Reverse–Spin® Technology — Innovative Principle of Microbial Cultivation

Medical–Biological Research & Technologies

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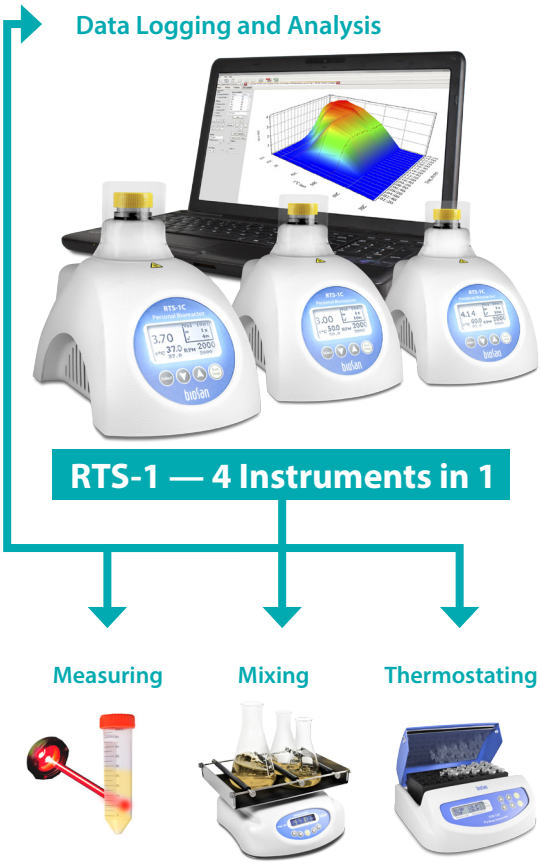
Introduction

This paper presents theoretical and experimental studies of microorganism growth using Reverse-Spin® mixing principle (RS). Reverse–Spinner — is a microbioreactor that applies non-invasive, mechanically driven, low energy consumption, original type of agitation where cell suspension is mixed by the single-use tube (bioreactor) rotation around its axis with a change of direction of rotation motion resulting in highly efficient mixing and oxygenation for cell growth.

Present work is the first to show experimental results of cell growth kinetics obtained by using single-use falcon tubes agitated on a Reverse–Spin® mixing principle. Growth conditions for several model microorganisms like facultative anaerobic *E.coli* and *B.subtilis*, extreme aerobic microorganism *Thermophillus sp.*, microaerophilic *L.acidophilus*, and methylotrophic yeast *P.pastoris* have been optimized. Scientific and applied valuable aspects of single–use personal bioreactors and their potential niche in different biotechnological fields are discussed.

The principles of mixing solutions are among one of the key fields in Bioengineering science. Area of mixing is not limited to bioreactors — mixing is also essential in the study of biochemical and molecular biological processes. Noninvasive mixing technology includes a different way of tubes agitation as shown in the Table 1.

Absence of invasive agitators inside the reactor enables to use Reverse Spinner as a rotating biomass registration device, which measures turbidity of the sample in real time. Intuitive software makes it possible to set optimal parameters of fermentation, registers and logs all parameters (mixing intensity in rpm and Reverse Spin Time (RST), temperature, specific growth rate and biomass in OD_{600} or other units, e.g. g/l).



#	Icon	Motion	Instrument	Max. V
1		Orbital		0.1–5 l
2		Rocking		1–100 l
3		Overhead Rotation		1–50 ml
4		Reciprocal (Hand-type)		1–50 ml
5		Vortex		1–50 ml
6		Reverse Spinning		1–2000 ml

Table 1. Comparison of Non invasive mixing methods

Initiation of the Reverse–Spin® mixing (RS) and depth of the Vortex cave depend on - 1) angular speed of the bioreactor vessel 2) time from initiating rotation 3) RS 4) growth media viscosity 4) temperature. These parameters, also, determine the angular speed of rotating Vortex Layer (VL) and tran¬sition state from the Irrotational Vortex (IRV), when angular speed of the VL is proportional to the radius, to the Rotational Vortex, when the angular speed of the VL is uniform and looks like a monolithic Vortex cavity, as shown in Figures 1-2. Common rules regulating Vortex type mixing processes may be stated as follows: the more time has passed since Vortex formation, the more obvious is the transition from IRV to the RV. In other words, mixing of the fluid media is carried out by rotation, and periodical change of the rotation direction. Rotation multiple times increases the contact area of liquid/gaseous phase and change of the direction of rotation acts as a uniform flow disruptor. These factors significantly increase the efficiency of the liquid mixing as well as liquid-gas interface. Thus, the liquid saturation with gas and gas solubility takes place with greater efficiency than in most standard mixing devices. The concept of the Reverse–Spin® mixing is based on these assumptions.

By exploiting centrifugal forces, bubbles that are created by mixing are pushed into the interface between liquid and gaseous phases, as illustrated in Figure 3, as well as other RTS advantages over shake flasks are described in Figure 4. Small amount of bubbles and the Reverse–Spin® mixing principle allows to use RTS as a biomass register/monitoring device. The final concentrations of *E.coli* cells in rich broth media's significantly exceed 1 OD_{600}, which requires stopping the process of growing cells, with further sterile aliquoting and dilution. This makes the process of growing cells and controlling their concentration very difficult to reproduce. The problem lies in the fact that the turbidimetric coefficients, unlike molar extinction coefficients, are not linear. The behavior of light in dense cell suspensions in 50 ml falcon tubes, as shown in Figure 5, is very interesting and at more than 2 OD_{600} it is almost impossible to measure the concentration of cells directly (unless the Rayleigh scattering is measured). We approached this problem from a different side. The same as in a 10 mm cuvette, when a certain sample concentration is reached, light cannot pass to the photometer's detector and it is required to dilute the sample to the range of 0-0,4 OD_{600}. As shown in figure 6, in the case of RS mixing and the generated monolithic liquid layer, depending on the working volume, serves as a mechanical dilution decreasing the optical path for the measurement to take place, enabling to register turbidity up to ~45 OD_{600} for rod shaped bacteria, e.g. *E.coli, B.subtilis, B.bifidum* and ~75 OD_{600} for yeast *(S.cerevisiae, P.pastoris)*, which is enough for most applications. In other words, bioreactor tubes containing different volumes of medium are intensely rotated (2,000 min^{-1}) and as a result, a monolayer of medium is generated, which thickness

Reverse–Spin® mixing principle

Spread of the broth media inside of rotation tube as a function of rotation intensity

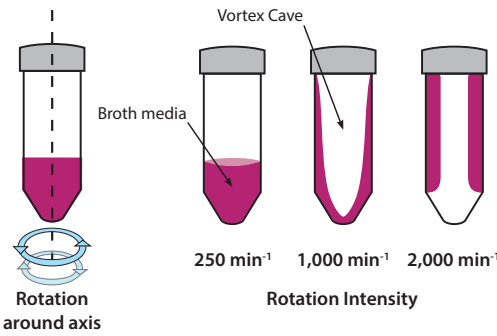


Figure 1

Reverse–Spin® life cycle

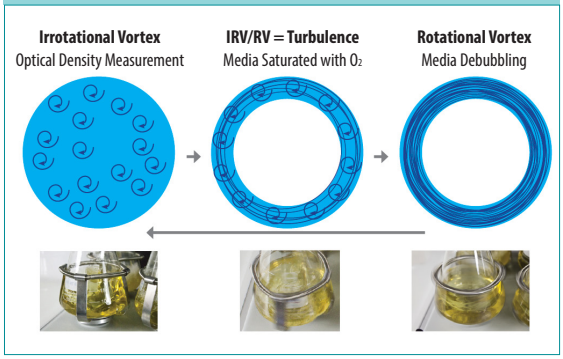


Figure 2

Centrifugal Forces as a Mechanical Defoamer

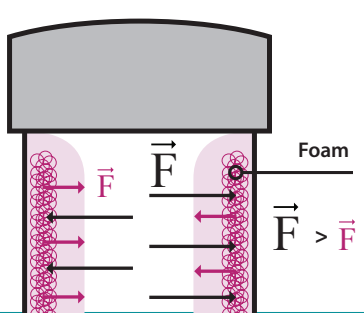
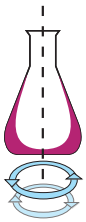


Figure 3

is directly proportional to the volume of culture medium in the tube, as shown in Figure 6, A-B. Consequently, the squared linear correlation coefficient (R^2) between RTS and off-line OD_{600} was 0.99 (Figures 6 and 7) measuring from 0 to 20 OD_{600} for rod shaped bacteria and 0 to 35 OD_{600} for yeast. Higher OD values can be calibrated choosing non-linear calibration models, which can be done automatically during calibration process in RTS software.

Reverse Spinning vs Orbital Shaking
Symmetrical vs Asymmetrical broth media distribution

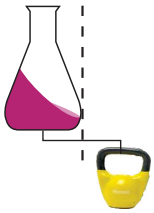
Reverse Spinning



Features:

- Natural centric auto–balancing
- Simplicity
- No power consumption for contra–balancing
- Self-cleaning optical cells
- Mechanical auto defoamer
- Single-use
- Centrifuge ready
- Lightweight

Orbital Shaking



Features:

- Proportionality between orbital diameter and the diameter of the moving vessel
- Artificial hula–hoop auto–balancing
- Complexity
- Extra power consumption for contra-balancing
- Heavy

Figure 4

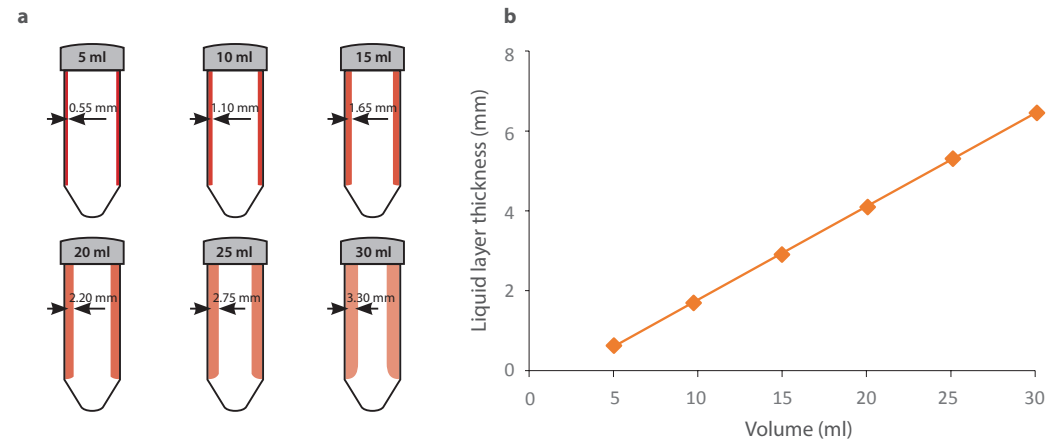


Figure 6 (a, b)

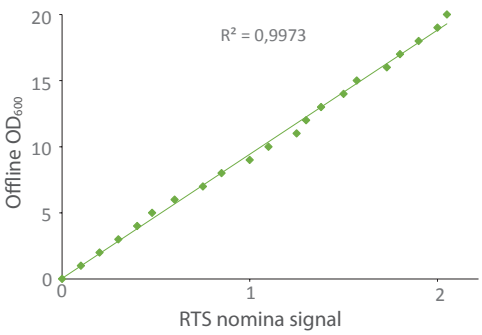


Figure 7. Squared linear correlation coefficient (R^2) between RTS and off-line OD_{600} for rod shaped bacteria.

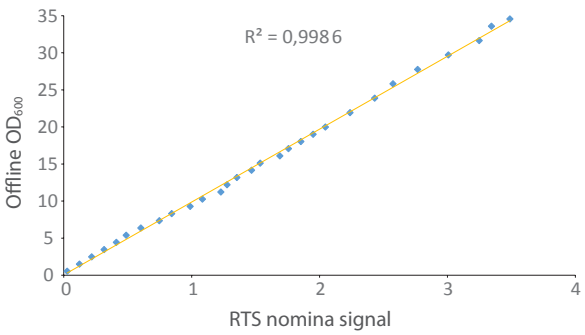


Figure 8. Squared linear correlation coefficient (R^2) between RTS and off-line OD_{600} for yeast.

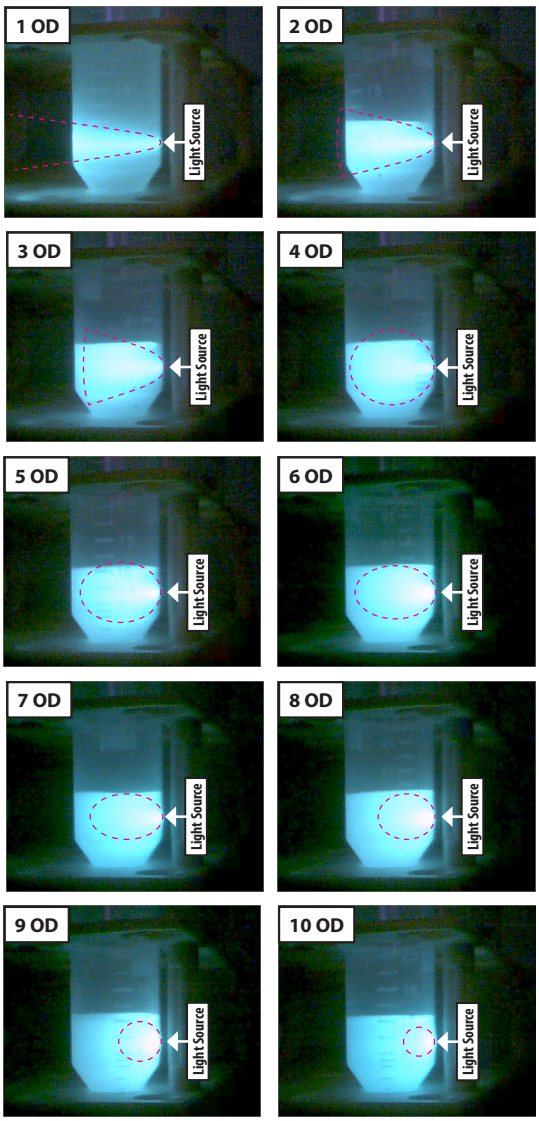
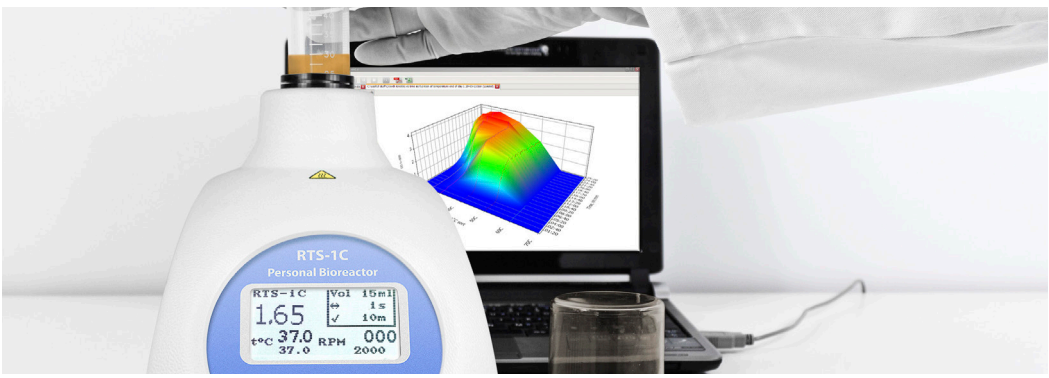


Figure 5. Experiment of Behaviour of Light in the Environment of Different Densities was carried out. Green (535 nm) laser was used in the Saccharomyces Cerevisiae of different optical densities (OD) in the range from 1 to 10 with 1 OD increment.

Examples of RTS bioreactor application

In development of biotechnological process, the need for initial screening of clone candidates and determination of optimal cultivation parameters is essential. Shake flasks are well established mainly due to their historical commonality, flexibility, low cost and ease of operation [1]. Nevertheless, at this initial and crucial stage of bioprocess development it is challenging to monitor or control important cultivation parameters such as biomass, specific growth rate and temperature. Offline sampling for establishing growth kinetics in flasks is troublesome, lacks data density, can create anaerobic stress and carries a risk of contamination. Incubator-shakers do not have individual temperature control for each flask which limits the possibility to have more temperature conditions e.g. for temperature sensitive protein expression. Moreover, the possibility of inadequate supply of oxygen through the gas–liquid interface and cap/closure in shake flasks can result in anaerobic stress and inefficient substrate accumulation resulting in low yield of the desired product [1]. Consequently, there is a niche for new mixing principles that should be introduced on the market as an alternative to solve the limitations of orbital shaken flasks. In comparison, the ability of RTS to register biomass online non-invasively as frequently as 20 seconds between measurements, the possibility to individually rapidly control temperature (0.7°C/min, direct sample temperature) and possibility to match even the most vigorous orbital mixing and consequently k_La conditions is clearly advantageous. Experimental results for k_La (h^{-1}), growth kinetics, relative level of recombinant protein accumulation by SDS-PAGE gel analysis and specific enzymatic activity for *E.coli* and *P.pastoris* (the protein and strain names are confidential by desire of the providing party) cultivated on commercially available broth media's for RTS as an alternative cultivation system that can solve the known limitations of shake flasks.



k_La (h^{-1}) results in RTS-1/C

The k_La was measured in 5, 10, 20, 30 mL of deionized water in 50 ml TPP Bioreaktor tubes at agitation rate of 2000 rpm and 1 s RST, this agitation rate was found optimal for Reverse–Spin® mixing principle during initial optimization studies. Over the working volume range, the k_La increased with the decrease of liquid volume (Figure 10). At working volume of 5 ml, the highest k_La of 350 h^{-1} ± 26 was reached. We think that by selecting lower working volume it is possible to increase the k_La even more since for Reverse–Spin® mixing principle, overall oxygen transfer is proportional to the surface to volume ratio, thus by decreasing the working volume gas–liquid mass transfer rate reaches higher values. All things considered, it was not possible to measure lower working volume conditions due the construction of the optical axis which is located at 5 ml mark of the tube.

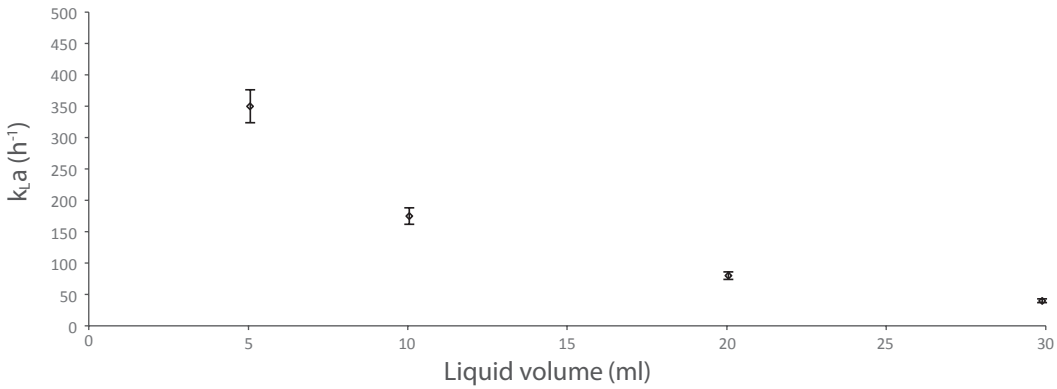


Figure 9. Determination of k_La in 50 ml TPP Bioreaktor tubes. The bioreactor vessels were filled with 5, 10, 20, 30 ml deionized water and measurements were made by non-invasive O_2 sensors and optics (PreSens, Regensburg, Germany) at 30 °C using a gassing-out method. Mean and standard deviation of at least five independent experiments are shown.

E.coli and *P.pastoris* cultivation and recombinant protein production

Non-limiting oxygen availability during screening is required for feasible bioprocess development [1,2] and OTR_{max} and k_La are crucial parameters in scaling up and scaling down the process conditions between shaking vessels and laboratory-scale mechanically stirred bioreactors [3,4,5]. Common conditions for cultivating *E.coli* and *P.pastoris* are orbital mixing, 25-50 mm orbit, 250 RPM, 250 ml shake flasks and 10-30% filling volumes. The k_La estimations for 250 ml flask, 10% filling volume, 25-50 mm orbit and 250 RPM can vary in published literature because of differences in methods and models of calculation. Studies using non-invasive O_2 sensors and optics (PreSens, Regensburg, Germany) have estimated a value of 100 h^{-1} [6,7], yet other studies at similar conditions showed lower k_La [8], which relies on different methodology. In these experiments 250 ml flasks with 10-30% filling volumes were used throughout. In the E.coli experiment of thermosensitive recombinant protein production optimization between RTS and shake flasks at different fermentation temperatures, the influence of k_La on growth kinetics can be clearly seen from Figure 10 a-b and Table 2, where the highest k_La condition of RTS at optimal growth temperature of 37 °C, in comparison to shake flask at the identical temperature, achieved greater biomass yield (24%) and higher specific growth rate (19%). Bacterial cultivation was performed using semi-synthetic medium supplied with 1% glucose, IPTG as expression inductor at 37, 30 and 25 °C throughout. Similiarly, in *P.pastoris* experiment (Table 3), the biomass yield was also 27% higher. Yeast cultivation was performed growing on BMGY medium at 30 °C, with further harvesting and centrifugation for the purpose of 5 times concentrating the cells (up to 200 ± 50 OD_{600}) for later recombinant thermosensitive protein expression using methanol as the carbon source and as a protein expression inductor, performed at 28 °C throughout, using BMMY medium with feeding pulses varying from 0.25 to 1.25% of methanol with pauses of different duration throughout the protein expression process.

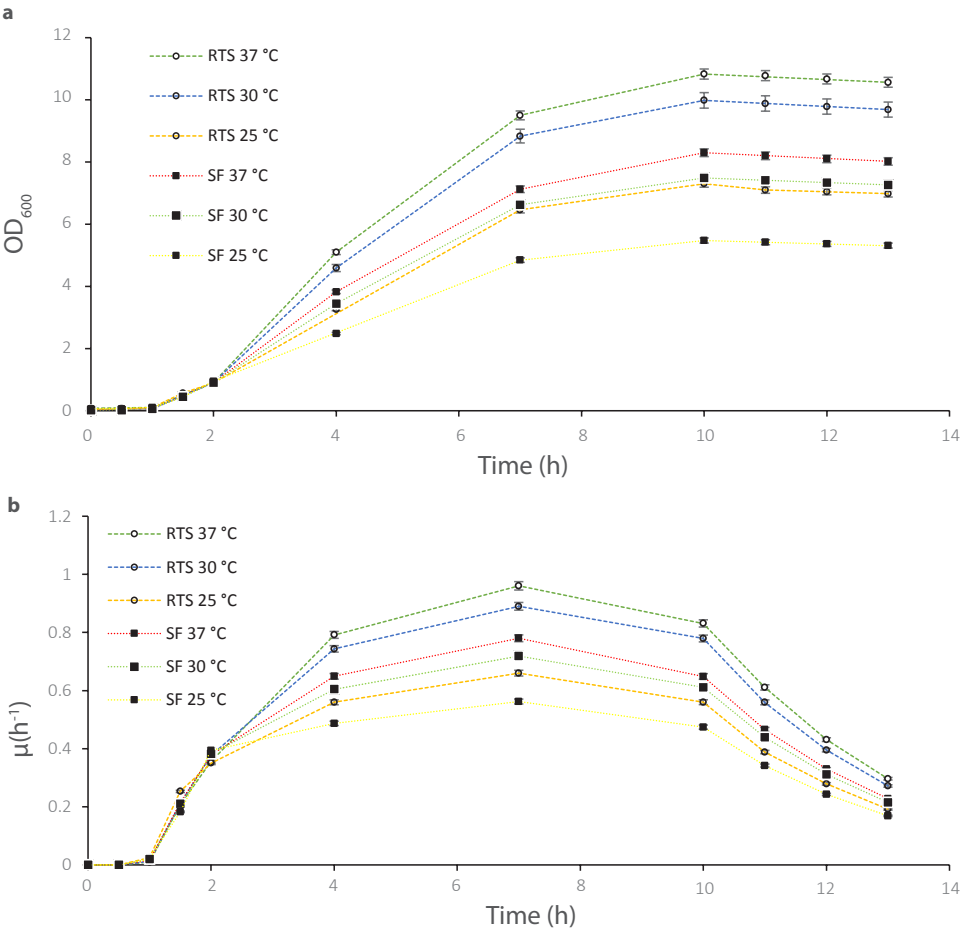


Figure 10 (a,b). Effect of temperature, cultivation method and vessel type on OD_{600} of *E.coli* expressing recombinant thermosensitive 37 kDa protein grown in flasks shaken by incubator-shaker and RTS-1. (RTS) RTS-1 TPP bioreactor tubes; (SF) Shake Flasks; throughout cultures grown in non-baffled shaken flasks (nominal size of 250 ml with 10% of filling volume) with 25 mm 240 rpm incubator-shaker and RTS-1 (50 ml TPP Bioreaktor tubes with 20% filling volume) with 2000 rpm 1 s RST, cultivated using Rich medium (buffered double concentrated LB). Mean and standard deviation of three independent experiments are shown.

Yield	RTS 37 °C	RTS 30 °C	RTS 25 °C	SF 37 °C	SF 30 °C	SF 25 °C
[a]OD_{600}	10.80 ± 0.16	9.98 ± 0.15	7.29 ± 0.11	8.3 ± 0.12	7.48 ± 0.11	5.5 ± 0.1
$\mu_{max}(h^{-1})$	0.96 ± 0.02	0.89 ± 0.014	0.66 ± 0.01	0.78 ± 0.014	0.71 ± 0.01	0.56 ± 0.15

Table 2. *E.coli* end point biomass yield in OD_{600} and maximum specific growth rate results

[a] – Measured in overnight cultures before cell harvest. Mean and standard deviation of three independent experiments are shown.

Yield	RTS 10%	RTS 20%	RTS 30%	SF 10%	SF 20%	SF 30%
[a]OD_{600}	65 ± 1.5	59 ± 2	51 ± 1.5	47.5 ± 2	30 ± 1.3	21 ± 0.7

Table 3. *P.pastoris* end point biomass yield in OD_{600} results

[a] – Measured in overnight cultures before cell harvest, cultivated at 30 °C O/N. Mean and standard deviation of three independent experiments are shown.

A well-known technique to limit the in vivo aggregation of recombinant proteins consists of cultivation at reduced temperatures [9]. This strategy has proven effective in improving the solubility of a number of difficult proteins [10]. In both *E.coli* and *P.pastoris* experiments, relative level of recombinant thermosensitive protein accumulation in the total, insoluble and soluble fractions of the cell lysate under induced cultural conditions was observed by 12% SDS–PAGE (Figure 11). Moreover, specific enzymatic activity U mg^{-1} of biomass was identified. In *E.coli* experiment the effect of temperature on the level of soluble recombinant protein is clearly seen and was the highest at 30 °C in both RTS bioreactors and shake flasks. Yet, the difference could not be clearly observed which mixing principle resulted in the highest relative soluble protein yield. Furthermore, specific enzymatic activity measurements (Figure 12) resulted in 18% higher specific enzymatic activity in RTS 30 °C. In contrast, in the *P.pastoris* experiment, the difference between RTS bioreactors and shake flasks in relative level of recombinant thermosensitive protein could be better observed and was significant (Figure 13). Moreover, specific enzymatic activity results (Figure 14) repeated this correlation, where 20% filling volume RTS was 62% higher than shake flask with identical filling volume percentage. The variables that could be involved in the substantial protein yield difference must be identified and studied further.

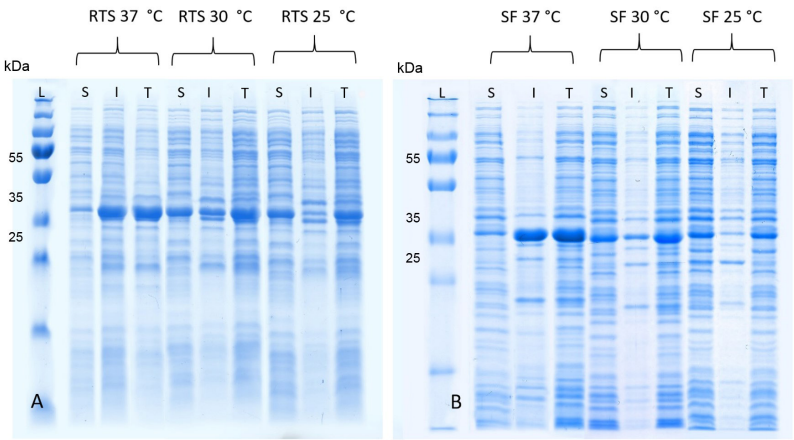


Figure 11. SDS-PAGE images (A and B) of total, solube and insolube recombinant thermosensitive protein fractions of samples of RTS and SF cultures cultivated at different temperature and cultivation vessels. L - Protein size standard (PageRuler™ Plus Prestained Protein Ladder, Thermo Fisher Scientific), T - Total protein fraction, S – Soluble protein fraction and I – Insoluble protein fraction.

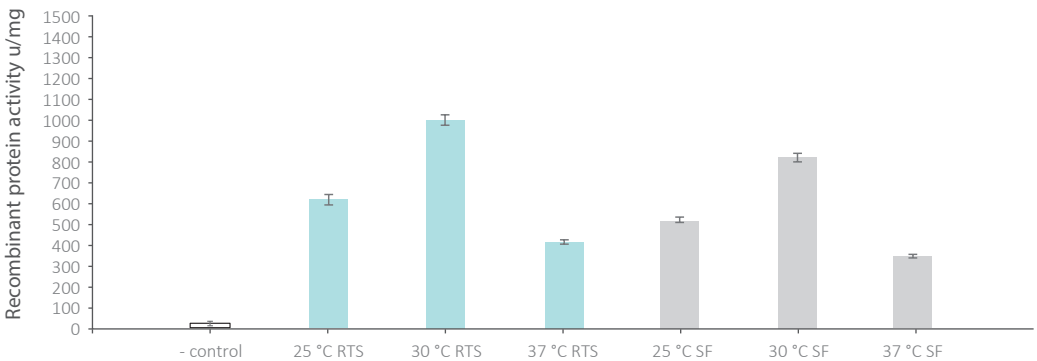


Figure 12. Effect of temperature on specific enzymatic activity (U mg^{-1}) of *E.coli* expressing recombinant thermosensitive protein grown in flasks shaken by incubator-shaker and RTS-1. (RTS) RTS-1 TPP Bioreaktor tubes; (SF) Shake Flasks; (- Control) *E.coli* biomass before induction. Mean and standard deviation of three independent experiments are shown.

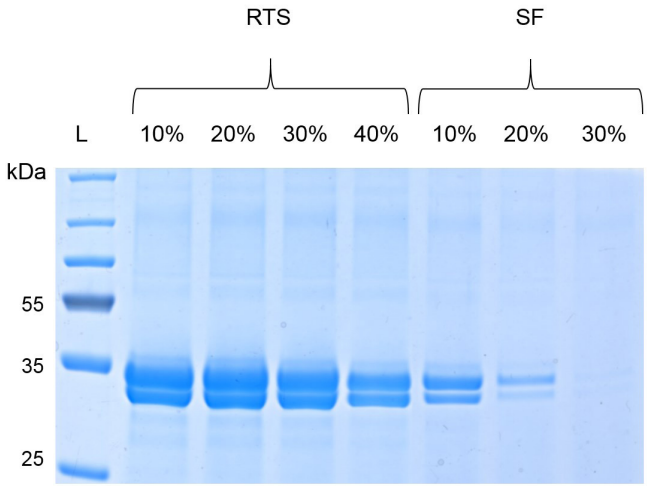


Figure 13. 12% SDS-PAGE image of *P.pastoris* supernatant samples expressing recombinant thermosensitive 37 kDa protein in (RTS) RTS-1 TPP bioreactor tubes; (SF) Shake Flasks; throughout the expression was performed in non-baffled shaken flasks (nominal size of 250 ml with 10, 20, 30% filling volumes) with 25 mm 250 rpm incubator-shaker and RTS-1 (50 ml TPP Bioreaktor tubes with 10, 20, 30 and 40% filling volumes) with 2000 RPM 1 s RST, using BMMY medium with various methanol feeding pulses of 0.25-1.25% with subsequent various feeding pauses. Mean and standard deviation of three independent experiments are shown. L - Protein size standard (PageRuler™ Plus Prestained Protein Ladder, Thermo Fisher Scientific). Protein bands are formed as "doublets" because of different carbohydrate groups attached during secretion.

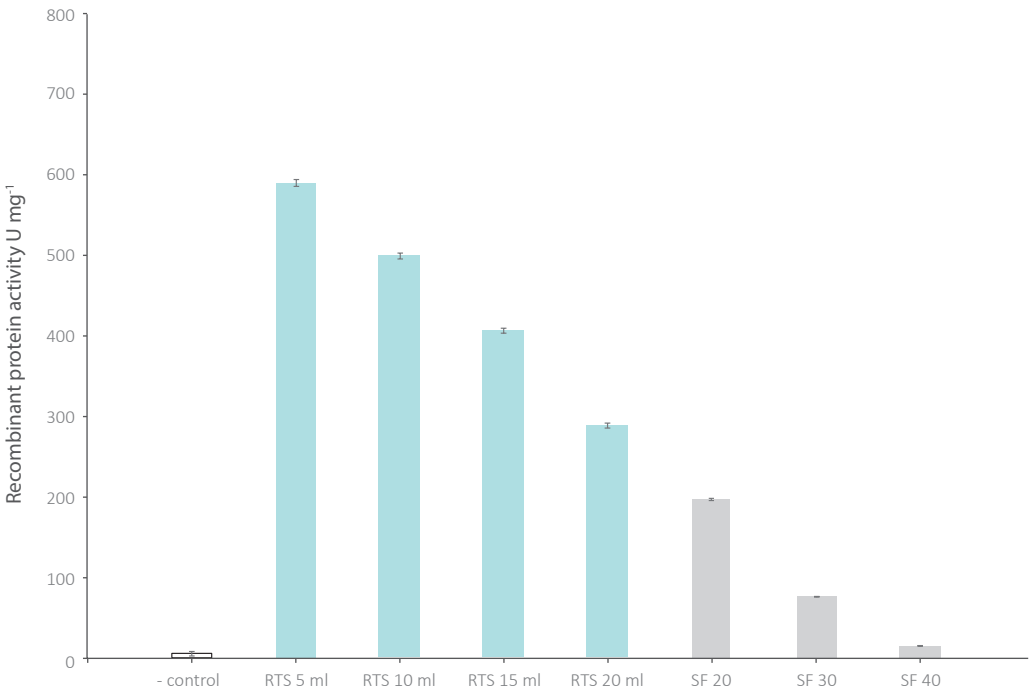


Figure 14. Effect of different filling volumes on specific enzymatic activity (U mg^{-1}) of *P.pastoris* expressing recombinant thermosensitive protein grown in flasks shaken by incubator-shaker and RTS-1. (RTS) RTS-1 TPP Bioreaktor tubes; (SF) Shake Flasks; (- Control) *P.pastoris* biomass before induction. Mean and standard deviation of three independent experiments are shown.

Conclusion

Previous version of RTS article concentrated only at estimating optimal growth conditions until 10 ml working volume. Yet, after additional investigations and modifications leading to the results that are provided in the article it has been found that at 5 ml working volume the k_La dramatically increased by 50%, consequently it is possible to increase the mass transfer coefficient further lowering the working volume. Moreover, as seen from experimental results, with increasing product popularity and received critical feedback it was experimentally proven that RTS system can be successfully used not only for day to day cell cultivation with real time growth kinetics but as an alternative initial screening bioreactor for protein production. Notably, this is not the only possible application for this system because of the possibility to register real time growth kinetics and individual rapid temperature control, which enables RTS to be used in temperature stress and fluctuation, e.g. adaptive laboratory evolution and heat-shock experiments, inhibition and toxicity tests, e.g. lactic acid bacteria inhibition by bacteriophages and media and growth optimization. Further studies to increase the potential of Reverse–Spin® mixing principle will be performed in the future. Additionally, pO_2 and pH noninvasive measurement will be available in the next generation of RTS devices that is planned to be released in the 3rd or 4th quarter of 2017.

Acknowledgments

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References

1. Büchs, J. Introduction to advantages and problems of shaken cultures. Biochemical Engineering Journal 7, 91–98 (2001).
2. Zimmermann, H. F., Anderlei, T., Büchs, J. & Binder, M. Oxygen limitation is a pitfall during screening for industrial strains. Applied Microbiology and Biotechnology 72, 1157–1160 (2006).
3. Konz, J., King, J. & Cooney, C. Effects of Oxygen on Recombinant Protein Expression. Biotechnology Progress 14, 393–409 (1998).
4. Freyer, S. A., König, M. & Künkel, A. Validating shaking flasks as representative screening systems. Biochemical Engineering Journal 17, 169–173 (2004).
5. Islam, R., Tisi, D., Levy, M. & Lye, G. Scale-up of Escherichia coli growth and recombinant protein expression conditions from microwell to laboratory and pilot scale based on matched k_La. Biotechnology and Bioengineering 99, 1128–1139 (2008).
6. Reynoso-Cereceda, G. I., Garcia-Cabrera, R. I., Valdez-Cruz, N. A. & Trujillo-Roldán, M. A. Shaken flasks by resonant acoustic mixing versus orbital mixing: Mass transfer coefficient k_La characterization and Escherichia coli cultures comparison. Biochemical Engineering Journal 105, 379–390 (2016).
7. Schiefelbein, S. et al. Oxygen supply in disposable shake-flasks: prediction of oxygen transfer rate, oxygen saturation and maximum cell concentration during aerobic growth. Biotechnology Letters 35, 1223–1230 (2013).
8. Klöckner, W. & Büchs, J. Advances in shaking technologies. Trends in Biotechnology 30, 307–314 (2012).
9. Schein, C. H. Production of Soluble Recombinant Proteins in Bacteria. Nature Biotechnology 7, 1141–1149 (1989).
10. Vasina, J. A. & Baneyx, F. Expression of Aggregation-Prone Recombinant Proteins at Low Temperatures: A Comparative Study of theEscherichia coli cspAandtacPromoter Systems. Protein Expression and Purification 9, 211–218 (1997).