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Full Length Research Paper

Effects of agitation speed, temperature, carbon and nitrogen sources on the growth of recombinant *Lactococcus lactis* NZ9000 carrying domain 1 of aerolysin gene

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***Lactococcus lactis* is a Gram-positive bacterium widely used in the production of buttermilk and cheese. Recently, the bacterium becomes famous as the genetically modified organism can be used alive for the treatment of disease. In this study, different cultural conditions based on agitation speed and temperature on the growth of recombinant *L. lactis* NZ9000 harboring domain 1 of aerolysin gene (NHD1Aer) were investigated using shake flask experiment. The effect of different carbon (glucose, sucrose and lactose) and nitrogen (yeast extract, peptone, NH_4Cl, $(NH_4)_2SO_4$, and urea) sources in M17 medium on the cell accumulation were also tested. The results showed that the highest cell concentration (3.22 g/L, μ_m = 0.58 h^{-1}) was obtained when the cultivation was incubated at 27°C and at agitation of 100 rpm. The cells growth was markedly improved when utilizing glucose and peptone/yeast extract as carbon and nitrogen sources, respectively. The aerolysin gene in the cells after four generation time was extracted and then analyzed using agarose gel electrophoresis. The results obtained showed a 250 bp band amplified of domain 1 of the aerolysin gene.**

Key words: Aerolysin, *Lactococcus lactis*, fermentation, one-factor-at-a-time.

INTRODUCTION

Aerolysin is a well-known pore-forming toxin and major virulence factor that was purified from *Aeromonas hydrophila,* a human pathogen that produces deep wound infection and gastroenteritis. *A. hydrophila* is also known as fish pathogen that causes high mortality and great economic losses in freshwater fish farming worldwide. It is capable of killing target cells by forming channels in their membrane after binding to glycosylphosphati-dylinositol-anchored receptors (Abrami et al., 2000). The toxin is activated either by soluble digestive enzymes or by host endoprotease furin which remove a C-terminal fragment about 40 amino acids long. The active aerolysin will then oligomerize in order to form a stable oligomer structure on the surface of the host cells, which inserts into the membrane to form a pore (Buckley, 1992; Fivaz et al., 2001).

Lactococcus lactis is a Gram-positive lactic acid bacterium (LAB) that is generally recognized as safe (GRAS). The potential of *L. lactis* as a vaccine vector has been demonstrated in several publications and one of the report showed that *L. lactis* used as an antigen delivery vehicle is for vaccination against tetanus (Grangett et al., 2002). The gene coding for domain 1 of aerolysin gene

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Abbreviations: LAB, Lactic acid bacterium; **GRAS,** generally recognized as safe; **DNS,** dinitrosalicylic acid; **HPLC,** high performance liquid chromatography; **PCR,** polymerase chain reaction**.**

from *A. hydrophila* AHMP isolated from diseased fish have been cloned and expressed in *L. lactis* NZ9000 by using pNZ8048 recombinant plasmid (Hossein, 2007). The sequences of domain 1 of aerolysin gene that has been cloned have 97% similarity with the published sequence in the GenBank (accession code: AY611033.1). The recombinant *L. lactis* was orally fed to fish in order to study their growth and mortality rates. Reduced mortality rates of the fish fed with recombinant *L. lactis* was observed when challenged with pathogenic *A. hydrophila* (Hossein, 2007).

Various stress response for the growth of different strains of *L. lactis* have been extensively studied such as pH (Simsek et al., 2008; Hofvendahl et al., 1999), temperature (Simsek et al., 2008; Hofvendahl et al., 1999; Kim et al., 2001; Ahmed et al., 2006) and aeration (Nordkvist et al., 2003; van Niel et al., 2002; Pedersen et al., 2008; Christopher et al., 2006). The effect of medium composition on the growth and product formation of *L. lactis* have also been discussed in previous study (Gaudreau et al., 1997; Razvi et al., 2008; Stoyanova and Levina, 2006). Genetically engineered *L. lactis* has a potential to be used as delivery system of live vaccines. However, the poor growth of *L. lactis* becomes a major drawback of this application. In order to obtain high cell density of recombinant *L. lactis* carrying domain 1 of aerolysin gene, optimisation of culture conditions and medium composition for this strain is essential. Therefore, in this study, the effect of various culture conditions such as agitation speed, temperature, carbon and nitrogen sources on the growth of the recombinant *L. lactis* NZ9000 were investigated.

MATERIALS AND METHODS

Microorganism and maintenance

Recombinant *L. lactis* NZ9000 carrying domain 1 of aerolysin gene (NHD1Aer) obtained from Microbiology Laboratory, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia was used in this study. The recombinant strain contains chloramphenicol resistance gene. The strain was propagated in M17 medium (Terzaghi and Sandine, 1975) supplemented with 7.5 µg/ml chloramphenicol and maintained at 4°C (subcultured every week). The stock culture was kept frozen at -80°C in 80% (v/v) glycerol.

Cultivation

The strain was grown in GM17 medium containing 0.5% glucose (w/v) with the following compositions (g L^{-1}): Peptone soya, 5.0; peptone from meat, 2.5; peptone from casein, 2.5; yeast extract, 2.5; meat extract, 5.0; ß-glycerophosphate, 19.0; ascorbic acid, 0.5; lactose, 0.5; $MgSO_4.7H_2O$, 0.25. The cultivation medium was supplemented with 7.5 µg/ml chloramphenicol. The medium (200 ml in 500 ml Erlenmayer flaks) was inoculated with 10% (v/v) inoculum and incubated at different temperature (27 - 33°C) and agitation (50 - 250 rpm).

For testing the effect of different carbon sources; 10 g/L of glucose, lactose and sucrose were added into the modified M17 medium. As for testing the effect of different nitrogen sources; peptone, yeast extract, urea, ammonium chloride and ammonium sulphate were used at the concentration of 17.5 g/L in the modified M17 medium containing (gL^{-1}): glucose, 10.0; ß-glycerophosphate, 19.0; ascorbic acid 0.5; $MgSO_4.7H_2O$, 0.25. The amount of nitrogen sources used was based on the total concentration of nitrogen sources in the original M17 medium (Terzaghi and Sandine, 1975). The experiments were carried out in triplicates for each parameter tested.

Analytical methods

The cell turbidity was measured at 600 nm (GENESYS 20 – Thermo Spectronic). A linear correlation of OD_{600} to biomass concentration relationship was established. This correlation was used to convert OD_{600} values to biomass concentrations.

The supernatant was collected for residual sugar analysis. Glucose and lactose concentration was determined by the dinitrosalicylic acid (DNS) method (Miller, 1959). Sucrose concentration was analyzed using high performance liquid chromatography (HPLC) fitted with an NH_2 column (Bio-Rad) fed with 80% acetonitril solution at a flow rate of 1 ml min^{-1}.

Plasmid DNA extraction and polymerase chain reaction (PCR) amplification of aerolysin gene

Recombinant plasmid pNZ8048D1Aer containing domain 1 of aerolysin gene from the recombinant *L. lactis* was extracted by using high yield plasmid extraction kit (Real Genomics™). The plasmid was then subjected to PCR amplification using a pair of primers: D1AerF (5' at gct gca gaa atg atg aat aga ata att acc gc-3') and D1AerR (5'- at gca agc ttg ccc cat aat ctc cca gcg at-3') to amplify the 250 bp fragment of aerolysin gene. The amplified PCR products were then analysed using 1% agarose gel electrophoresis.

RESULTS AND DISCUSSION

Effect of agitation speed

Optimisation of the growth of recombinant *L. lactis* carrying domain 1 of aerolysin was carried out using traditional method of one-factor–at-a-time. Table 1 shows the results obtained under different agitation speed at 30°C in shake flasks. The fermentation profile is shown in Figure 1a. The strain took four to six hours to reach maximum cell concentration. The maximum cell concentration (2.93 g/L) was obtained for the fermentation with an agitation speed of 100 rpm. The cell concentration obtained was slightly decreased when the agitation speed is increased to 200 rpm (2.82 g/L). *L. lactis* was classified as a facultative anaerobe (Neves et al., 2005) and oxygen has a strong regulatory effect. Cell growth under aerobic condition is typically low due to product inhibition such as hydrogen peroxidase or oxygen stress (Ishizaki and Ueda, 1995). However, from the results on different aeration speed, the high oxygen supply to the strain did not suppress the growth of NHD1Aer. Lower agitation at 50 rpm gave a relatively low value of cell accumulation (2.39 g/L). According to Van Niel et al. (2002), H_2O_2 accumulation might occurred especially when the lactococci are grown under growth-limiting conditions. This could be

Table 1. Effect of agitation speeds on the growth kinetics of recombinant *L. lactis* NHD1Aer at 12 h fermentation.

Growth kinetics	Agitation speed				
	50 rpm	100 rpm	150 rpm	200 rpm	250 rpm
X (g/L)	2.42 ± 0.01	2.93 ± 0.01	2.69 ± 0.08	2.82 ± 0.19	2.73 ± 0.27
μ_m (h^{-1})	0.58	0.57	0.64	0.66	0.59
$Y_{x/s}$ (g/g)	0.46	0.65	0.41	0.75	0.71
S_i-S_o	4.86	4.53	6.14	3.85	3.92

S_i, Initial substrate concentration (g/L); S_o, final substrate concentration (g/L); S_i-S_o, substrate utilization; $Y_{x/s,}$ cell yield coefficient (g/g); μ_m, maximum specific growth rate (h^{-1}); X, maximum cell concentration (g/L).

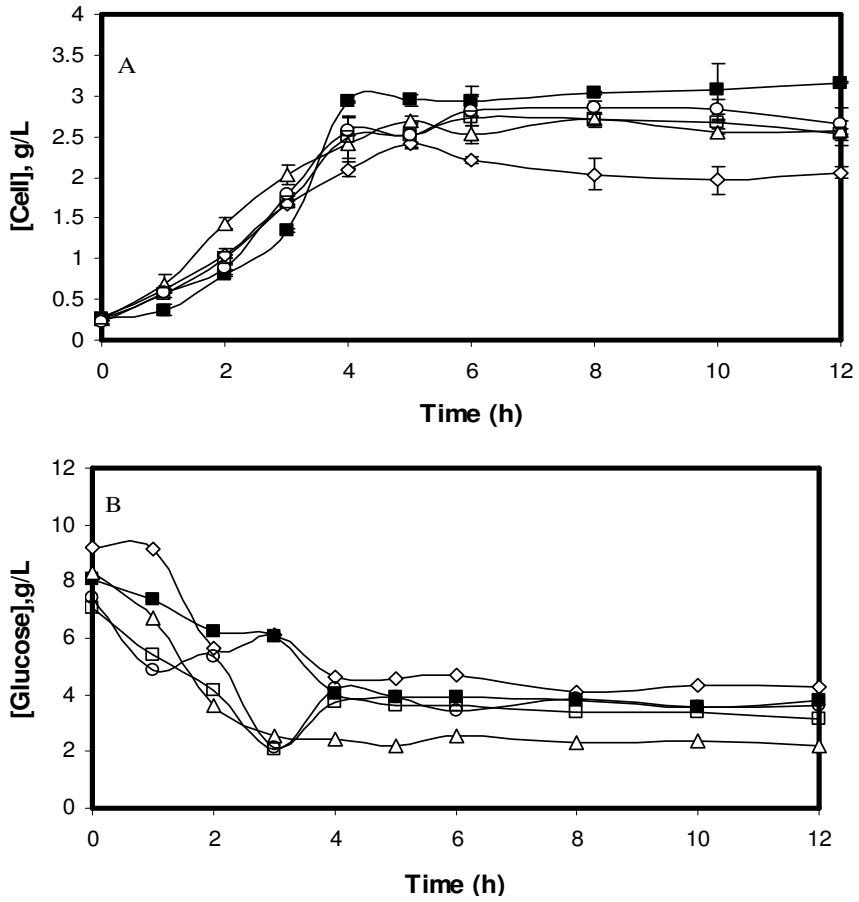

Figure 1. Growth profile of NHD1Aer (A) and residual sugar (B) at different agitation speeds. (◊) 50 rpm, (■) 100 rpm, (Δ) 150 rpm, (o) 200 rpm and (□) 250 rpm.

avoided by using rich medium or medium added with catalase, yeast extract or reducing agents. The sustain growth of NHD1Aer in this study might be due to the presence of complex nitrogen sources in the media used (GM17).

Figure 1b shows that the sugars supplied in the medium (10 g/L) were not fully utilized in all conditions. Rapid utilization of the sugar can be observed at early growth of this strain until it reached late exponential phase. However, bacterial growth showed plateau after 6 h fermentation.

Effect of temperature

The fermentation of NHD1Aer carried out at 27°C gave the highest value of cell concentration (3.22 g/L) as compared to the other two conditions (Table 2). Lactic acid bacteria are mesophilic in nature. They were grown rapidly at temperatures between 20 - 30°C and the growth

Table 2. Effect of temperature on the growth kinetics of recombinant *L. lactis* NHD1Aer at 12 h fermentation agitated at 100 rpm.

Growth kinetics	Temperature		
	27ºC	33ºC	37ºC
X (g/L)	3.22 ± 0.04	2.74 ± 0.15	2.81 ± 0.11
μ_m (h^{-1})	0.58	0.74	0.66
$Y_{x/s}$ (g/g)	0.59	0.41	0.42
S_i-S_o	5.34	6.39	6.47

S_i, Initial substrate concentration (g/L); S_o, final substrate concentration (g/L); S_i-S_o, substrate utilization; $Y_{x/s}$, cell yield coefficient (g/g); μ_m, maximum specific growth rate (h^{-1}); X, maximum cell concentration (g/L).

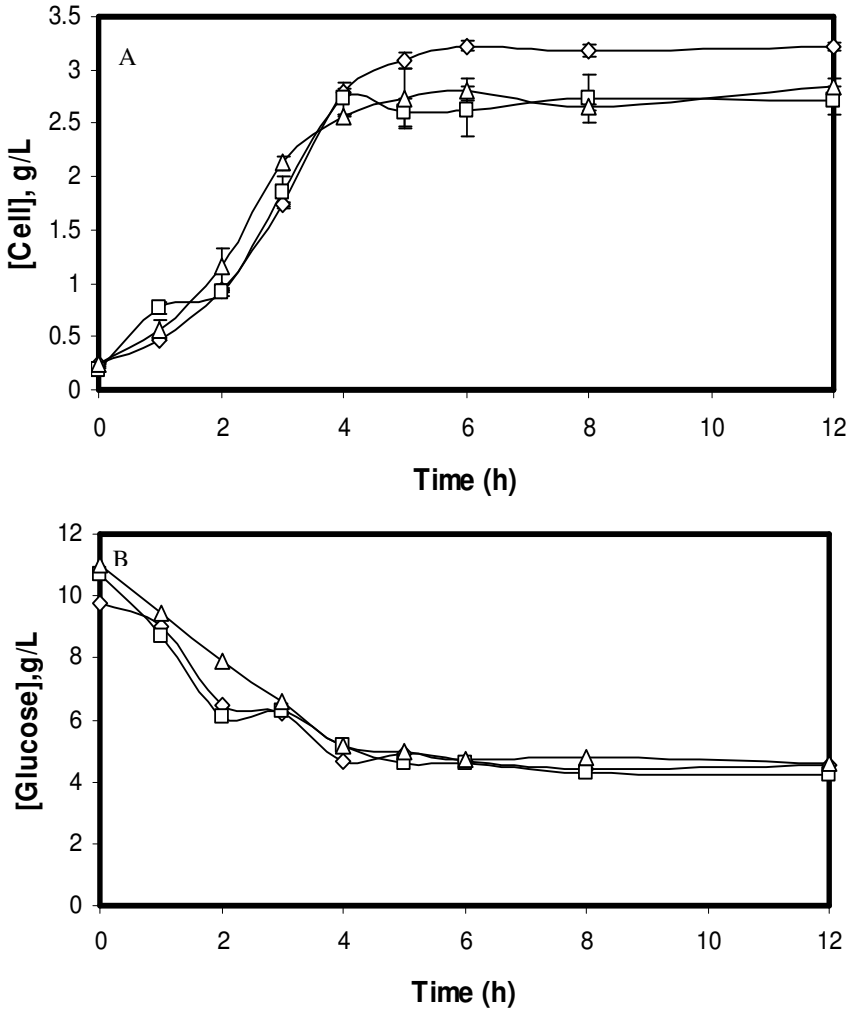

Figure 2. Growth profile of NHD1Aer (A) and residual sugar (B) at different cultural temperature (◊) 27°C, (□) 33°C and (Δ) 37°C.

is inhibited at temperatures greater than 40°C (Ahmed et al., 2006). According to Ademberg et al. (2003), the optimal temperature range for different strains of *L. lactis* was 27-33°C. Simsek et al. (2008) reported that accelerated cell growth of two different strains of *L. lactis* spp. *lactis* can be observed at 25°C up to 40°C.

Sugar utilization by NHD1Aer in all three different cultural temperatures was not much different. A total of 5 - 6 g/L glucose has been consumed for the growth of this strain. Figure 2a and b show less sugar consumed after

Table 3. Growth kinetics of recombinant *L. lactis* NHD1Aer grown on different carbon and nitrogen sources at 27º C.

Growth kinetics	Carbon source (M17 medium)			Nitrogen source				
	Glucose	Sucrose	Lactose	Yeast extract	Peptone	NH_4Cl	$NH_4(SO_2)_4$	Urea
X (g/L)	2.57 ± 0.03	2.23 ± 0.09	0.79 ± 0.02	3.17 ± 0.03	3.18 ± 0.01	1.23 ± 0.00	1.25 ± 0.14	0.41 ± 0.01
μ_m (h^{-1})	0.57	0.56	0.37	0.56	0.56	0.35	0.39	0.14
$Y_{x/s}$ (g/g)	0.52	0.62	0.24	0.58	0.58	0.34	0.31	0.14
S_i-S_o	5.12	4.33	2.34	5.22	5.48	3.52	3.92	1.59

S_i, Initial substrate concentration (g/L); S_o, final substrate concentration (g/L); S_i-S_o, substrate utilization; $Y_{x/s,}$ cell yield coefficient (g/g); μ_m, maximum specific growth rate (h^{-1}); X, maximum cell concentration (g/L).

the cells reached stationary phase.

Effect of carbon and nitrogen sources

The effects of different carbon and nitrogen sources on the growth of this recombinant strain were also tested. Table 3 shows the results of different substrates and nitrogen sources used. The other media components, except for the carbon source were added based on the components of M17 media. M17 medium was commonly used for the growth of *L. lactis* (Novak et al., 1997; Christopher et al, 2006).

Effect of carbon source

The media added with glucose as the carbon source resulted in the highest cell concentration (2.57 g/L), followed by sucrose (2.23 g/L) and lactose (0.79 g/L) (Figure 3). However, the specific growth rate of the strain grown on glucose and sucrose were comparable (Table 3). Stoyanova and Levina (2006) reported the effect of fermentation medium components on the growth and bacteriocin production of recombinant strain *L. lactis* subsp. *lactis* F-116. Their results showed that D-sucrose was the most suitable carbon source for the growth of strain F-116, followed by D-glucose and D-lactose. The low growth rate of NHD1Aer was observed in the medium containing lactose. High concentration of remaining lactose can be observed (Figure 1b) at the end of the fermentation. This indicated that the bacterium was unable to utilize the lactose. This result is in agreement to Stoyanova and Levina (2006).

Effect of nitrogen source

The effect of different nitrogen sources was tested in order to select the best nitrogen source for the growth of NHD1Aer. There were three inorganic nitrogen tested for the growth of this strain; urea, NH_4Cl and $(NH_4)_2SO_4$. The results showed that the cell concentration of the recombinant *L. lactis* grown in the medium containing inorganic nitrogen was much lower compared to the medium using organic nitrogen; yeast extract and peptone. The values of cell concentration of NHDIAer grown in the medium containing yeast extract and peptone were 3.17 and 3.18 g/L, respectively (Figure 4). The cell concentration values were higher compared to cell concentration of recombinant *L. lactis* containing *lux*AB gene (1.43 g/L) grown on GM17 (Griffiths et al., 2006) and recombinant F-116 (1.61 g/L) grown on semi-defined media containing sucrose, yeast autolysate, salt, ammonium sulphate and KH_2PO_4 (Stoyanova and Levina, 2006). Complex nitrogen sources enhanced the growth of NHD1Aer, while inorganic nitrogen sources especially urea did not support the growth of this strain. According to van Niel and Hahn-Hagerdal (1999), LAB is incapable of growing in medium using mineral nitrogen without exogenous amino acids. *L. lactis* requires a large number of essential nutrients to grow. It includes some essential amino acids and vitamins for accelerated cell growth (Razvi et al., 2008). Rich media such as yeast extract and peptone contain adequate amounts of minerals needed for the growth of LAB. In addition, buffering capacity of yeast extract could prevent excessive drops in pH, which would inhibit cell growth (Gaudreau et al., 1997). *L. lactis* NHD1Aer consumed about 50% of the glucose provided when grown in the medium containing yeast extract or peptone as carbon source (Table 3). The slow growth of *L. lactis* NHD1Aer in the medium containing urea as nitrogen source resulted in the less amount of glucose consumed.

Plasmid extraction and PCR confirmation of aerolysin gene

The stability of plasmid in a recombinant strain during fermentation especially for industrial application is very important. Therefore, in this study the presence of the plasmid in the strain was verified. The recombinant plasmid pNZ8048D1Aer from the cells of *L. lactis* transformant after four generations time was extracted using high yield plasmid extraction kit. The amplified PCR product was analysed using agarose gel electrophoresis. Lane 3 and 4 (Figure 5) show the plasmid band at the expected size

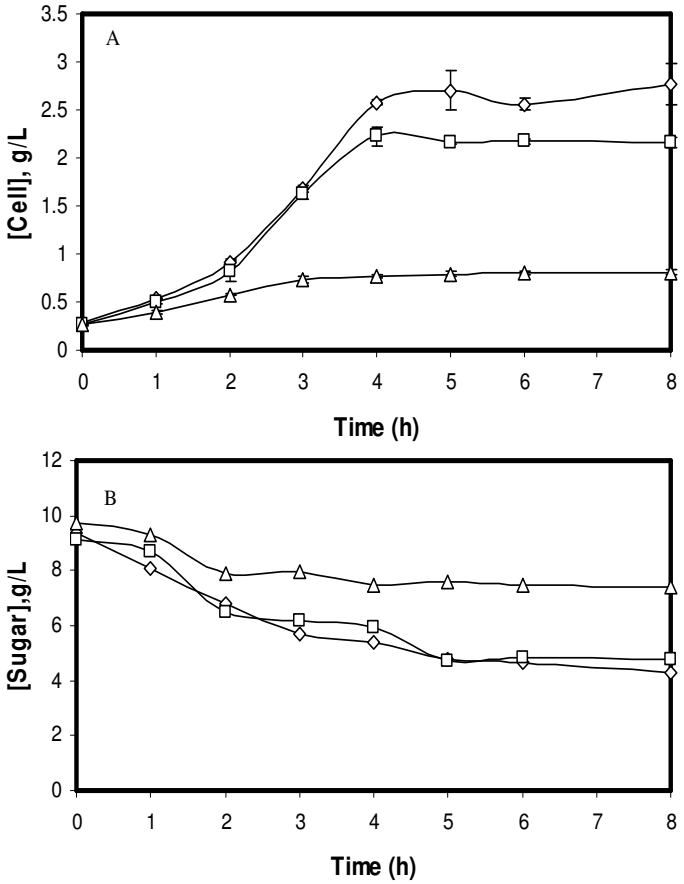

Figure 3. Growth profile of NHD1Aer (A) and residual sugar (B) on M17 media containing different carbon sources. (◊) Glucose, (□) sucrose and (Δ) lactose.

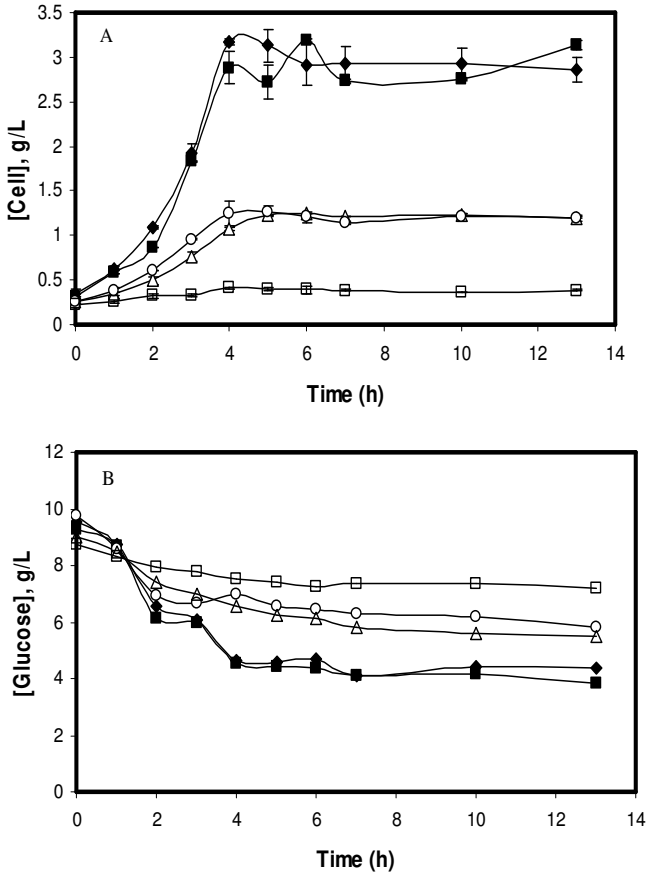

Figure 4. Growth profile of NHD1Aer (A) and residual sugar (B) on modified M17 media containing different nitrogen sources. (♦) Yeast extract, (■) peptone, (Δ) ammonium chloride, (o) ammonium sulphate and (□) urea.

of around 3.5 Kbp. After the plasmid has been extracted, the product was subjected to PCR amplification. The size of the interest gene, domain 1 of aerolysin was approximately 250 bp. Amplified PCR products were analysed with 1% agarose gel electrophoresis and the results obtained show a 250 bp band amplified of domain 1 of the aerolysin gene (lane 1 and 2 of Figure 4), which is in accordance with the expected size (250 bp).

Conclusion

The highest cell concentration of recombinant *L. lactis* NHD1Aer (3.22 g/L) was obtained when the fermentation was carried out at 27°C, agitated at 100 rpm. Glucose and yeast extract/peptone showed the best carbon and nitrogen sources for the growth of the recombinant strain. Verification for the presence of the gene fragment coding for domain 1 of aerolysin gene up to four generations in the cells was done by PCR amplification and agarose gel electrophoresis. The fragment amplified showed the expected size of 250 bp.

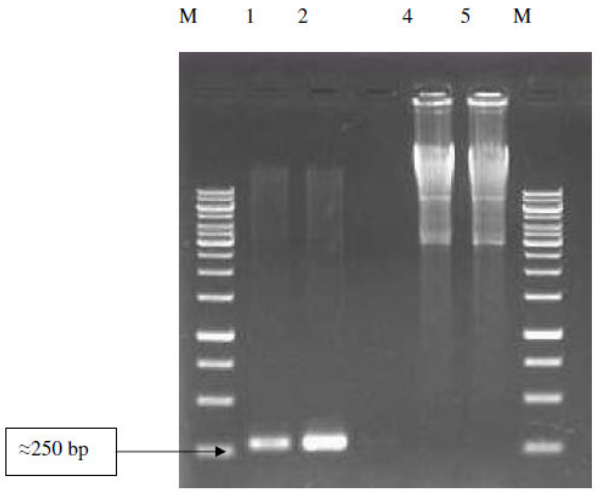

Figure 5. Agarose gel electrophoresis of plasmid extraction and PCR amplification products of Domain 1 of aerolysin gene. M: 1 Kb GeneRuler DNA ladder; lanes 1 and 2: PCR amplification product of domain 1 of aerolysin gene; lanes 4 and 5: product of plasmid extraction from the recombinant *L. lactis* harboring domain 1 of aerolysin gene.

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