J Ind Microbiol Biotechnol (2009) 36:1363–1368
DOI 10.1007/s10295-009-0621-0

ORIGINAL PAPER

L(+)-Lactic acid production by co-fermentation of glucose and xylose with *Rhizopus oryzae* obtained by low-energy ion beam irradiation

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Received: 20 May 2009 / Accepted: 9 July 2009 / Published online: 4 August 2009

Abstract Low-energy ion beam irradiation (10–200 keV) has been proved to have a wide range of biological effects in recent years. When *Rhizopus oryzae* PW352 was irradiated with a 15-keV low-energy ion beam an L(+)-lactic acid high-yield mutant, RQ4015, was obtained. When 150 g/l glucose was used as the sole carbon source, L(+)-lactic acid of RQ4015 reached 121 g/l after 36 h shake-flask cultivation. However, the highest lactic acid concentration 74 g/l was obtained when 100 g/l xylose was present in the medium as the sole carbon source. When mixed xylose (25 g/l) and glucose (75 g/l) were present in a bubble column, L(+)-lactic acid production of RQ4015 reached 83 g. A high mutation rate and a wide mutation spectrum of low-energy ion implantation were observed in the experiment, suggesting that ion implantation can be a highly efficient mutagenic means for microorganism breeding in many commercial applications.

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Keywords Ion implantation · L(+)-Lactic acid · *Rhizopus oryzae* · Co-fermentation · Bubble column

Introduction

Since Muller's discovery of X-rays in 1927, various kinds of mutagenic source have been developed and successfully used for breeding many different kinds of organisms. The biological effects of low-energy ion irradiation were largely recognized and demonstrated experimentally in the mid 1980s [1]. Compared with irradiation with gamma rays and high-energy particles [2], ion implantation has many advantages, for example higher linear energy transfer (LET) [3–5] and relative biological effectiveness (RBE). These biological effects may result from energy absorption, mass deposition, and charge exchange of energetic ions [6, 7]. Now, low-energy ion radiation has been used to bombard microorganisms for industrial strain purposes; examples include *E. coli* [8], *Aspergillus niger*, *Mortierella alpine* [9], *Gluconobacter oxydans*, and *Bacillus megaterium*.

L(+)-Lactic acid is a typical organic acid widely applied in food, chemical, and pharmaceutical industries [10]. It can also be used to synthesize polylactic acid (PLA), for use in the manufacture of new biodegradable plastic [11]. PLA is one of the most promising polymers which may play an important role in solving the world-wide environmental problems. However, commercial replacement of plastic with PLA depends on whether L-(+)-LA can be produced at a lower cost than petrochemical derivatives [12].

Previous studies on lactic acid production mainly used glucose as a substrate [13]. In recent years, inexpensive and widely available lignocellulosic biomass materials, for example wheat straw, have become potential feedstocks for

production of bulk chemicals. Lignocellulose mainly consists of cellulose, hemicellulose, and lignin. Both hemicellulose and cellulose can be hydrolyzed into fermentable monosaccharides, for example glucose and xylose. For example, complete hydrolysis of corn stalks generates a solution primarily containing glucose and xylose in an approximate ratio of 3:1 (*w*/*w*). Co-fermentation of lignocellulose-based carbohydrates is a potential solution to improving the economics of L(+)-lactic acid production [14].

The purpose of this work was to offer more support for mutation effects induced by low-energy ion beam irradiation and to screen an excellent industrial strain using glucose and xylose as carbohydrate feedstocks to produce L(+)-lactic acid.

Materials and methods

Strains

Rhizopus oryzae PW352 was cultivated in 3× PDA medium containing 60% potato extract, 2.0% glucose, 1.0% $CaCO_3$, and 2.0% agar.

Radiation facility

Implantation sources were produced by an ion beam bioengineering instrument (patent no. ZL93103361.6, Zengliang Yu et al. 2000. PR China) devised by ASIPP (Chinese Academy of Sciences, Institute of Plasma Physics). In this device, ions were produced by a radio frequency ion source, electrostatically extracted and accelerated, focused, and finally transported to the target chamber where a special bio-sample holder was installed.

The pulse implantation technique was used with pulse time 5 s and interval time 30 s; the fluence of each pulse to the sample was 2.6×10^{13} ions/cm^2. During ion bombardment, the pressure in the target chamber was kept around 10^{-5} Pa by a turbomolecular pump; the temperature of the target in this environment was estimated to be about 0.

Irradiation procedures

After being cultured on slant agar medium (2.0% glucose, 60% potato extract, 1.0% $CaCO_3$, and 2.0% agar) at 37°C for 1 day, the spores of *Rhizopus oryzae* PW352 were collected and suspended in sterilized physiological salt solution and 0.1 ml of the spores solution with 10^2 dilution was spread on a Petri dish. It was then implanted with nitrogen ion beam under a dry vacuum station. At the same time, in order to evaluate the effects of vacuum on mutation, the spores of control group without N^+ beam implantation were also placed in the target chamber.

Mutant selection

After implantation, the sample was washed with distilled water, and then smeared on the plates (2.0% glucose, 20% potato extract, 1.0% $CaCO_3$, 2.0% agar, and 0.1% Bromocresol green) and incubated at 37°C for 24 h. The colonies on the plates were transferred to 3× PDA slants. After incubation in flasks (5% xylose 0.3% $(NH_4)_2SO_4$, 0.015% $ZnSO_4.7H_2O$, 0.05% KH_2PO_4, 0.075% $MgSO_4.7H_2O$, 5% $CaCO_3$), the colonies producing L(+)-lactic acid were screened by yield.

Analytical methods

L(+)-Lactic acid was quantified by use of a biosensor (SBA-40C, Shangdong Academy of Sciences) specifically sensitive to L(+)-lactic acid. The optical purity of lactic acid was measured by HPLC using a μBondpak C18 column (Waters, USA). The mobile phase was CH_2OH–H_2O–H_3PO_4 10:90:0.3 (v/v) and the flow rate was 0.8 ml/min. Detection was conducted at a wavelength of 210 nm at 25°C. The standard L(+)-lactic acid was purchased from Sigma (St Louis, USA). Residual glucose was quantified by use of a biosensor (SBA-40C). The concentration of xylose was measured by DNS.

Results and discussion

Survival fraction of *Rhizopus oryzae* PW352

Survival is a prerequisite for finding ion-induced mutants as it is only *Rhizopus oryzae* PW352 that received at least one hit and survived that can be scored as mutation, on the basis of phenotypic expression.

For ion implantation, mutation frequency and screen efficiency are closely related to energy and the dose of ions. In this experiment (Fig. 1), N^+ was chosen as the ion source and the fluence of nitrogen was $50 \times 2.6 \times 10^{13}$ ions/cm^2. The survival rate decreased slowly when energy was below 6 keV, however, it decreased sharply from 6 to 10 keV. When energy surpassed 10 keV, the rate of decrease slowed again. To obtain the maximum positive mutation, 10 keV was chosen as the optimum energy according to the theory stated in the Ref. [15].

The fluence of the nitrogen ion beam ranged between 0 and $220 \times 2.6 \times 10^{13}$ ions/cm^2. The reduction in the survival rate did not follow the exponential law which is also referred to as the log–linear model. The survival rate was related to the fluence of N^+ implantation showed

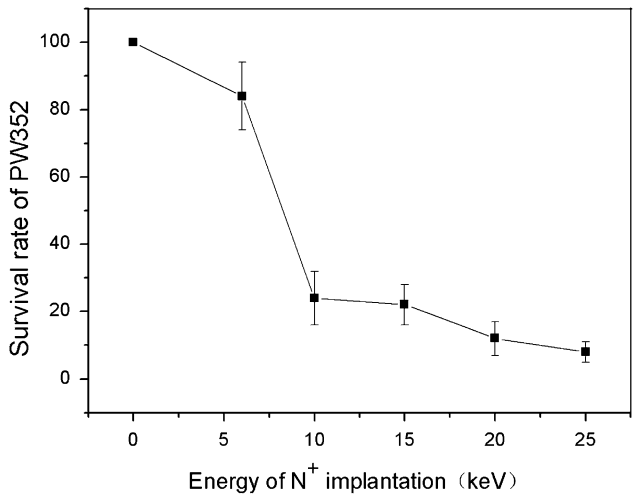

Fig. 1 Effect of N^+ energy on survival rate of *Rhizopus oryzae* PW352

a characteristic curve with a "saddle" shape (Fig. 2). A sharp decrease in the survival rate of cells was observed for fluences from 0 to $15 \times 2.6 \times 10^{13}$ ions/cm^2, then a slow increase in the narrow fluence range $15 \times 2.6 \times 10^{13}$ to $30 \times 2.6 \times 10^{13}$ ions/cm^2 followed by a slow decrease with increasing fluence. The shape of trend for fluence ranging from $15 \times 2.6 \times 10^{13}$ to $80 \times 2.6 \times 10^{13}$ ions/cm^2 looks like a "saddle", therefore we use the "saddle" curve to denote the abnormal radiation damage induced by low-energy ions [6].

When the microorganism was mutated by UV, diethyl sulfate (DES) and ^{60}Co, spore survival was reduced by increasing the period of exposure to the mutagenic agents

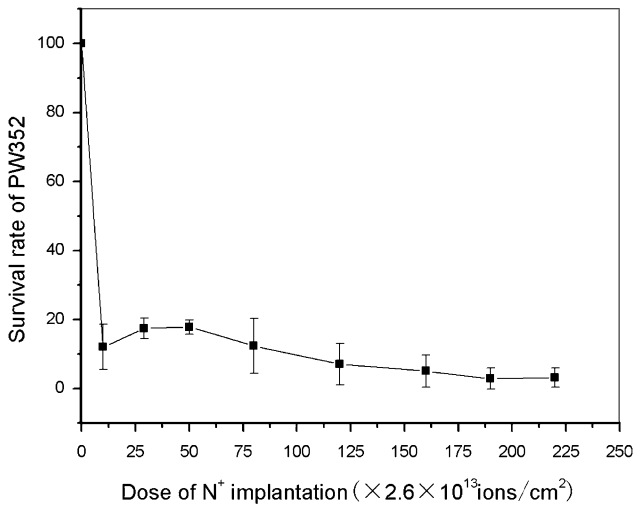

Fig. 2 Fluence response of nitrogen ion beam-irradiated *Rhizopus oryzae* PW352. The survival fraction can be calculated from the formula: Survival fraction $= C_1/C_0$ where C_1 is the number of clones scored on treated plates and C_0 is the number of clones scored on control plates without ion implantation

[10]. This pattern of survival is different from the above-mentioned mutagen. Therefore, the interaction between low-energy ions and organisms may be more complex than that of other radiation with organisms. The biological effect may not only be induced by energy absorption, but also results from mass deposition and charge exchange. At the beginning of ion implantation, more ions reach the cellular cytoplasm; deposition of energy and mass in the cytoplasm might play an important role in breakage of the cytoskeleton and indirect induction of nucleolus damage, which could lead directly to the death of a cell. As the fluence of ion beam is sequentially increased, more deposition of both energy and mass taking place in the nucleolus is likely to be the main factor that causes cellular damage, and activates the cell repair system [6, 16, 17]. No process other than the activation could bring about an increase in the survival fraction. As a result, the survival rate gradually decreases with the increasing ion beam fluence [18].

Mutation induced by ion implantation

The mutation rates induced by nitrogen ion implantation are shown in Fig. 3. At the beginning of ion implantation, with the fluence of nitrogen ions ranging from 0 to $30 \times 2.6 \times 10^{13}$ ions/cm^2, we observed comparably lower positive and negative mutation rates. For doses ranging between $50 \times 2.6 \times 10^{13}$ and $120 \times 2.6 \times 10^{13}$ ions/cm^2, the positive mutation was higher than the negative one, and the range of dose was located right in the "saddle". This range of dose can help us improve the efficiency of mutant screening. As the flux of nitrogen ion implantation was continuously increased, a wide range of effect of fluence was observed; a tendency for gradual decrease of positive mutation was observed with continuously increasing negative mutation rate as the cellular damage became more and more serious.

Energy absorption, mass deposition, and charge exchange of energetic ions possibly lead to mutation effects of ion implantation with remarkable mutagenic efficiency and broad spectrum [19]. Low-energy ion implantation has a distinct mutation mechanism and more complicated biological effects than those induced by convenient mutagens such as X-rays, gamma-rays, and other irradiation.

Characteristics of mutant RQ4015 with high L(+)-lactic acid

Some high-yield mutants were obtained after *Rhizopus oryzae* PW352 was implanted by 10-keV nitrogen ions at 1.30×10^{15} ions/cm^2. The mutant with L(+)-lactic acid yield was continually implanted by nitrogen ions with energy 10 keV and fluence from 3.12×10^{14} to

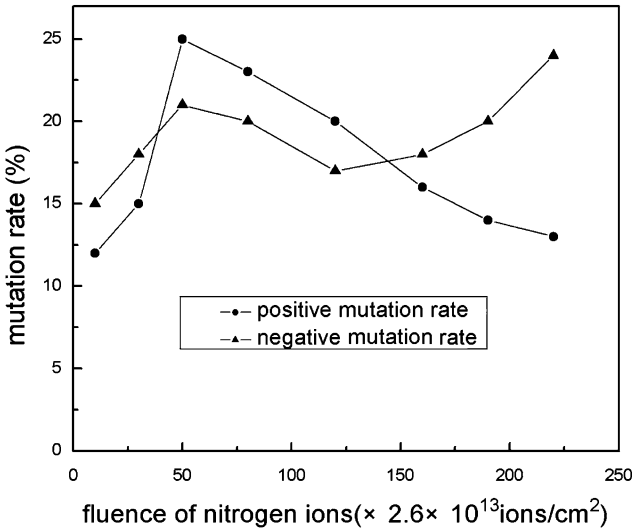

Fig. 3 Relationship between N^+ implantation fluence and mutation rate. About 80 colonies were chosen randomly from survival conidia for each implantation fluence to test the *Rhizopus oryzae* content of cell mass after shake-flask fermentation. The strains were regarded as positive mutation when their yield was higher by 10% than the control strains, whose productivity was less than 10% of negative mutation strains; there were no other differences between positive mutant and negative mutant and other strains. The mutation rate for each implantation fluence was obtained by use of the expressions: Positive mutation rate: $\frac{M^+}{N} \times 100\%$, Negative mutation rate: $\frac{M^-}{N} \times 100\%$ where M^+ is number of positive mutation clones and M^- is the number of negative mutation clones; *N* is the total number of strains screened. Positive mutation rate is indicated by *filled circles* and negative mutation rate by *filled triangles*

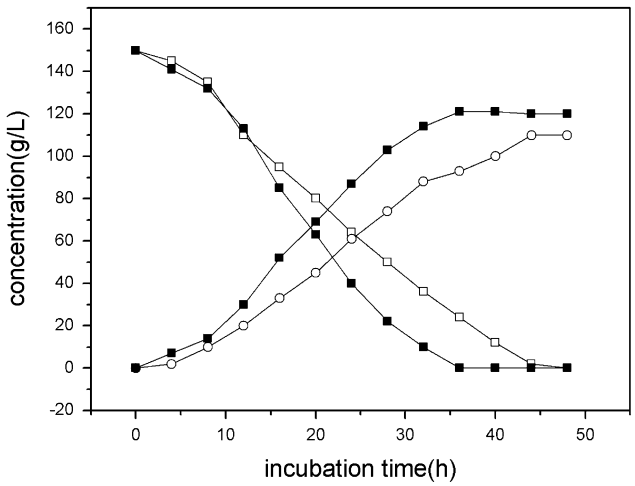

Fig. 4 Fermentation curves for L(+)-lactic acid and glucose for strains PW532 and RQ4015. The fungus was inoculated into a 250-ml shaking flask containing 50 ml medium, and shaken at 200 rpm on an orbital-shaker. pH adjustment was by addition of powdered $CaCO_3$. Glucose concentration in PW352 is indicated by *open squares*, that in RQ4015 by *filled squares*; L(+)-lactic acid concentration in PW352 is indicated by *open circles*, that in RQ4015 by *filled circles*

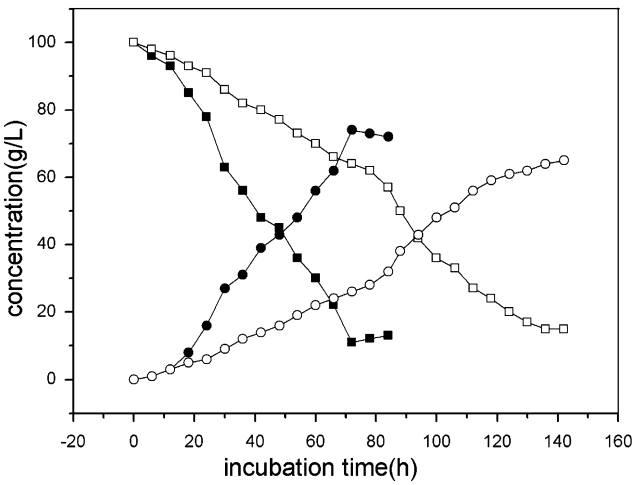

Fig. 5 Fermentation curves for L(+)-lactic acid and 100 g/l xylose for strains PW532 and RQ4015. The fungus was inoculated into a 250-ml shaking flask containing 50 ml medium, and shaken at 200 rpm on an orbital-shaker. pH adjustment was by addition of powdered $CaCO_3$. Xylose concentration in PW352 is indicated by *open squares*, that in RQ4015 by *filled squares*; L(+)-lactic acid concentration in PW352 is indicated by *open circles*, that in RQ4015 by *filled circles*

2.08×10^{15} ions/cm^2. After many serial mutations, the mutant RQ4015, which could produce more L(+)-lactic acid from glucose and xylose, was obtained.

Figure 4 shows the characteristics of mutant RQ4015 and original strain PW532 for lactic acid from glucose. After shake-flask cultivation for 36 h, L(+)-lactic acid production of RQ4015 reached 121 g/l and the productivity was 3.36 g/l h. Compared with the original strain PW532, mutant RQ4015 expressed 10 and 46.7% increases in maximum L(+)-lactic acid concentration and productivity, respectively. The higher yield and shorter fermentation period suggest that *Rhizopus oryzae* RQ4015 is a good microbial producer of L(+)-lactic acid from glucose (Fig. 5) [20].

Effect of different proportions of glucose and xylose

Because glucose and xylose are the main monosaccharides in lignocellulose hydrolysate, these hydrolysates were simulated by mixing xylose and glucose in the synthetic media. To investigate the effect of xylose ratios in sugar mixtures on lactic acid production, RQ4015 was cultivated in fermentation medium with different initial concentrations of xylose in a range from 0 to 100 g/l. Increasing the glucose concentrations resulted in the rise of L(+)-lactic acid yields. With concentrations of glucose at 75 g/l and xylose at 25 g/l mixed in the synthetic medium, the yield of L(+)-lactic acid reached 83% (Fig. 6), which was slightly lower than with glucose as the sole source of carbon.

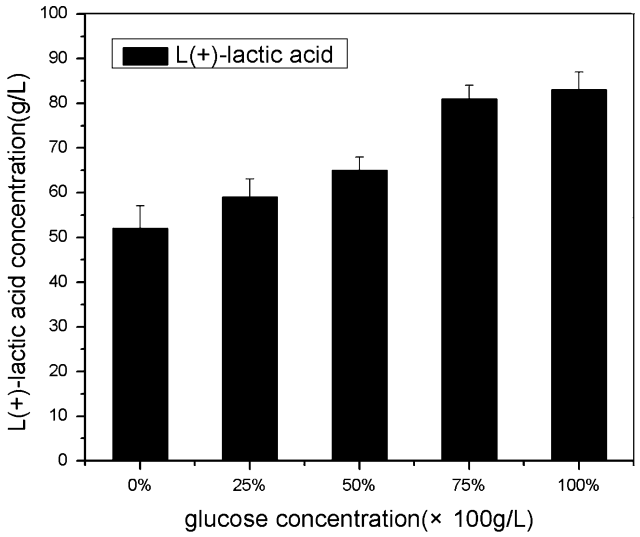

Fig. 6 Effect of different proportions of glucose and xylose mixture on L(+)-lactic acid production by RQ4015. The fungus was inoculated into a 250-ml shaking flask containing 50 ml medium, then shaken at 200 rpm on an orbital-shaker for 72 h. pH adjustment was by addition of powdered $CaCO_3$

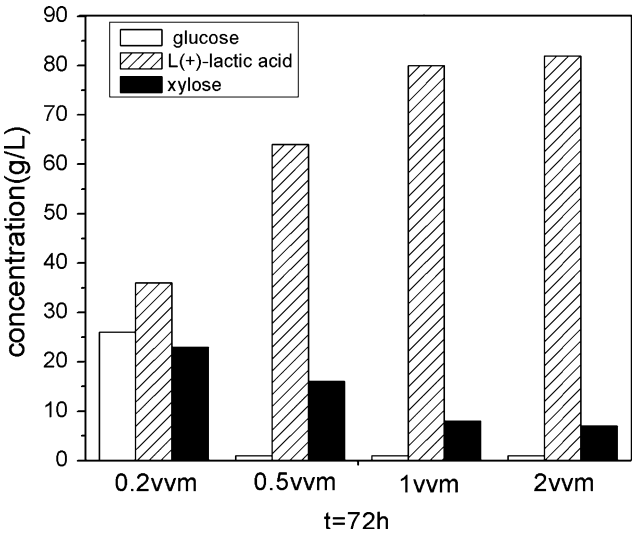

Fig. 7 Effect of aeration on L(+)-lactic acid fermentation. The fungus was inoculated into a 3.7-l bubble column containing 2.5 l fermentation medium and cultured for about 48 h. pH was adjusted by addition of Na_2CO_3

Effect of aeration and pH on L(+)-lactic acid fermentation in a bubble column

Oxygen plays an important role in lactic acid production by *Rhizopus oryzae*. The aeration rates were varied from 0.25 to 2 vvm in a bubble column and the highest yield of lactic acid was obtained for aeration rates ranging from 1 to 2 vvm. As shown in Fig. 7, the concentration of L(+)-lactic acid reached 82 g/l at an aeration rate of 1 vvm, which was adopted for use in subsequent fermentations.

In order to determine the impact of pH on the production of lactic acid by RQ4015 in a bubble column, the pH values were set at 2.5, 4.5, and 6.5. It is recognized that a favorable pH range for lactic acid fermentation in *Rhizopus oryzae* is 5.0–6.0 [21]. Figure 8 shows that yields of L(+)-lactic acid at pH 4.5 and 6.5 were higher than those at pH 2.5. The yield of L(+)-lactic acid at pH 4.5 reached 81% at 72 h. The data revealed that RQ4015 could produce lactic acid effectively at pH ranging from 4.5 to 6.5. RQ4015 might produce lactic acid effectively from lignocellulosic hydrolysate at low pH [22]. Because the quantity of neutralizing agents and the cost of lactic acid purification can be reduced by decreasing the pH, we chose pH 4.5 in the next fermentation.

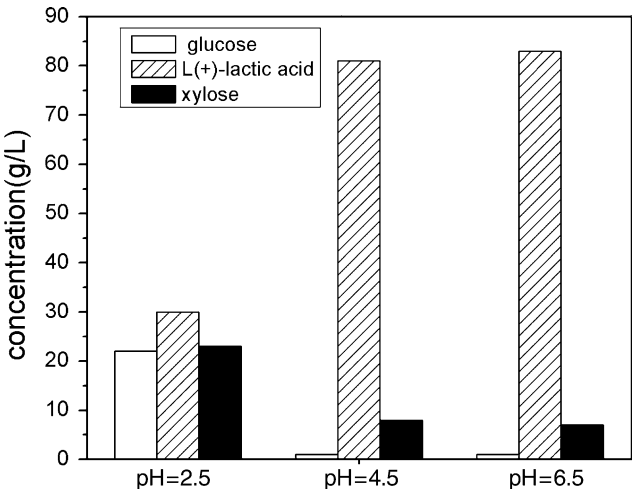

Fig. 8 Effect of pH on L(+)-lactic acid production by RQ4015. The fungus was inoculated into a 3.7-l bubble column containing 2.5 l fermentation medium and cultured for about 48 h. pH was adjusted by addition of Na_2CO_3

L(+)-Lactic acid productivity by co-fermentation of glucose and xylose by *Rhizopus oryzae* RQ4015

To explore the effect of sugar mixtures in a bubble column on lactic acid production by *Rhizopus oryzae* RQ4015, a synthetic medium containing 75 g/l glucose and 25 g/l xylose was used. As shown in Fig. 9, glucose was consumed after 6 h incubation and depleted after 30 h with the xylose being consumed thereafter. The rate of substrate consumption was significantly higher during glucose consumption (2.75 g/l h) compared with xylose consumption (0.5 g/l h). The rates of lactic acid production were 2.1 g/l h during the glucose-consumption phase and 0.4 g/l h during the xylose-consumption phase (Fig. 9). This result indicates that consumption of xylose by *Rhizopus oryzae* is inhibited by glucose, which is called bi-phasic or diauxic growth [23]. There was no statistically significant difference between shake flasks and bubble column. For mixed substrates,

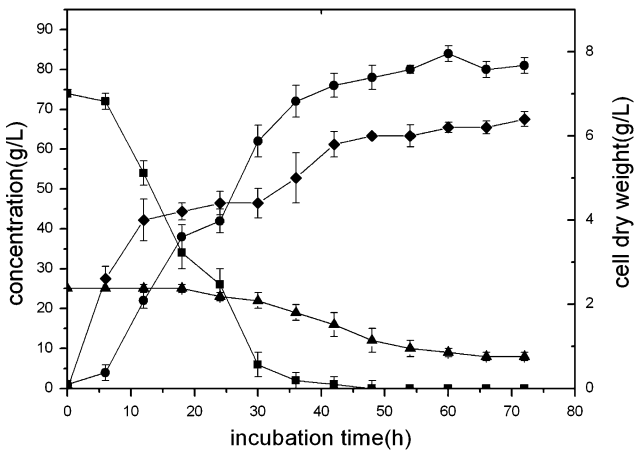

Fig. 9 Curves for production of L(+)-lactic acid from xylose and glucose by strain RQ4015. Culture conditions: aeration rate, 1 vvm; temperature 37°C; pH 4.5. pH adjusted by addition of Na_2CO_3. Glucose concentration is indicated by *filled squares*, xylose concentration by *filled triangles*, L(+)-lactic acid concentration by *filled circles*, and dry weight biomass per liter by *filled diamonds*

available xylose (25 g/l) and glucose (75 g/l) present in shake flasks and bubble column, both concentrations of L(+)-lactic acid from RQ4015 exceeded 80 g/l. In the bubble column we used sodium carbonate as a neutralizing agent to avoid over-consumption of $CaCO_3$ and crystallization of calcium lactate.

Conclusion

This work was performed to obtain a promising industrial strain of *Rhizopus oryzae* to produce high yields of L(+)-lactic acid using glucose and xylose as carbohydrate feedstock. With the proper energy and dose of the ion beam, the mutant RQ4015 was screened as a promising microbial producer which effectively converted glucose and xylose into lactic acid. The use of sodium carbonate as a neutralizing agent in a bubble column avoided over-consumption of $CaCO_3$ and crystallization of calcium lactate. In addition, producing lactic acid by *Rhizopus oryzae* RQ4015 from wheat straw with conversion of glucose and xylose was also an economically feasible process.

Acknowledgments This work is sponsored by The National High Technology Research and Development Program ("863" Program) of China no. 2009AA02Z305. The authors thank Mr Yuan Hang in the Key Laboratory of Ion Beam Bioengineering of the Chinese Academy of Science for his help in performing the ion implantation.

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