

# Nitrogen, glucose and chitin interactively regulate the expression of chitinolytic enzyme encoding genes *ech30*, *ech42* and *nag1* in *Trichoderma atroviride* P1 under varying growth conditions

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## Background

*T. atroviride* P1 (ATCC 74058), previously known as *T. harzianum* P1, has been known as an effective biocontrol agent of several plant diseases. One of the processes involved in mycoparasitism is the excretion of cell wall degrading enzymes such as chitinases, glucanases and proteases. Due to their important role in biocontrol, chitinase encoding genes have been studied in certain circumstances. The regulation of *ech42* and *nag1* expression in *T. atroviride* P1 has been studied in considerable detail.

However, due to the different methods applied, results were varying. Thus, we have included both genes in the current study.

Furthermore, there is no published report describing the expression of gene *ech30* under different growth conditions. Moreover, combining the quantitative real-time PCR method and statistical analyses was a new approach and enabled us to distinguish factors that had significant effect on gene expression. Such information could not otherwise be obtained by northern blot analysis or enzyme assays.

Here, we report the quantification of expression and regulation of the *ech30*, *ech42* and *nag1* genes in *T. atroviride* P1 under 12 media combinations. Principal component analyses were used to distinguish the factors that had the greatest effect on gene expression.

## Methods

*Trichoderma atroviride* strain P1 was cultured on potato dextrose agar for 10-14 days at 21°C. Conidial spores were harvested from the PDA plates with sterile deionized water and were inoculated to a final concentration of  $1 \times 10^6$  spores/ml, in a 100 ml liquid synthetic medium containing the following (a) 13.6 g/l  $\text{KH}_2\text{PO}_4$ , (b) 10 g/l  $\text{NH}_4\text{NO}_3$ , (c) 0.2 g/l  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , (d) 2 g/l KCl, 2g/l  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 2g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.02 g/l  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ , 0.02 g/l  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  and 10 g/l sucrose. The pre-culture was carried out at 22°C on a reciprocal shaker at 100 rpm for 48h in the dark. The mycelium was collected by centrifugation.

To study the impact of chitin, carbon and nitrogen sources on *ech30*, *ech42* and *nag1* gene expression, colloidal chitin, glucose and ammonium acetate at different concentrations were added to the culture medium resulting in 12 different growth media with varying nutrient concentrations as shown in Table 1. The culture conditions were the same as described above. Two replications and three incubation times (18 h, 36 h and 72 h) for each experiment. In total, 72 cultures were included.

According to the time scale set up for the growth experiments, mycelia were harvested, centrifuged, filtered and washed as described above. After harvest, the mycelia were frozen in liquid nitrogen and stored at -80 °C until RNA extraction. Total RNA was isolated using Fast RNA™ Kit-Green, BIO101, according to the manufacturer's instructions.

Table 1. List of media combinations used in the present study

Glucose (%)	Colloidal chitin (dry weight/V)	Ammonium (mM)		
		0	10	100
0.1	2	1	3	5
	0	2	4	6
3	2	7	9	11
	0	8	10	12

PCR primers and probes for the endogenous control, 18S rRNA, and the *ech30*, *ech42* and *nag1* genes were designed using Primer Express® software 1.0. The probes were labeled at the 5' ends either with reporter dye FAM (6-carboxyfluorescein) or VIC™, and at the 3' ends with quencher dye TAMRA (6-carboxytetramethylrhodamine). The primers were purchased from Eurogentec while the probes were made by Applied Biosystems. The sequences of primers and probes and the expected size of PCR products are listed in Table 2.

The real-time quantitative RT-PCR was carried out in MicroAmp optical 96-well or MicroAmp optical 8 strip PCR tubes using the TaqMan ABI Prism 7700 sequence detection system.

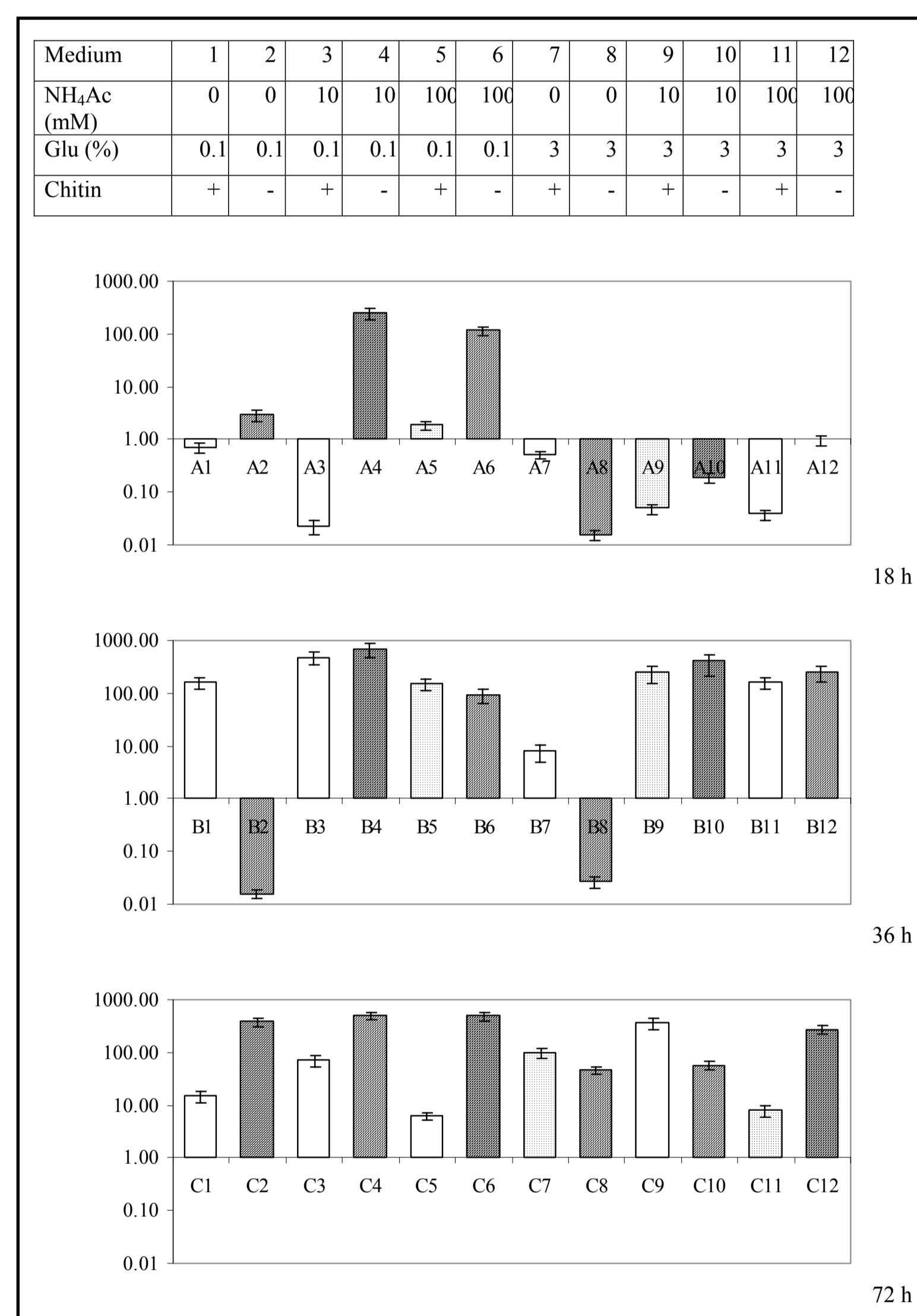


Fig. 1. Expression of *ech30* relative to the internal control 18SrRNA and calibrated to the expression level of A12. X-axis: 1-12 = medium names; A, B and C = 18 h, 36 h and 72 h cultures respectively. Y-axis: expression level on log scale, mean  $\pm$  standard deviation.

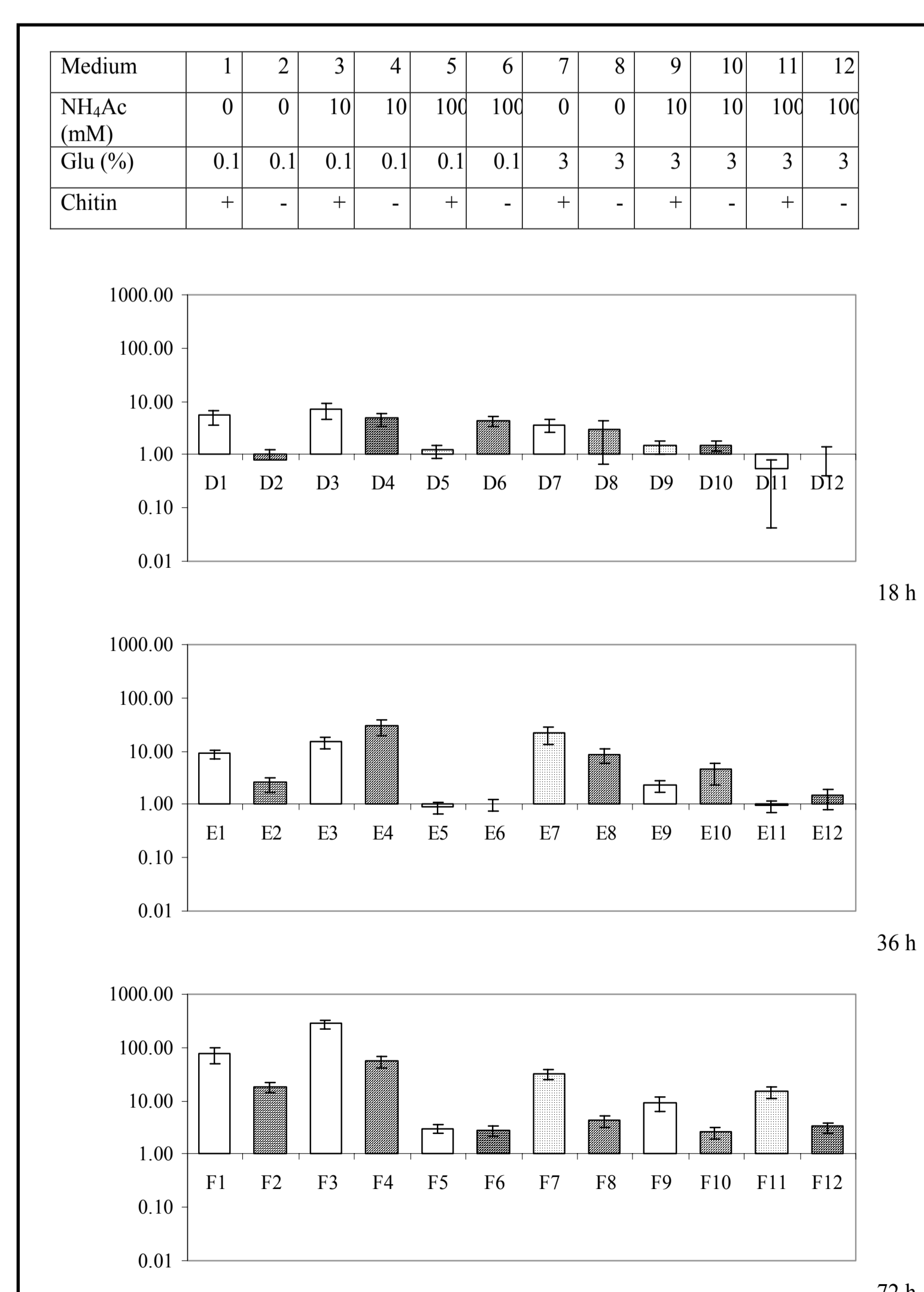


Figure 2. Expression of *ech42* relative to the internal control 18SrRNA and calibrated to the expression level of D12. X-axis: 1-12 = medium names; D, E and F = 18 h, 36 h and 72 h cultures respectively. Y-axis: expression level on log scale, mean  $\pm$  standard deviation.

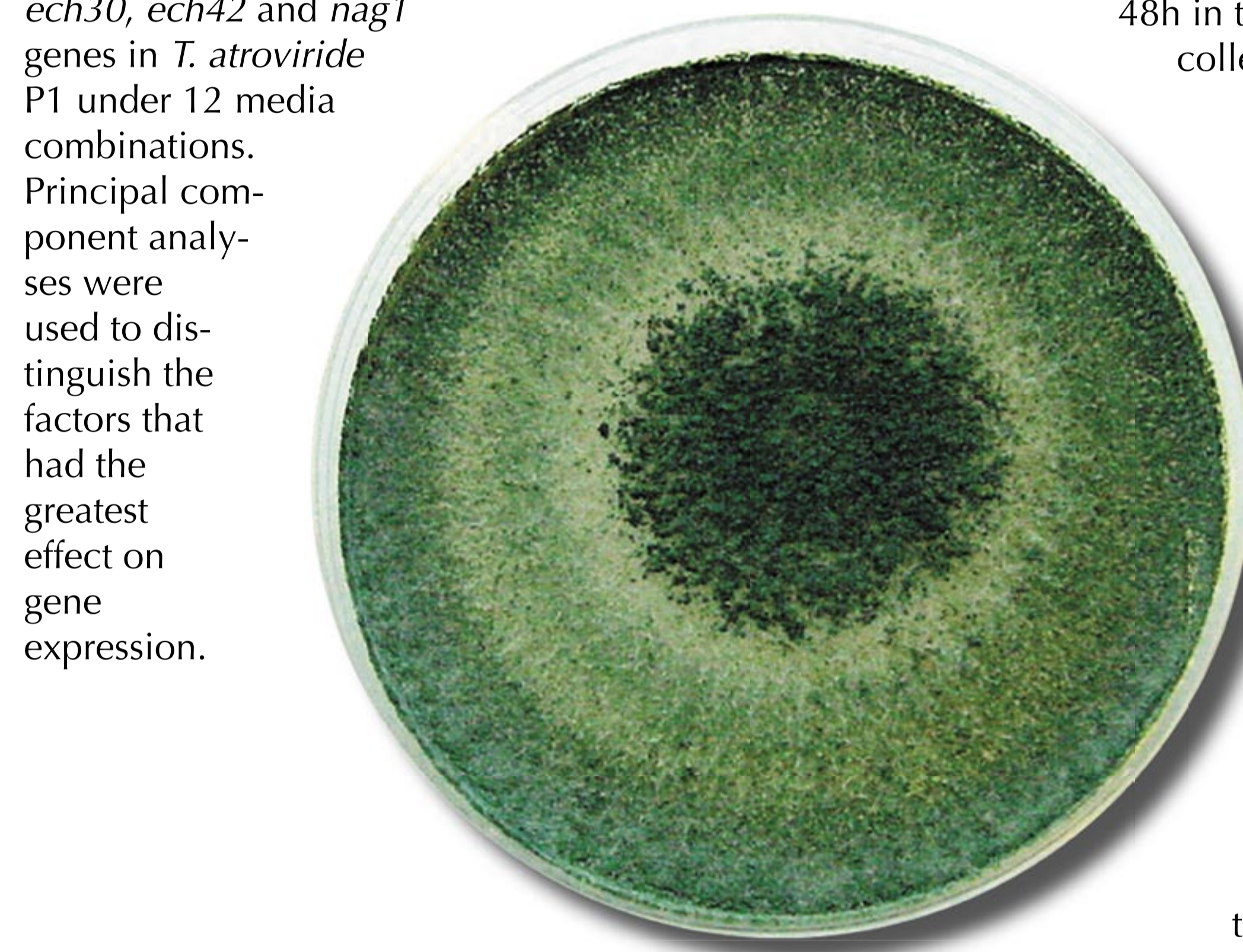


Table 2. Sequences of PCR primers and TaqMan fluorescent probes for the genes 18S rRNA, *ech30*, *ech42* and *nag1*

Gene	PCR product size (bp)	Forward primer (5'-3')	Reverse primer (5'-3')	Probe (5'-3')
18S rRNA	73	GAACCAGCGGAGGGATCAT	CGAGGCAACAGTTGGTATGG	ACCGAGTTTACAACCTCCAAACCAATGTG
<i>ech42</i>	72	GACGACGATTCTTGAACGAC	TTCTTCAGTTGACAGCTGCT	CACACAGCCGTACGCATTGTTACCGA
<i>nag1</i>	69	CCGATCATCAATCGGCTA	GCCCTGAACAATCTCTTGC	CCGCTGCTGGTTCACCACTCAAC

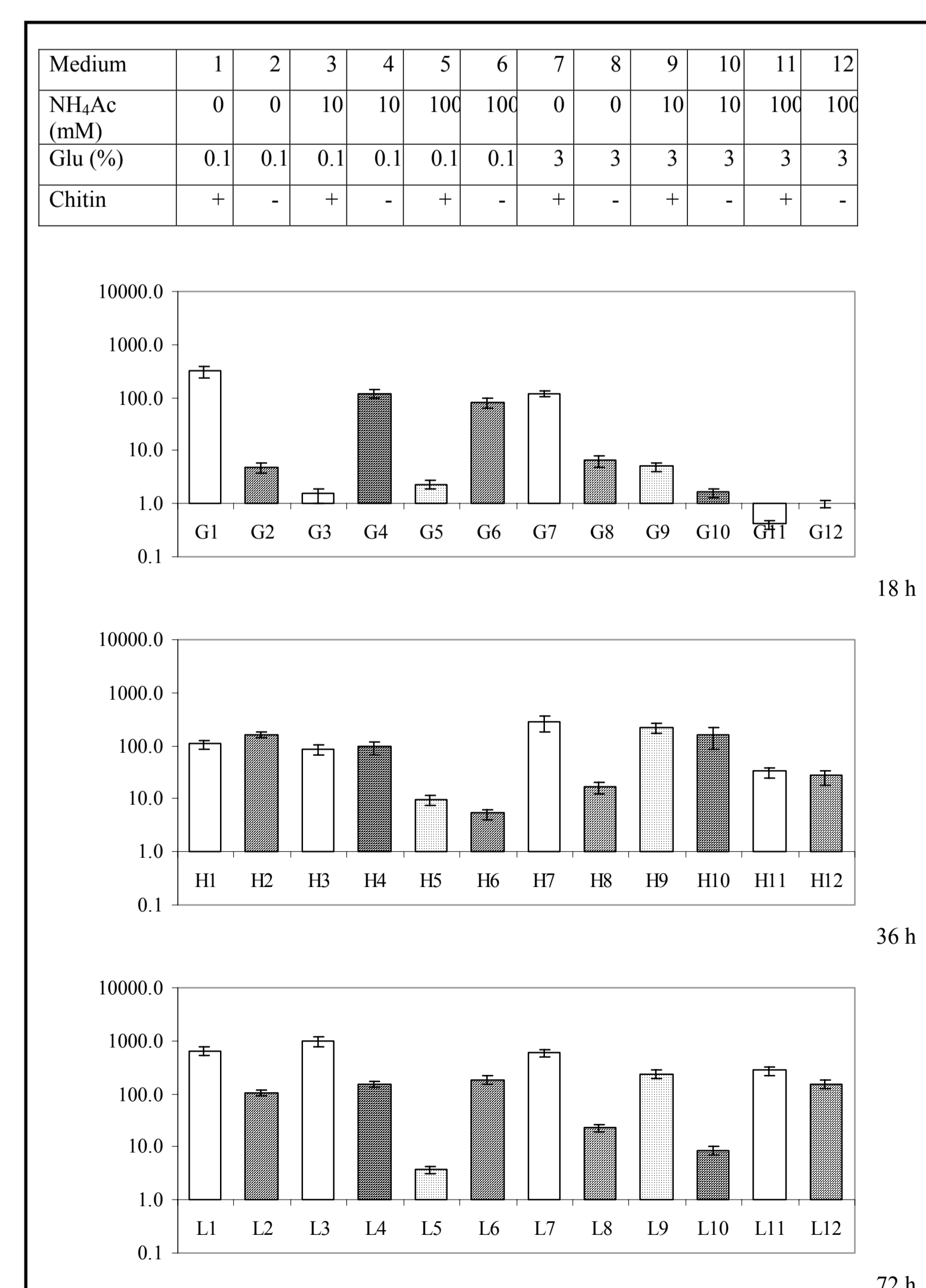


Figure 3. Expression of *nag1* relative to the internal control 18SrRNA and calibrated to the expression level of G12. X-axis: 1-12 = medium names; G, H and L = 18 h, 36 h and 72 h cultures respectively. Y-axis: expression level on log scale, mean  $\pm$  standard deviation.

Table 3. Principal component analysis factor loadings (Varimax normalized). Loadings >0.5 are shown in bold type.

Time (hours)	Variable	Factor 1	Factor 2	Factor 3
18	Chitin	0.21	<b>0.92</b>	0.05
	Glucose	<b>-0.82</b>	0.15	-0.22
	NH <sub>4</sub> acetate	-0.11	0.01	<b>0.95</b>
	<i>nag1</i>	<b>0.72</b>	0.16	-0.32
	<i>ech30</i>	<b>0.53</b>	<b>-0.71</b>	0.09
	<i>ech42</i>	<b>0.80</b>	<b>0.02</b>	-0.35
36	Chitin	0.10	0.02	<b>0.96</b>
	Glucose	0.26	<b>0.76</b>	-0.20
	NH <sub>4</sub> acetate	<b>-0.88</b>	0.09	0.04
	<i>nag1</i>	<b>0.87</b>	0.23	0.24
	<i>ech30</i>	0.20	<b>-0.73</b>	-0.24
	<i>ech42</i>	<b>0.67</b>	<b>-0.57</b>	-0.06
72	Chitin	0.25	<b>0.85</b>	-0.06
	Glucose	<b>-0.85</b>	0.28	0.16
	NH <sub>4</sub> acetate	-0.06	0.04	<b>-0.96</b>
	<i>nag1</i>	<b>0.65</b>	<b>0.57</b>	0.37
	<i>ech30</i>	0.21	<b>-0.84</b>	-0.04
	<i>ech42</i>	<b>0.77</b>	0.37	0.37

## Results

The real-time RT-PCR analysis showed that the expression of *ech30*, *ech42* and *nag1* was regulated by the interaction of nitrogen, glucose and chitin under different growth conditions (Fig 1-3). The highest and earliest expressions of *ech30* were induced by glucose and nitrogen starvation i.e. 0.1% glucose and 10 mM NH<sub>4</sub>Ac in the growth media. This was also the case for *ech42* and *nag1* but at a relatively low level (Fig 1-3). In contrast, high (3%) glucose and high (100 mM) NH<sub>4</sub>Ac concentrations repressed the expression of all the genes studied. These results were confirmed by principal component analyses (Table 3). The axis of maximum variance (36%) in the data set for the 18h culture was connected with glucose, indicating that glucose was the main factor negatively regulating the expression of *ech30*, *ech42* and *nag1* (Table 3). The impact of ammonium was related with the axis of maximum variance (35%) in the 36 h culture, suggesting that it was a main factor (besides glucose) regulating the expressions (Table 3). The effect of chitin on *ech30*, *ech42* and *nag1* expression varied depending on the concentrations of glucose and NH<sub>4</sub>Ac.