented screening platform evaluated hydrolysates in three different aspects, namely protection, growth promotion and growth inhibition, to predict their cell culture in fed-batch bioreactors.

Our results (Fig. 3) showed that if unprotected, the cells were susceptible to the fatty acid. Essential fatty acids are critical to mammalian cell proliferation in serum-free medium (Butler and Huzel 1995; Butler et al. 1999). However, delivery and safety are two major issues when using fatty acids in the medium. With the addition of TC soy, the cells can be protected from the cytotoxicity from cyclodextrin-delivered fatty acids. The protection test proposed in this work was able to semi-quantify the protection potential of different hydrolysates.

The protection could be from hydrolysates' anti-oxidative activity that protected the fatty acids from oxidation. It could also be from their growth/survival

factor-like anti-apoptotic activity that protected the cells. It has been reported decades ago about the anti-oxidative activity of soy protein and its hydrolysates (Pratt 1972; Yamaguchi et al. 1975; Hayes et al. 1977). Anti-oxidative peptides were isolated from soy hydrolysates and demonstrated activity against the peroxidation of linoleic acid (Chen et al. 1995). Anti-apoptotic effects of plant hydrolysates were also observed. This effect could be attributed to the growth-, survival-, or protection-factor-like functions of the bioactive peptides in the hydrolysates (Franek and Katinger 2002; Burteau et al. 2003). The proposed protection test did not differentiate which mechanism (anti-oxidative and/or anti-apoptotic) is active. It showed the overall protection effect of a hydrolysate.

Besides the protection effect, our results showed that the growth promotion is another important benefit of hydrolysates to the process. It is well known that protein hydrolysates contain complex low molecular-weight components, including peptides, amino acids, vitamins and trace elements. In some reported applications, the beneficial effects of hydrolysates were interpreted as the source of nutrient supplement, mostly the amino acids (Jan et al. 1994; Schlaeger 1996; Nyberg et al. 1999; Heidemann et al. 2000). Heidemann et al. (2000) investigated a couple of plant hydrolysates in a baby hamster kidney cell perfusion process. The authors concluded that the hydrolysates increased the amino acid contents in the culture. As such, this benefit can be fully delivered by replacing the hydrolysates with fortified medium. Some other studies attributed the growth promotion of hydrolysates more to their bioactive peptides. (Franek et al. 2000; Franek and Katinger 2002; Burteau et al. 2003). In these studies isolated or synthetic peptides were shown to enhance cell growth and productivity. The enhancement was believed to be largely associated with the presence of the intact peptides in the culture. Replacing the peptides with their amino acid counterparts only had a slight promotion of the cell growth.

The mechanism of growth promotion exerted by TC soy in our process was likely more sophisticated than a simple nutritive source can explain. The soy hydrolysate candidates (Soy 01 to Soy 10) were developed purposely to have similar free/total amino acid profiles and carbohydrate profiles with TC soy. Replacing TC soy with some of them (Soy 04, Soy 06 and Soy 08) provided similar amino acid and

carbohydrate contents to the culture. Yet they did not yield the same cell growth (Figs. 2, 9). In all of the 3-l evaluation runs, amino acids, vitamins and trace metals were included in the supplementation cocktail fed to the culture. The spent medium analysis of these runs showed no amino acid deficiency. Therefore, in our case, it is likely the different peptide contents that determined the different growth promotion potential of these hydrolysates.

Each hydrolysate contains hundreds of peptides with varied lengths and amino acid sequences, which makes the identification of the effective peptides impossible. Fractionation of the hydrolysate could narrow down the range of the peptide length but each fraction still contains lots of peptides. Reverse phase chromatography can be used to separate peptides and generate peptide profile by UV detectors. But the separation is typically poor because of the rich contents of hydrolysate peptides. As a result of many overlapping peptide peaks, similar peptide profile does not necessarily represent the similar peptide compositions. Soy 06 and Soy 08 in this study were the examples. They had a similar peptide profile with TC soy but did not demonstrate the equal growth promotion. Therefore, using cell growth as an indicator was a fast and practical approach to differentiate the growth promotion potential of hydrolysates. In this study, the ingenious design of the growth promotion test using spent medium from the bioreactor made the screening of growth promotion potential of hydrolysates easy and quick.

The inhibition of hydrolysates on the cell growth at high concentration was also observed in this study. The accumulated growth-inhibitory portion of a hydrolysate in the fed-batch culture will potentially damage the cells. This demonstrated one of the disadvantages of a complex medium like hydrolysates: their growth-promotion part and the growth-inhibition part cannot be segregated. The growth inhibition of protein hydrolysates at high concentration was reported in literature. In certain cases, suppressing the cell growth at a certain level may even enhance the cell specific productivity (Zhang et al. 1994; Franek and Katinger 2002; Franek et al. 2003). Therefore, the amounts of any hydrolysate added should be carefully optimized so that the growth-inhibitory effect was appropriately balanced with the growth promotion counterpart.

The information gained from these three T-flask tests can be used to guide/predict the bioreactor process. Because of the smaller volume and simpler experimental requirements than bench scale bioreactors, a high throughput screening from a large candidate pool became possible. In this case, a suitable hydrolysate replacement was quickly identified from 14 candidates. It not only facilitated the screening process, but also discovered the properties of each hydrolysate it evaluated. The information is especially useful if a blending strategy must be adopted under the circumstance that no single hydrolysate can bring satisfactory results. Hydrolysates with complementary properties may blend together to achieve synergetic effects. For example, yeastolate provided good growth promotion but it had a high growth inhibition. One may reduce the feeding amount of yeastolate to reduce its inhibition. The resultant reduction in growth promotion and protection capacity then could be compensated from blending with Soy 06. Because of the low growth inhibition nature of Soy 06, the final blending might achieve the same level of overall growth promotion and protection but with lower growth inhibition. Our preliminary results with one blending ratio showed it was promising (data not shown). Different hydrolysates blended together with different ratios could generate a large amount of combinations, which can be pre-screened by the platform quickly.

The platform could also be applied in the quality control area, testing the lot-to-lot variation of hydrolysates. The methodology in developing this platform can apply to other cell lines and processes under appropriately optimized experimental conditions. Depending on the cell line and available techniques, this platform could easily be adapted to shaker flasks or multi-well plates.

Acknowledgement We sincerely thank BD Biosciences and Kerry Bio-Science for the supply of hydrolysate samples for evaluation.

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