Preparative Biochemistry and Biotechnology

ISSN: 1082-6068 (Print) 1532-2297 (Online) Journal homepage: http://www.tandfonline.com/loi/lpbb20

Scale-up of naringinase production process based on the constant oxygen transfer rate for a novel strain of Bacillus methylotrophicus

Keyur Raval, Kartik Gehlot & Prasanna B. D.

To cite this article: Keyur Raval, Kartik Gehlot & Prasanna B. D. (2017) Scale-up of naringinase production process based on the constant oxygen transfer rate for a novel strain of Bacillus methylotrophicus, Preparative Biochemistry and Biotechnology, 47:2, 192-198, DOI: 10.1080/10826068.2016.1201680

To link to this article: http://dx.doi.org/10.1080/10826068.2016.1201680

PREPARATIVE BIOCHEMISTRY AND BIOTECHNOLOGY
2017, VOL. 47, NO. 2, 192–198
http://dx.doi.org/10.1080/10826068.2016.1201680

Taylor & Francis
Taylor & Francis Group

Scale-up of naringinase production process based on the constant oxygen transfer rate for a novel strain of *Bacillus methylotrophicus*

Keyur Raval, Kartik Gehlot, and Prasanna B. D.

Department of Chemical Engineering, National Institute of Technology Karnataka, Surathkal, Mangalore

ABSTRACT

Naringinase bioprocess based on *Bacillus methylotrophicus* was successfully scaled up based on constant oxygen transfer rate (OTR) as the scale-up criterion from 5-L bioreactor to 20-L bioreactor. OTR was measured in 5 and 20-L bioreactor under various operating conditions using dynamic method. The operating conditions, where complete dispersion was observed were identified. The highest OTR of 0.035 and 0.04 mMol/L/s was observed in 5 and 20-L bioreactor, respectively. Critical dissolved oxygen concentration of novel isolated strain *B. methylotrophicus* was found to be 20% of oxygen saturation in optimized medium. The *B. methylotrophicus* cells grown on sucrose had maximum oxygen uptake rate of 0.14 mMol/L/s in optimized growth medium. The cells produced the maximum naringinase activity of 751 and 778 U/L at 34 hr in 5 and 20-L bioreactors, respectively. The maximum specific growth rate of about 0.178/hr was observed at both the scales of operations. The maximum naringinase yield of 160 and 164 U/g biomass was observed in 5 and 20-L bioreactors, respectively. The growth and production profiles at both scales were similar indicating successful scale-up strategy for *B. methylotrophicus* culture.

KEYWORDS

Naringinase; OTR similarity; scale-up; stirred-tank bioreactor

Introduction

Naringin (4′,5,7-trihydroxyflavonone-7-rhamnoglycoside) is a flavonone glycoside and imparts bitterness to several citrus fruit juices. The citrus fruit juice industries are currently dealing with removal of naringin and, thereby, improve the quality of the fruit juice. The threshold value of the naringin to impart bitterness is about 50 mg/L.[1] Although, naringin can be removed from fruit juices by chemical methods, it has inherent disadvantages of the change of the properties of the fruit juice, chemical impurities, and so on. Therefore, there is a need to remove naringin by other means. An enzymatic approach of degradation of naringin seems sustainable and promising.[2] Naringin is hydrolyzed by an enzyme naringinase (EC 3.2.1.40) which is an α-rhamnopyranosidase, possess α-L-rhamnosidase (E.C. 3.2.1.40) and β-D-Glucosidase (E.C. 3.2.1.21) activities.[3,4] Moreover, it is also being used to enhance aroma of wine, production of chlorosporin antibiotics, biotransformation of steroids, preparation of rhamnose, pruning, and glycolipids.

Naringinase was discovered in 1938, in the isolates from celery leaves[5] and in grapefruit leaves.[4] Later, this promising enzyme was isolated from various microbial sources. Puri published a detailed review of biotechnological aspects of naringinase.[6] However, the reports related to bioprocess development and scale-up for naringinase production are mostly related to fungal sources. Bram and Solomons used 10-L bioreactor to enhance the production of naringinase enzyme by *Aspergillus niger*.[7] Puri et al. produced extracellular naringinase from *A. niger* MTCC 1344 in a complex medium with molasses and peptone as carbon and nitrogen sources, respectively in a 7-L stirred-tank reactor.[8] Birgisson et al. immobilized recombinant *Escherichia coli* producing thermostable α-L-rhamnosidase and investigated naringin hydrolysis in a bioreactor.[9]

The literature available on the production of naringinase in stirred-tank bioreactors from bacterial sources is scarce. Puri and Banerjee[10] and Puri et al.[11] investigated naringinase production from *Staphylococcus xylosus* MAK2 in a stirred-tank reactor and reported the maximum naringinase production of 8.25 IU/mL at 34th hr. Usually, the yield of biological compounds is good at small scale and tends to decrease as the scale of production increases, which ultimately increases the cost.[12] The cost of production can be decreased by a suitable scale-up strategy in such a way that the yield of the biological compound is same at large-scale. Moreover, the scale-up from laboratory scale to pilot scale and further to industrial scale requires scale-up parameters to be well established at each level of scale-up. Deive et al. and Lopez et al. demonstrated the importance of scale-up parameters very well in their research work on producing biomolecules using extremophiles.[13,14]

When shear stress is not a cause of concern, such as in the case of most of the bacterial cultures; the performance of aerobic bioprocess depends solely upon oxygen supply to the broth because of dependency of growth rate on oxygen transfer rate (OTR).[12] Moreover, the OTR in the broth is governed by volumetric mass transfer coefficient. The rate at which various metabolic pathways function in aerobic systems largely depend on oxygen availability to the system which is well explained by Fu et al. who demonstrated the hypoxia effect of scale-up of a *Saccharomyces cerevisiae* high-cell density

CONTACT Keyur Raval keyurnraval@nitk.ac.in Department of Chemical Engineering, National Institute of Technology Karnataka, Surathkal, P.O. Srinivasnagar, Mangalore - 575025, India.

Supplemental data for this article can be accessed on the publisher's website at http://dx.doi.org/10.1080/10826068.2016.1201680.

fed-batch biopharmaceutical process. They concluded that homogeneous distribution of dissolved oxygen plays a crucial role for growth and product formation.[15] Constant OTR as the scale-up criterion imparts similar OTRs at two different scales of operation.[16] The constant OTR as scale-up criterion has been successfully applied for Xanthan gum production,[17] biosurfactant production, biodesulfurization, high-cell density industrial-scale biopharmaceutical process development,[15] and other bioprocesses.[14,18] Thus, constant OTR is indispensable parameter for successful scale-up of aerobic bioprocesses.

Mukund et al. discovered and characterized gram positive, rod shaped, and sporulating bacterium *Bacillus methylotrophicus*.[19] It is generally found in rhizospheres of soil. It imparts in the growth of plant and enhances nitrification–denitrification process.[20] Mukund et al. optimized culture conditions and obtained the highest naringinase activity of 12 U/L which was 50% more than the activity obtained without optimization.[19] In this manuscript, the research of Mukund et al. is extended and the naringinase bioprocess is scaled up to 20-L stirred-tank bioreactor. Constant OTR is chosen as the scale-up criterion for naringinase production using previously isolated *B. methylotrophicus*.

Materials and methods

All the chemicals were purchased either from Merck India Ltd, HiMedia Ltd, or Sigma Aldrich India. The bacterial source *B. methylotrophicus* was obtained from Biotechnology Laboratory of Department of Chemical Engineering, NITK, Surathkal. Pure culture of the isolate was maintained in Sabouraud dextrose agar/broth at 4°C. All experiments were conducted in duplicate, and average values are plotted in graphs. Whenever errors are exceeding more than 10% of average value; it is explained in the text. Error bars are shown, as and when required, to reduce crowding effect in the plots.

Shake-flask studies

The shake-flask studies were performed as mentioned by Mukund et al.[19] Two loops of pure culture from petri plate were added in a 100-mL conical flask having 30-mL medium. The flask was incubated at 30°C and 150 rpm for 24 hr. Seed culture of *B. methylotrophicus* was prepared by adding 1.5 mL of 24-hr-old pure culture in 30-mL Sabouraud dextrose broth. Naringinase production from *B. methylotrophicus* was observed in an optimized medium that comprised of the following components: Naringin, 0.68 g/L; Sucrose, 12.5 g/L; Yeast Extract, 4 g/L; NaCl, 0.5 g/L; $MgSO_4$, 0.5 g/L; KH_2PO_4, 1 g/L. Initial pH of the medium was 6. Thirty milliliters of production medium was inoculated with 4% of 12-hr-old seed culture in a 100-mL conical flask at 150 rpm. Samples were withdrawn at every 4 hr interval for determination of biomass concentration and enzyme activity.

Stirred-tank bioreactor studies

The available 5 and 20-L *in situ* sterilizable stirred-tank bioreactors (Scigenics India Ltd.) were used for establishing batch culture for production of *B. methylotrophicus*. Inoculum percentage and age were kept same as that mentioned by Mukund et al.,[19] i.e., 2% and 48 hr, respectively. The dimensions of both the bioreactors are listed in Table 1.

Table 1. Dimensions of 5 and 20-L stirred-tank bioreactors.

Dimension	5-L Bioreactor	20-L Bioreactor
Total volume, V_T (L)	5	20
Working volume, V_L (L)	3	14
Vessel height, H_T (m)	0.23	0.48
Liquid height, H_L (m)	0.15	0.32
Vessel diameter, D_T (m)	0.16	0.237
Aspect ratio (H_T/D_T)	1.4375	2.025
Impeller type	Flat blade disc turbine	Flat blade disc turbine
Number of impellers	2	3
Impeller diameter, D_i (mm)	52	65
Impeller thickness, T_i (mm)	3.56	16.2
Impeller width, W_i (mm)	11	12.9
Ratio impeller to vessel diameter	0.325	0.274
Top impeller distance from top plate (m)	0.26	0.47
Spacing between impeller (m)	0.125	0.129
Maximum agitator speed (rpm)	1,320	1,320
Sparger type	Ring	Ring
Sparger diameter, Ds (m)	0.044	0.063
Sparger distance from bottom impeller (m)	0.01	0.034
Baffles	4	4

Determination of critical dissolved oxygen concentration

Critical dissolved oxygen concentration was determined, because it is a prerequisite of dynamic gassing-out method that the dissolved oxygen of the fermentation broth should not drop below critical value, so the constant microbial oxygen uptake is maintained. Dissolved oxygen of broth was measured in real time using sterilizable galvanic oxygen electrode (Mettle Toledo, series 6800). An average electrode response time of 9 ± 0.5 s was observed. The critical dissolved oxygen concentration was determined as per the method given by Bandyopadhyay et al.[21] A 5-L stirred-tank bioreactor was used with 3 L volume of optimized medium as mentioned above under the heading of "shake-flask studies". The *B. methylotrophicus* strain was grown at 500 rpm and 1 vvm. The dissolved oxygen was maintained at 80% saturation. The aeration was stopped when cells were in the exponential growth phase and decrease in dissolved oxygen profile was observed. The profile of the decrease in dissolved oxygen was linear with respect to time up to a certain concentration of dissolved oxygen. Below this concentration, the decrease in dissolved oxygen became nonlinear. The value of dissolved oxygen concentration at which the decrease in dissolved oxygen concentration became nonlinear was chosen as the critical dissolved oxygen concentration.

Determination of volumetric mass transfer coefficient

Dynamic method was used for the determination of OTR in stirred-tank bioreactors.[12,21] The rate of change of dissolved oxygen concentration in a broth can be explained by

$$\frac{dC_l}{dt} = k_L a(C^* - C_l) - q_{O_2} X \quad (1)$$

where C^* = saturation oxygen concentration (mMol/L)

C_l = actual oxygen concentration at time t (mMol/L)
X = dry cell weight (g/L)
q_{O_2} = specific oxygen uptake rate [(mMol of oxygen/gram of biomass)/s]

k_La = volumetric oxygen mass transfer coefficient (1/s) where the first term on the right-hand side of Eq. (1) is the OTR and the second term is the oxygen uptake rate (OUR) of the culture. The OTR and OUR were measured in two-step process. In the first step, the aeration was stopped and the decrease in dissolved oxygen profile with time was measured by oxygen electrode. The OUR was determined using the measured dissolved oxygen profile by rearranging Eq. (1) as given below

$$\frac{dC_l}{dt} = -q_{O_2}X = \text{OUR} \tag{2}$$

In the second step, the culture broth was aerated again at desired flow rate, and the increase in the dissolved oxygen concentration was monitored. Under these conditions Eq. (1) can be rearranged to following linear relationship

$$C_l = C^* - \left[\left(\frac{1}{k_La}\right)\left(\frac{dC_l}{dt} + q_{O_2}\cdot X\right)\right] \tag{3}$$

From Eq. (3), the plot of C_l v/s $\left(\frac{dC_l}{dt} + q_{O_2}\ldots X\right)$ will result in a straight line which has the slope of $(-1/k_La)$ and the Y-axis intercept of C^*. The OTR then can be determined from the values of k_La. This technique was used to measure volumetric mass transfer coefficient under different operating conditions.

Estimation of naringinase activity

The naringinase activity was estimated using a modified Davis method[22] as described by Mukund et al.[19] The culture broth was centrifuged at 10,000g for 10 min at 4°C, and the supernatant was collected. The supernatant (0.1 mL) was mixed with 0.9 mL of 0.05% (w/v) naringin dissolved in 0.1 M sodium acetate buffer of pH 4.0. The solution was mixed for 30 min at 50°C, and the reaction was terminated by the addition of 0.5 mL of trichloroacetic acid (10%, w/v). Then 0.1 mL of the reaction mixture was added to 5 mL of 90% (v/v) diethylene glycol followed by the addition of 0.1 mL NaOH (4 N). This solution was kept for color development at room temperature for 10 min. The resulting yellow color was measured at 420 nm. One unit (U) of naringinase activity was defined as the amount of enzyme that hydrolyze 1 mMol naringin per minute under the assay conditions.

Estimation of biomass concentration

Biomass was estimated from a standard graph of optical density versus cell mass. Optical density of culture was measured using UV–vis spectrophotometer (LABOMED Inc.) at 600 nm. Biomass was measured by centrifuging the fermented broth in preweighed centrifuge tubes at 10,000 rpm at 4°C for 15 min. Tubes containing the cell pellet was dried at 60°C for 12 hr and dry biomass was weighed.

Results and discussion

Figure 1 represents dissolved oxygen profile of *B. methylotrophicus* strain in optimized medium after aeration was stopped. The OUR of cells was linear till 18–20% dissolved oxygen as indicated by the arrow in Figure 1. The OUR became non-linear immediately after this value because of the onset of dissolved oxygen values reaching below critical value. Therefore, to avoid cell respiration experiencing the critical region, 20% was considered as the critical dissolved oxygen requirement for *B. methylotrophicus*.

Figure 2 represents k_La values obtained under different operating conditions in 5 and 20-L bioreactor. For both reactor sizes, as the agitation speed increased, the volumetric mass transfer coefficient increased. The k_La values ranged from 0 to 0.04/s. The k_La values obtained here are comparable with the values obtained in other literature based on dynamic gassing-out method.[16] All data points followed a pattern of increase in k_La with the increase in revolution speed, except few data points which are not connected with other data points by line. These scattered data points represented the condition where incomplete dispersion of air was observed. Nienow et al. established the following correlation to identify the impeller speed above which the complete dispersion was observed.[23]

$$(Fl_g)_{CD} = 0.2\cdot Fr_{CD}^{0.5}\cdot\left(\frac{D}{T}\right)^{0.5} \tag{5}$$

where
$(Fl)_g$ = gas flow number = Q_g/ND^3
$(Fr)_{CD}$ = Froude number = N^2D/g
Q_g = gas flow rate (m^3/s)
N = revolutions per second
D = diameter of impeller (m)
g = gravitational constant (9.81 m/s^2)

Based on the above equation, operating conditions at which complete dispersion of air bubbles in broth may not prevail were identified. The scattered data of Figure 2, which are not connected with line represent these operating conditions. The irregular hydrodynamic behavior for these operating conditions can be explained by the correlation developed by Kapic and Heindel based on 282 data sets of well-known publications on volumetric mass transfer coefficient using dynamic gassing-out method. Kapic and Heindel developed the following correlation for scale-up based on constant volumetric mass transfer coefficient,[16]

$$\frac{k_La}{U_g^{0.93}} = 1.59\cdot\left(\frac{N}{N_{CD}}\right)^{1.342}\cdot\left(\frac{T}{D}\right)^{0.415} \tag{6}$$

where
U_g = superficial gas velocity (m/s)
N = impeller revolutions per second
N_{CD} = impeller revolutions required per second for complete dispersion
T = Tank diameter (m)
D = impeller diameter (m)

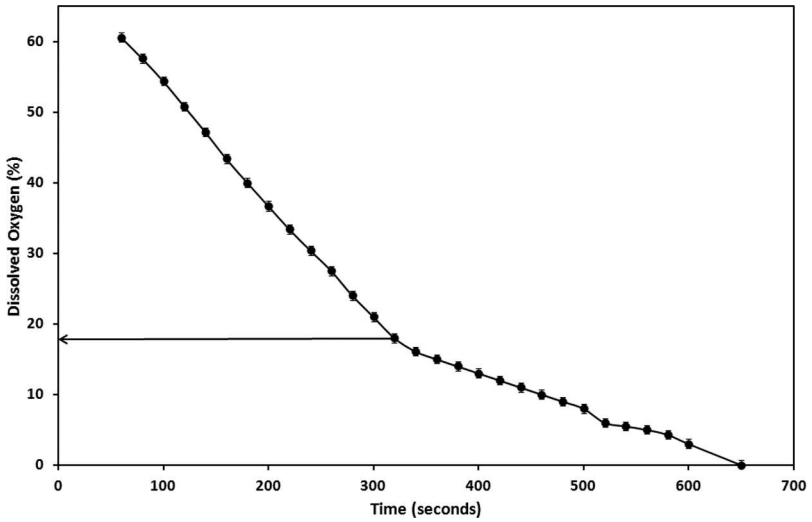

Figure 1. Decrease in dissolved oxygen profile of *B. methylotrophicus* strain after aeration was stopped. The horizontal line shows the point from where the decrease was nonlinear. The value of dissolved oxygen at this point was considered as the critical dissolved oxygen concentration.

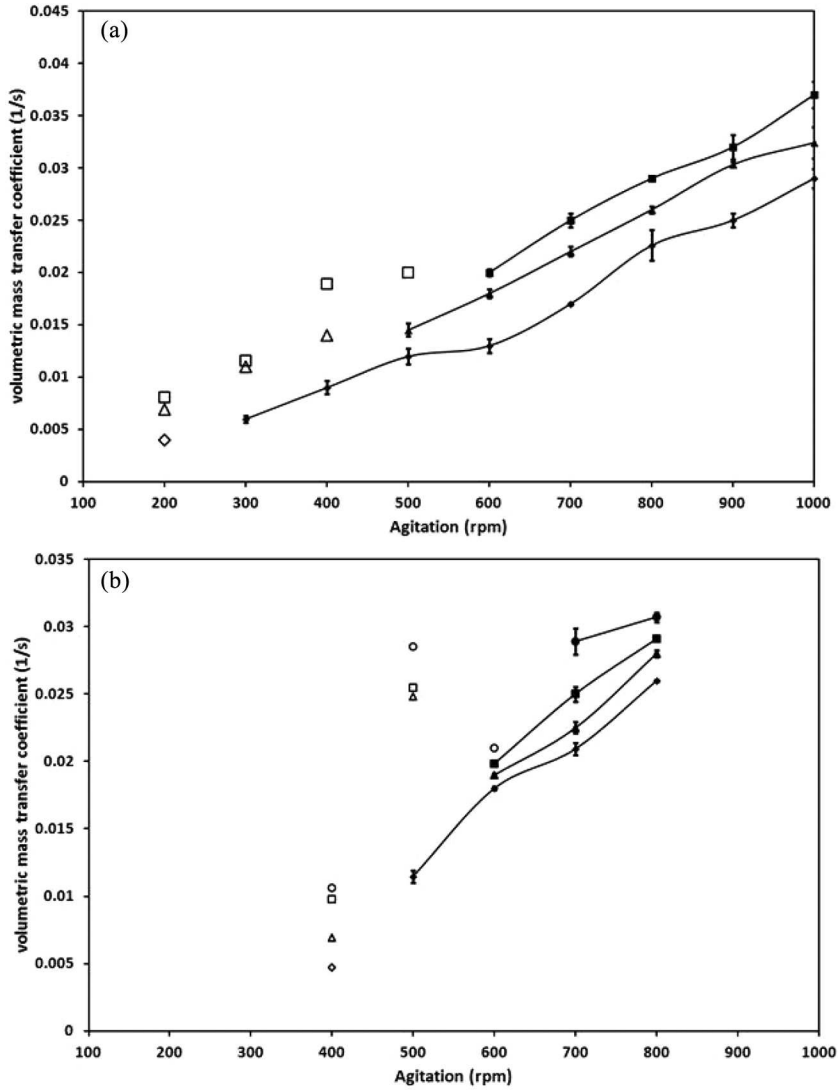

Figure 2. k_La values in 5 and 20-L bioreactor. (a) shows values of k_La at 1.5 LPM (◆), 3.5 LPM (▲), and 4.5 LPM (■) and (b) shows values of k_La for 20-L bioreactor at 8 LPM (◆), 10 LPM (▲), 12 LPM (■), and 14 LPM (●). The data points at which complete dispersion was not observed as per Nienow et al. are marked with open symbols and are not connected with lines.

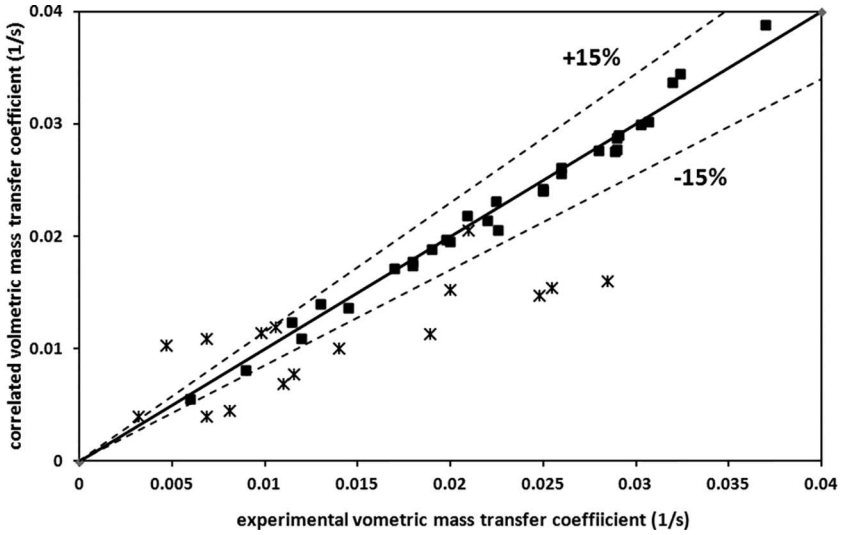

Figure 3. Parity plot of k_La obtained as per Eq. (5) versus experimentally determined k_La. The data points marked with cross (*) sign are the ones where nonuniform dispersion can be observed as per Eq. (5).

Equation (6) is valid for tank diameters up to 2.3 m and for impeller speeds greater than that required for complete dispersion as calculated by Eq. (5). Above correlation was used to compare k_La for all operating conditions investigated in this study. The calculated values of k_La and experimentally obtained k_La values are compared in Figure 3.

All the data points represented by "solid squares" correlate very well within ±15% error suggesting that the k_La obtained in this work was similar to published research work. Moreover, k_La was proportional to $N^{1.34}$ in Eq. (6). In the current research work, dependency of k_La was found out to be $N^{1.35}$ (supplementary Figure 1). However, as shown in Figure 3, few data points represented by "*" shape, where complete dispersion was not observed did not correlate well with the Eq. (6). Therefore, these operating conditions were omitted for scale-up studies.

The growth, naringinase production, and OUR were then investigated in optimized medium developed by Mukund et al. using 5-L bioreactor.[19] For simplicity, the growth profile and the OUR of *B. methylotrophicus* are represented in Figure 4.

The OUR increased during exponential growth phase and then decreased during late growth phase and stationary phase. An ideal OUR profile is bell shaped.[24] In this study, a couple of interesting phases were observed along with a bell-shaped curve. As Figure 4 depicts, the increase in OUR was gradual in between 3rd and 11th hr which is a characteristic of either product inhibition or less amount of inoculums.[24] A bell-shaped curve was observed between 12th and 20th hr after which a slow decrease in OUR was observed. This slow decrease in stationary phase can be characterized by substrate limitation other than the carbon source.[24] Interestingly, the inoculum and media were already optimized for this organism by statistical method in one of the previously published articles, where biomass and naringinase activity were considered as optimization parameters.[19] As discussed in

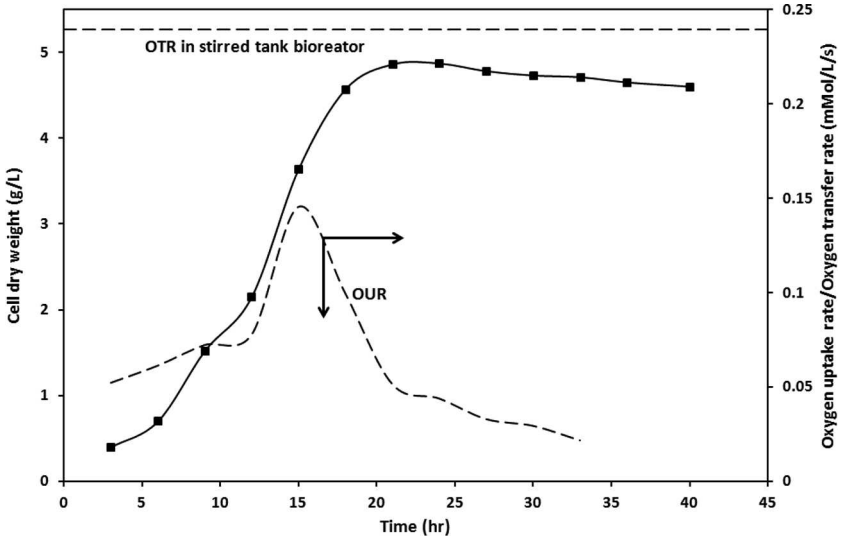

Figure 4. Growth profile and oxygen uptake rate of *B. methylotrophicus* in 5-L bioreactor at 3.5 LPM aeration rate, 3 L of filling volume, 500 rpm. The solid line indicates biomass, the bell-shaped broken line indicates oxygen uptake rate (OUR), and horizontal broken line indicates maximum oxygen transfer rate obtained at given operating condition. Arrow lines indicate reference of OUR to the secondary *Y*-axis.

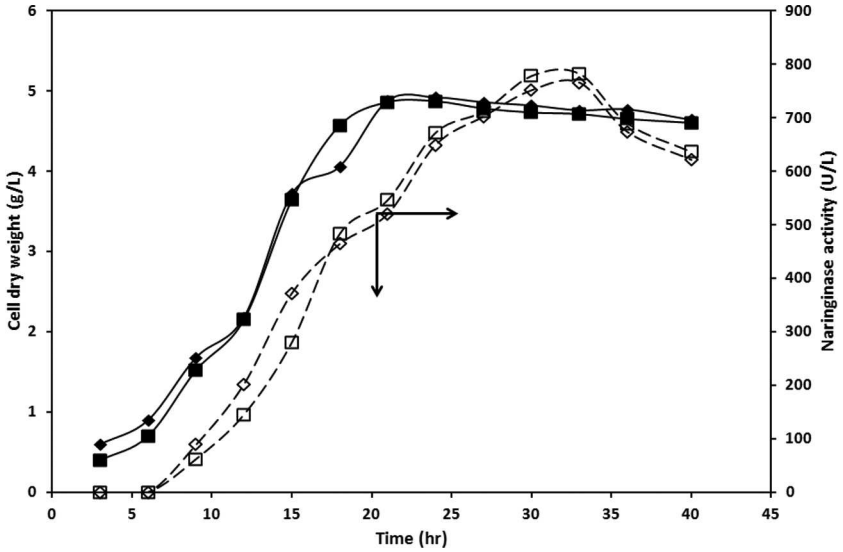

Figure 5. Biomass (solid symbols) and enzyme activity (open symbols) profile in 5-L (■, (□) and 20-L (◆, ◇) stirred-tank bioreactor at constant k_La. Arrow lines indicate naringinase activity reference to the secondary *Y*-axis.

the text above, the presented results reveal that the shape of OUR curve can give valuable insight into microbial growth. The shape of the OUR curve can be compared with the standard bell-shaped curve as mentioned by Anderlei et al.[24] If the OUR curve is not bell shaped then the probable reasons can be identified as mentioned by Anderlei et al. and rectified. Thus, shape of OUR curve can be utilized as one of the response parameters for optimization of medium and operating conditions.[24] The horizontal dotted line in Figure 4 indicates maximum OTR obtained in stirred-tank bioreactors of size 5 and 20 L. The maximum OTR was well above the maximum OUR of *B. methylotrophicus* indicating culture was maintained well above critical dissolved oxygen level.

As mentioned above in the text, the OTR had to be maintained well above maximum OUR so that microbial culture does not enter into critical dissolved oxygen region. As shown in Figure 2, k_La of about 0.03/s was obtained in the 5-L bioreactor at 900 rpm and 3.5 LPM aeration giving maximum OTR of 0.24 mMol/L/s. Almost similar k_La was maintained in 20-L bioreactor by operating at 700 rpm and 14 LPM. The maximum OTR obtained at this k_La value was well above the maximum OUR of 0.145 mMol/L/s obtained in the given medium. Therefore, to eliminate possibility of *B. methylotrophicus* culture growing below critical dissolved oxygen region; these operating conditions were chosen at 20-L scale bioreactor.

Figure 5 compares the growth and production profile of naringinase from *B. methylotrophicus* at 5 and 20-L bioreactor. The growth and production profiles were similar and bioprocess was successfully scaled up. The maximum specific growth rate achieved in optimized medium was 0.178/hr. The maximum yield of naringinase on biomass was 160 and 164 U/g for 5 and 20-L bioreactor, respectively. Bram and Solomons reported that naringinase production process scale up to 10-L bioreactor using *A. niger*.[7] Puri et al. produced naringinase using *Stephylococcus xylosus* MAK2. However, they used citrus peel powder as one of the inducers in the 5-L bioreactor and obtained maximum specific naringinase activity of 1,730 U/g dry weight.[11] Mukund et al. also reported the scale-up of naringinase production process to 3-L scale using the *B. methylotrophicus* and obtained 12 U/L naringinase activity at 48 hr.[19] However, naringinase production was scaled up directly from shake flask to stirred-tank bioreactors in all the three reports mentioned above without choosing any scale-up criteria. This is the first report of naringinase production up to 20-L-scale bioreactor based on the standard scale-up criterion of constant OTR, to the best of our knowledge. The yields reported here are less than that reported by Puri et al.[11] However, the determination of critical dissolved oxygen concentration and OUR for *B. methylotrophicus* and approach of using OTR and OUR for scale-up will help in future investigations.

Conclusion

This is the first report of successful scale-up approach of naringinase production process using *B. methylotrophicus* from 5-L bench-top stirred-tank bioreactor to 20-L stirred-tank bioreactor. Constant OTR was chosen as the criterion for scale-up from 5 to 20-L-scale bioreactor. Critical dissolved oxygen level of *B. methylotrophicus* was investigated. OTR under different operating conditions was measured. The OUR of *B. methylotrophicus* was also measured at different time intervals of the growth. The results revealed that OUR can give a valuable insight to the growth of organism and can be utilized as one of the parameters for medium development and optimization. The bioprocess was successfully scaled up and maximum specific naringinase activity of 164 U/g dry weight of the cells was obtained after 34 hr of culture time in the stirred-tank reactor of the size 20 L.

References

[1] Puri, M.; Marwaha, S.; Kothari, R. Studies on the Applicability of Alginate-entrapped Naringiase for the Debittering of Kinnow Juice. *Enzyme Microb. Technol.* **1996**, *18*(4), 281–285.

[2] Nunes, M.A.; Rosa, M.E.; Fernandes, P.C.; Ribeiro, M.H. Operational Stability of Naringinase PVA Lens-shaped Microparticles in Batch Stirred Reactors and Mini Packed Bed Reactors-one Step Closer to Industry. *Bioresour. Technol.* **2014**, *164*, 362–370.
[3] Roitner, M.; Schalkhammer, T.; Pittner, F. Characterisation of Naringinase from *Aspergillus niger*. *Monatshefte für Chemie/Chem. Monthly* **1984**, *115*(10), 1255–1267.
[4] Thomas, D.; Smythe, C.; Labbee, M. Enzymatic Hydrolysis of Naringin, the Bitter Principle of Grapefruits. *J. Food Sci.* **1958**, *23*(6), 591–598.
[5] Hall, D. A New Enzyme of the Glycosidase Type. *Chem. Ind.* **1938**, *57*, 473.
[6] Puri, M. Updates on Naringinase: Structural and Biotechnological Aspects. *Appl. Microbiol. Biotechnol.* **2012**, *93*(1), 49–60.
[7] Bram, B.; Solomons, G. Production of the Enzyme Naringinase by *Aspergillus niger*. *Appl. Microbiol.* **1965**, *13*(6), 842–845.
[8] Puri, M.; Banerjee, A.; Banerjee, U. Optimization of Process Parameters for the Production of Naringinase by *Aspergillus niger* MTCC 1344. *Process Biochem.* **2005**, *40*(1), 195–201.
[9] Birgisson, H.; Wheat, J.O.; Hreggvidsson, G.O.; Kristjansson, J.K.; Mattiasson, B. Immobilization of a Recombinant *Escherichia coli* Producing a Thermostable α-L-Rhamnosidase: Creation of a Bioreactor for Hydrolyses of Naringin. *Enzyme Microb. Technol.* **2007**, *40*(5), 1181–1187.
[10] Puri, M.; Banerjee, U.C. Production, Purification, and Characterization of the Debittering Enzyme Naringinase. *Biotechnol. Adv.* **2000**, *18*(3), 207–217.
[11] Puri, M.; Kaur, A.; Barrow, C.J.; Singh, R.S. Citrus Peel Influences the Production of an Extracellular Naringinase by *Staphylococcus xylosus* MAK2 in a Stirred Tank Reactor. *Appl. Microbiol. Biotechnol.* **2011**, *89*(3), 715–722.
[12] Bailey, J.E.; Ollis, D.F. *Biochemical Engineering Fundamentals*; Chemical Engineering Education, McGraw-Hill Book Company, New York, USA, 1976.
[13] Deive, F.J.; López, E.; Rodriguez, A.; Longo, M.A.; Sanromán, M. Targeting the Production of Biomolecules by Extremophiles at Bioreactor Scale. *Chem. Eng. Technol.* **2012**, *35*(9), 1565–1575.
[14] López, E.; Domínguez, B.; Deive, F.J.; Sanromán, M.Á.; Longo, M.A. Scaling-up the Production of Thermostable Lipolytic Enzymes from *Thermus aquaticus* YT1. *Bioprocess Biosyst. Eng.* **2012**, *35*(6), 1011–1022.
[15] Fu, Z.; Verderame, T.D.; Leighton, J.M.; Sampey, B.P.; Appelbaum, E.R.; Patel, P.S.; Aon, J.C. Exometabolome Analysis Reveals Hypoxia at the Up-scaling of a *Saccharomyces cerevisiae* High-cell Density Fed-batch Biopharmaceutical Process. *Microb. Cell Fact.* **2014**, *13*(1), 1–22.
[16] Kapic, A.; Heindel, T. Correlating Gas-liquid Mass Transfer in a Stirred-tank Reactor. *Chem. Eng. Res. Des.* **2006**, *84*(3), 239–245.
[17] Garcıa-Ochoa, F.; Santos, V.; Casas, J.; Gomez, E. Xanthan Gum: Production, Recovery, and Properties. *Biotechnol. Adv.* **2000**, *18*(7), 549–579.
[18] Garcia-Ochoa, F.; Gomez, E. Bioreactor Scale-up and Oxygen Transfer Rate in Microbial Processes: An Overview. *Biotechnol. Adv.* **2009**, *27*(2), 153–176.
[19] Mukund, P.; Belur, P.D.; Saidutta, M. Production of Naringinase from a New Soil Isolate, *Bacillus methylotrophicus*: Isolation, Optimization and Scale-up Studies. *Prep. Biochem. Biotechnol.* **2014**, *44*(2), 146–163.
[20] Zhang, Q.-L.; Liu, Y.; Ai, G.-M.; Miao, L.-L.; Zheng, H.-Y.; Liu, Z.-P. The Characteristics of a Novel Heterotrophic Nitrification–aerobic Denitrification Bacterium, *Bacillus methylotrophicus* Strain L7. *Bioresour. Technol.* **2012**, *108*, 35–44.
[21] Bandyopadhyay, B.; Humphrey, A.; Taguchi, H. Dynamic Measurement of the Volumetric Oxygen Transfer Coefficient in Fermentation Systems. *Biotechnol. Bioeng.* **1967**, *9*(4), 533–544.
[22] Davis, W. Determination of Flavanones in Citrus Fruits. *Anal. Chem.* **1947**, *19*(7), 476–478.
[23] Nienow, A.; Wisdom, D.; Middleton, J. *The Effect of Scale and Geometry on Flooding, Recirculation and Power in Gassed Stirred Vessels*. 2nd European Conference on Mixing, Cambridge: England, 1977, pp. 1–16.
[24] Anderlei, T.; Zang, W.; Papaspyrou, M.; Büchs, J. Online Respiration Activity Measurement (OTR, CTR, RQ) in Shake Flasks. *Biochem. Eng. J.* **2004**, *17*(3), 187–194.