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Repeated fed-batch fermentation using biosensor online control for citric acid production by *Yarrowia lipolytica*

Lucie Moeller[a], Mario Grünberg[b], Andreas Zehnsdorf[a], Andreas Aurich[a], Thomas Bley[c], Beate Strehlitz[a,*]

[a] Helmholtz Centre for Environmental Research - UFZ, Centre for Environmental Biotechnology, Leipzig, Germany
[b] University of Applied Sciences Mittweida, Anhalt University of Applied Sciences Köthen, Köthen, Germany
[c] Dresden University of Technology, Institute of Food Technology and Bioprocess Engineering, Dresden, Germany

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ABSTRACT

Biosensor-controlled substrate feeding was used in a citric acid production process with the yeast strain *Yarrowia lipolytica* H222 with glucose as the carbon source. The application of an online glucose biosensor measurement facilitated the performance of long-time repeated fed-batch process with automated bioprocess control. Ten cycles of repeated fed-batch fermentation were carried out in order to validate both the stability of the microorganism for citric acid production and the robustness of the glucose biosensor in a long-time experiment. In the course of this fermentation with a duration of 553 h, a slight loss of productivity from 1.4 g/(L × h) to 1.1 g/(L × h) and of selectivity for citric acid from 91% to 88% was observed. The glucose biosensor provided 6,227 measurements without any loss of activity.

1. Introduction

Citric acid is a bulk chemical that is produced solely by a biotechnological process. Because of its versatile attributes, citric acid is applied in many branches of industry: food and beverages, pharmaceuticals and cosmetics, and the chemical and tobacco industries (Anastassiadis et al., 2008). The annual consumption of citric acid has increased from 400,000 t/a in 1992 (Rane and Sims, 1993) to more than 1,600,000 t/a more recently (Sauer et al., 2007).

Most citric acid is produced in a submerged process using the mould *Aspergillus niger* (Crolla and Kennedy, 2001). However, this production process possesses various drawbacks, which can be overcome by using the yeast *Yarrowia lipolytica*. The main advantages of this microorganism are its better stability (its toleration of non-optimum conditions as well as its genetic stability) and its ability to utilize a wide substrate spectrum (Grewal and Kalra, 1995; Mattey and Kristiansen, 1999; Stottmeister, 2003; Rymowicz and Rywińska, 2006; André et al., 2009; Papanikolaou and Aggelis, 2009; Papanikolaou et al., 2009; Rywińska et al., 2010). However, the application of this more environmentally friendly production process is still not attractive enough to replace the current method of citric acid production. To enhance the desirability of the yeast-based process for industry, this production process has to be optimized and the most efficient process strategy has to be found.

In our previous work, we tested various process strategies for citric acid production using the yeast strain *Yarrowia lipolytica* H222 from glucose (Moeller et al., 2010). Batch, fed-batch, repeated batch and repeated fed-batch cultivations were carried out. The substrate concentration in the fed-batch and repeated fed-batch fermentations was controlled using a glucose biosensor. The most suitable of these methods turned out to be the repeated fed-batch mode. However, to ensure applicability of this process strategy on an industrial scale, it is necessary to test the stability of both the microorganism and the glucose biosensor in long-term cultivations. For this purpose, in this paper we demonstrate the robustness of the glucose biosensor and the stability of *Yarrowia lipolytica* H222 in terms of cell viability as well as of the yeast activity for citric acid production in long-time repeated fed-batch fermentation using glucose as the carbon source.

Abbreviations: CA, citric acid; GL, glucose; ICA, isocitric acid; μ_{max}, maximum specific growth rate; n.d., not determinate; P_{CA}, volumetric productivity; p_{CA}, biomass specific productivity of citric acid; S_{CA}, selectivity of the bioprocess for the production of citric acid; slpm, standard liters per minute; X, biomass; Y_{CA}, citric acid yield coefficient.

* Corresponding author. Tel.: +49 341 235 1764; fax: +49 341 235 2492 451764.
E-mail address: beate.strehlitz@ufz.de (B. Strehlitz).

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Table 1
Yeast seed culture and cultivation medium (Thiersch, 1987).

Components	Yeast seed culture	Cultivation medium
NH_4Cl [g/L]	3.0	
$(NH_4)_2SO_4$ [g/L]		5.57
KH_2PO_4 [mg/L]	700	360
$MgSO_4 \times 7H_2O$ [mg/L]	350	525
$CuSO_4 \times 5H_2O$ [mg/L]	20	20
$MnSO_4 \times 5H_2O$ [mg/L]	20	20
$ZnCl_2$ [mg/L]	10.5	10.5
$CoSO_4 \times 7H_2O$ [mg/L]	2.5	
H_3BO_3 [mg/L]	28.5	
Yeast extract [g/L]	2.5	
$CaCO_3$ [g/L]	5	
$FeSO_4 \times 7H_2O$ [mg/L]	3.5	3.5
Thiamine HCl [mg/L]		1
$CaCl_2 \times 6H_2O$ [mg/L]		30
Glucose [g/L]	30	150

2. Material and methods

2.1. Microorganism

The wild type yeast strain *Yarrowia lipolytica* H222 was used in this work. This strain was obtained from the Institute of Microbiology, Dresden University of Technology, Dresden, Germany. For the purpose of short-time storage, Reader medium plates (composition as per (Moeller et al., 2007)) inoculated with *Yarrowia lipolytica* were incubated for 2 days at 30 °C and stored at 4 °C.

2.2. Cultivation medium

The seed medium and the cultivation medium were prepared with distilled water. Their compositions are given in Table 1.

The fermentation medium components were sterilized in an autoclave for 45 min at 121 °C. Yeast extract, thiamine hydrochloride and ferrous sulfate were sterilized by filtration through a sterile membrane filter (0.22 μm, Sartorius, Göttingen, Germany). Calcium carbonate was sterilized by dry heat at 160 °C for 4 h. The medium was mixed under sterile conditions. The bioreactor was sterilized in situ for 20 min at 121 °C.

2.3. Culture conditions

The seed culture was prepared in 500-mL shake flasks with a working volume of 100 mL and incubated for 72 h on a rotary shaker (Edmund Bühler GmbH, Tübingen, Germany) at 130 rpm and 30 °C.

Fermentations were carried out in a Biostat ED 15 bioreactor (Sartorius Stedim Biotech S.A., Aubagne, France) with a working volume of 10 L. The fermenter is equipped with temperature, pH and pO_2 control. The concentrations of oxygen and carbon dioxide in the exhaust gas were measured using a Xentra O_2/CO_2, Type 4100C gas analyzer (Servomex, England). The dissolved oxygen in the fermenter was controlled by a cascade (stirrer 675–1500 rpm → air flow rate 1–10 slpm). The concentration of glucose was measured continuously using the ProcessTRACE 1.21MT quasi online measuring system (Trace Analytics GmbH, Braunschweig, Germany), which is equipped with an amperometric enzyme biosensor for glucose detection. The measuring system was connected to the software control of the bioreactor (MFCS/win2.1, Sartorius Stedim Biotech S.A., Aubagne, France) via a serial port. This allows the glucose concentration in the fermenter to be kept constant by means of feed regulation. The biosensor that was used in the experiments described here was specified for 5000 measurements and for a measuring range of 0.5–40 g/L glucose. The desired set point of the glucose concentration was maintained by a *P* controller ($P = 15$).

The bioreactor filled with the appropriate starting volume of sterilized cultivation medium was inoculated with 10% (v/v) of a 3-day seed culture. The pH was stabilized using 30% NaOH (w/v) and 5% H_3PO_4 (w/v). The Tego KS 911 antifoaming agent (Degussa Care & Surface GmbH, Germany) was used. The pH was adjusted to 6.0 and the temperature was maintained at 30 °C. pO_2 was maintained at 50%.

Ten cycles of repeated fed-batch fermentation were carried out. The starting volume of each cycle amounted to 5 L. The glucose starting concentration for each cycle was 150 g/L; when decreased to 20 g/L, glucose concentration was held at this level by biosensor-based feeding using 500 g/L glucose solution. The first three cycles took 71 h each, and the duration of the remaining seven cycles was shortened to 48 h each.

2.4. Analytical methods

After taking fermentation samples, an aliquot was used for the determination of the biomass concentration. The remains were centrifuged for 5 min at 13,000 rpm (Eppendorf 5415D centrifuge, Hamburg, Germany) and analyzed.

Biomass concentration (i.e. dry cell weight) was measured with the aid of a MA40 Moisture Meter (Sartorius AG, Göttingen, Germany) at 105 °C after filtering 5–10 mL of culture broth through a membrane filter (cellulose acetate, 0.45 μm, Sartorius Stedim Biotech S.A., Aubagne, France). The glucose concentration was measured offline using the enzymatic spectrophotometric test kit based on glucose dehydrogenase (Enzytec™ D-glucose kit, Scil Diagnostics GmbH, Viernheim, Germany). Citric acid and isocitric acid were assayed using a DX-600 ion chromatography system (Dionex, Sunnyvale, USA): IonPac AS column 11–4 mm in diameter, eluent KOH. The ammonia and nitrogen concentrations were detected in a wet chemical manner by a relative method using a flow injection analyzer (Ismatec®, Glattburg, Switzerland). The analysis was performed photometrically at 605 nm by detecting the colour change of the bromocresol purple indicator.

2.5. Determination of cell growth kinetics, citric acid yield coefficient and productivity

The maximum specific growth rates (μ_{max}) were calculated using the program ORIGIN® 8. The biomass concentration vs. time curve was fitted with a polynomial function. The μ_{max} was determined as the first-order derivate of this function.

The total quantity of citric acid or isocitric acid was ascertained from the volume of the fermentation broth at the end of the fermentation and the present citric or isocitric acid concentration.

The selectivity of the bioprocess (S_{CA}) for the production of citric acid compared to the sum of citric acid and isocitric acid concentrations was calculated as follows:

$$S_{CA} = \frac{100 \times c_{CA}}{c_{CA} + c_{ICA}} \,[\%CA]$$

The citric acid yield coefficient (Y_{CA}) was calculated using the following equation with $c_{CA,produced}$ being the end concentration of citric acid and $c_{glucose,consumed}$ being the difference between the start and end glucose concentration:

$$Y_{CA} = \frac{c_{CA,produced}}{c_{glucose,consumed}} \,[g_{CA}/g_{CA}]$$

Volumetric productivity (P_{CA}) and biomass specific productivity of citric acid (p_{CA}) of the entire fermentation were calculated using the following equations with $c_{CA,produced}$ representing the final con-

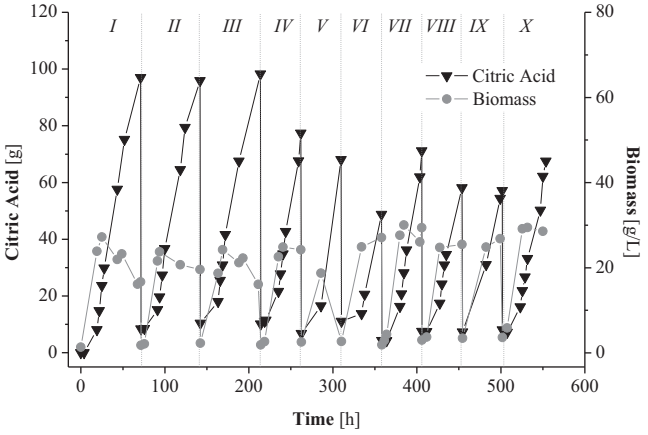

Fig. 1. Citric acid and biomass concentrations over the course of long-time repeated fed-batch fermentation. Conditions: $T = 30\,^{\circ}C$, pH = 6.0, pO_2 = 50%, medium (Thiersch, 1987): 5.56 g/L $(NH_4)_2SO_4$, 150 g/L glucose (after decreasing to 20 g/L, substrate feeding using 500 g/L glucose solution started), fermentation duration: 554 h (three cycles taking 71 h and seven cycles taking 48 h each).

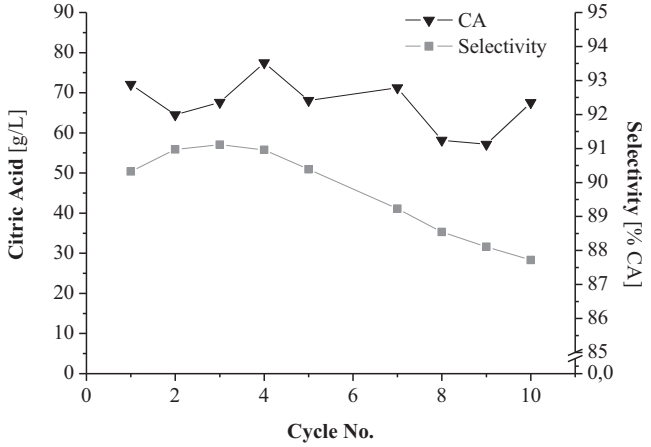

Fig. 2. Selectivity of the bioprocess for citric acid and citric acid concentration after every 48 h of cycle time for each cycle of the long-time repeated fed-batch cultivation. Condition: see Fig. 1.

centration of citric acid, t denoting fermentation time and $X_{average}$ denoting average biomass:

$$P_{CA} = \frac{c_{CA,produced}}{t}\ [g_{CA}/(L \times h)]$$

and

$$p_{CA} = \frac{P_{CA}}{X_{average}}\ [g_{CA}/(g_{biomass} \times h)]$$

3. Results and discussion

Three cycles of repeated fed-batch fermentation running 71 h each and seven cycles with a duration of 48 h each were carried out in the total time period of 554 h (Table 2, Fig. 1). During the sixth cycle, a technical defect caused an electrical power outage for a few minutes, which had a negative impact on citric acid production. However, in the following cycles, the yeast growth and citric acid production were not influenced by this occurrence.

In the first five cycles in the growth phase, the biomass concentration reached similar values of 26–28 g/L (Fig. 1). During the subsequent cycles, the biomass concentrations obtained increased from cycle to cycle, staying almost constant at 34–35 g/L in the last two cycles. The maximum growth rate decreased with rising cycle number from values of $\mu_{max} = 0.20\ h^{-1}$ up to $0.16\ h^{-1}$ in the fourth cycle (Table 2). In the next cycles, μ_{max} sank again to values of $0.12\ h^{-1}$ in the tenth cycle. Similar observations made also Rywińska and Wojtatowicz (2008), who studied the behavior of recombinant *Yarrowia lipolytica* A-101-1.31 growing on glucose during six cycles of repeated batch fermentation. The authors described, that the volumetric biomass productivity decreased from 0.54 g/(L × h) in the first cycle to 0.22 g/(L × h) in the last cycle, whereas the biomass end concentration clearly increased in the last two batches. The values of maximum specific growth rates obtained in the first cycles of repeated fed-batch fermentation presented in Table 2 are in agreement with the values reported by Wojtatowicz et al. (1991), who obtained a $\mu_{max} = 0.17–0.22\ h^{-1}$ by various strains of *Yarrowia lipolytica* grown on glucose hydrol.

In the first three cycles, running 71 h each, the highest citric acid end concentrations were achieved, yielding similar values of 96–98 g/L (Table 2). As regards the citric acid concentration curve in the first three cycles, a certain retardation of citric acid formation in the last 24 h of each cycle can be observed (Fig. 1). The reason for this is the product inhibition that has been described previously (Moeller et al., 2007). In the first seven cycles, the citric acid end concentration fluctuated at values between 65 and 78 g/L after 48 h of cycle time (in the first cycle 72.1 g/L, the second cycle 64.5 g/L and the third cycle 67.6 g/L citric acid were produced after 48 h of cycle time) (Fig. 2). In the eighth and ninth cycles, the citrate end concentration reached values lower than 60 g/L, which changed in the last cycle, achieving 62.2 g/L citrate after 48 h of cycle time. This fact does not indicate a loss in the activity of the yeast cells for citric acid excretion over ten cycles of repeated fed-batch fermentation. On the other hand, regarding the selectivity of the bioprocess for citric acid, a clear tendency of a shift in favor of isocitrate can be observed (Fig. 2). In the first three cycles, the highest end selectivity of about 92% citric acid was obtained, which was due to the longer cycle time. Nevertheless, considering the selectivity after 48 h of cycle time, there was no decisive change in the first five cycles. With further increasing cycle number, the end selectivity for citrate decreased to 88% citric acid in the last cycle. In conclusion, it can be stated that there was no decisive decline in the microbial activity for the citric acid production but that the product pattern changes in favor of isocitric acid with rising cycle number.

The results achieved are in accordance with the data presented by other researchers for various selected mutant strains of *Yarrowia lipolytica* growing on versatile carbon sources in fed-batch fermentations. Using plant oils, up to 150 g/L citric acid was produced by the yeast strain *Y. lipolytica* N15 in 144 h of cultivation, with insignificant isocitric acid production (Kamzolova et al., 2008). Rymowicz et al. (2008) reported the formation of 110 g/L citric acid by the acetate negative mutant of *Yarrowia lipolytica* Wratislavia K1 from crude glycerol as the substrate after 168 h of fermentation. 140 g/L citric acid was produced in a cultivation of the ICL1 multicopy strain *Yarrowia lipolytica* H222-S4(p67ICL1)T5 using sucrose as the carbon source with a duration of 191 h; the selectivity of the bioprocess reached 96.6% citric acid (Förster et al., 2007). Rane and Sims (1995) studied the effect of the cycle time on citric acid yields of *Candida lipolytica* Y 1095 growing on glucose in repeated fed-batch culture. They found out, that the optimum duration of the citric acid production phase, during which continuous feeding occurred, was 36 h. The citric acid yield coefficient of 0.56 g/g, which was achieved in this cycle, corresponds with the results presented in the present paper. Anastassiadis and Rehm (2006) studied the influence of the air saturation on the citric acid production by the yeast strain *Candida oleophila* ATCC 20177 using glucose as carbon source in repeated batch fermentation process. The results which they reached at optimum (80%) air saturation are somewhat higher than the results described in the present paper. The glucose start

Table 2
Parameters of single cycles of repeated fed-batch cultivation for the citric acid production process by *Yarrowia lipolytica* H222.

Cycle no.	Cycle duration [h]	Biomass [g/L]	μ_{max} [h^{-1}]	Total quantity		End concentration		Selectivity [% CA]	Yield [g_{CA}/g_{GL}]	Productivity of CA	
				CA [g]	ICA [g]	CA [g/L]	ICA [g/L]			Volumetric [$g_{CA}/(L \times h)$]	Specific [$g_{CA}/(g_{biomass} \times h)$]
1	71.2	27.0	0.20	654	55.4	97.0	8.2	92.2	0.54	1.36	0.05
2	70.3	26.3	0.19	589	48.5	95.9	7.9	92.4	0.59	1.25	0.05
3	71.3	26.2	0.13	679	58.8	98.3	8.4	92.1	0.69	1.23	0.05
4	47.6	28.2	0.16	391	39.8	77.5	7.7	91.0	0.52	1.41	0.05
5	47.2	28.1	n.d.	377	40.4	68.1	7.2	90.4	0.56	1.30	0.05
6	47.8	28.9	n.d.	237	56.1	52.8	11.2	81.3	0.47	0.79	0.03
7	47.5	32.5	0.16	392	45.4	71.2	8.6	89.2	0.49	1.41	0.04
8	47.6	33.3	0.14	292	38.5	58.2	7.5	88.5	0.39	1.06	0.03
9	47.4	35.2	n.d.	298	40.9	57.2	7.7	88.1	0.39	1.05	0.03
10	51.7	34.2	0.12	341	42.8	67.6	9.3	87.9	0.41	1.15	0.03

concentration of 336 g/L could be used thanks to the high osmotolerance of *C. oleophila*. The end concentrations of biomass and citric acid were 29–34 g/L and 132–167 g/L in 5–7 days cycle time, respectively. The volumetric productivity reached 1.5 g/(L × h).

Each cycle started in batch mode, switching to fed-batch after the glucose concentration decreased from 150 g/L to 20 g/L (Fig. 3). With the help of biosensor online control, the glucose concentration in the fermentation broth was held at 20 g/L, averaging around this value to within ±0.38 g/L (i.e. 1.9%) (according to the measurement data obtained from the glucose biosensor). Comparing the data provided by enzymatic test kit analysis with that of the glucose biosensor, a difference of 5.6 g/L was found on average taking into consideration the measurements inside the producer-defined measuring range. This difference is probably caused by the multiplication of errors from the individual measuring methodologies (e.g. the spectrophotometric enzymatic test kit analysis involves an error of 0.4–0.8 g/L (in samples after a dilution of 1:100)). Very good agreement was found outside the measuring range too. Throughout the whole fermentation time, the difference between these two methodologies reached 6.9 g/L. The glucose biosensor provided 6227 measuring data points, although specified for only 5000 measurements. Its activity did not diminish during the whole fermentation time. Also, there was no reduction of its activity when the glucose concentration was higher than the specified measuring range. This experiment demonstrates the applicability of this biosensor online measuring system for glucose measurements in long-time fermentations.

In order to test the reproducibility of the experiment, one more repeated fed-batch fermentation of three cycles was carried out

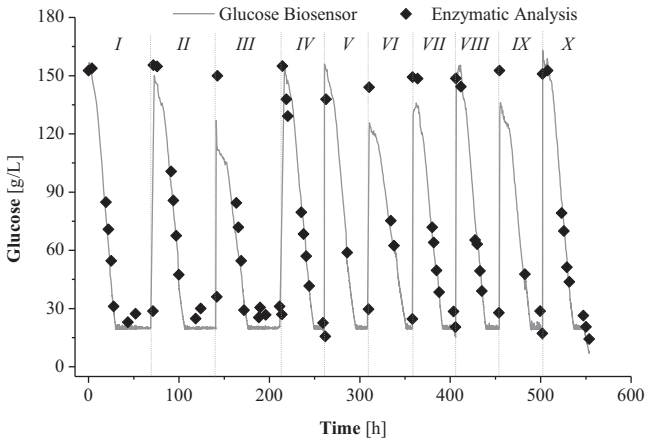

Fig. 3. Glucose concentration over the course of long-time repeated fed-batch fermentation, comparison of two analytical methods: glucose biosensor assay and spectrophotometric enzymatic test kit analysis. Condition: see Fig. 1.

(see Moeller et al., 2010). The cycle duration was 71–72 h, and the cultivation was carried out under conditions identical to those of the long-time repeated fed-batch. The results of this fermentation were in very good agreement with the first three cycles of the long-time experiment. The citric acid end concentration reached a mean value of 97.7 ± 6.0 g/L, the productivity of the bioprocess was 1.23 ± 0.16 g/(L × h), the yield amounted to 0.56 ± 0.08 g_{CA}/g_{GL} and the selectivity of the bioprocess for citric acid was 93 ± 1.3%. To confirm the stability of the microorganism in long-time cultivations, ten cycles of repeated batch cultivation in shake flasks were carried out (data not shown). The cycle duration was 5 days, and the glucose start concentration was 100 g/L. In this experiment, the same trend in the citric acid production was achieved as in the case of the repeated fed-batch experiment. The highest concentrations of up to 60 g/L citric acid were achieved in the first five cycles, fluctuating in the last two cycles between the values of 46 and 50 g/L citric acid. However, in contrast with the fermentation described above, the selectivity reached its highest values of 91% in the last three cycles, starting at 87% in the first two cycles.

The high stability of the citric acid production yeast over the course of cultivation in different modes has been already described in the literature. Anastassiadis and Rehm (2006) carried out a repeated batch fermentation of *Candida oleophila* ATCC 20177, where the microorganism showed no loss of activity over the course of twenty cycles. Rane and Sims (1995) reported on the stability of the yeast strain *Candida lipolytica* Y 1095 in a cell recycle bioreactor during fermentation with a duration of almost 700 h. *Saccharomycopsis lipolytica* D 1805 (Kim et al., 1987), *Saccharomycopsis lipolytica* Y7576 (Enzminger and Asenjo, 1986) and *Yarrowia lipolytica* VKM Y-2373 (Arzumanov et al., 2000) are also very stable in growth and citric acid production over long fermentation times.

Nevertheless, this is the first description of long-time biosensor-based repeated fed-batch fermentation for citric acid production by yeasts from glucose. The application of online measurement of glucose concentration by biosensors turned out to bring considerable advantages in long-time fermentations by enabling the process control based on substrate consumption.

4. Conclusions

We demonstrated that repeated fed-batch is a very effective fermentation methodology for the citric acid production process using the yeast *Yarrowia lipolytica* from glucose. The stability of the microorganism as regards citric acid production was confirmed in an experiment with duration of 553 h. The application of glucose biosensors in the online control of substrate concentration during the long-time repeated fed-batch fermentation was evaluated. The robustness of the biosensor online measuring system was demonstrated, providing more than 6,200 measurement data

points (nearly 25% higher number of measurements than specified) without any loss of activity and accuracy.

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