Supplementary Materials for

Screening for Type II L-Asparaginases: Lessons from the Genus *Halomonas*

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Volume 16, Issue 4 (Autumn 2017)

This PDF file includes:

Figures S1 to S6

Tables S1 and S2

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huangheensis    1 CGAGGATCATGCCAAAAGCCATGGGGTCCACCAGCGGATCACCAGGTTCCTTGCGAATGA
KO116           1 CCAGGATCATCCCGAACGCCATTGGATCGACGAGCGGTGTGCCGGGTTCGCGGCGAATCA
R57-5           1 -----ATCATTCCAAATGCCATCGGGTCGACGAGTGGCGTGCCTGGTTCGCGGCGAATCA

huangheensis   61 TGGTGCCCTGCCCGCGCTTCACTTCGACAATGCCGATGGCCTGCAGCATCTTGATGGCTT
KO116          61 TGGTGCCTTGGCCTCGTTTGACTTCGACAATCCCTATCGCCTGCAGCATCTTGATGGCTT
R57-5          56 TGGTGCCCTGGCCGCGTTTGACTTCCACAATGCCAATGGCTTGCAGCATTTTGATGGCTT
                                                                  ANSF ⇨

huangheensis  121 CTCGCACACTTGACTTTCCGATTCCCAGGCTTTGGGTCAGCTCTGTTTCCGAAGGCAGGT
KO116         121 CGCGCACACTGGATTTACCAATGCCCAGGGTGTGAGTGAGCTCCGTTTCTGAGGGTAGGT
R57-5         116 CACGCACGCTCGATTTGCCAATCCCCAAACTGTGGGTCAATTCAGTTTCTGAAGGTAGAT

huangheensis  181 AGTCACCTGGCTGAAGCTCGCCGCTGATCAGCGCGCTCTTGATACGCTCGAGCAGTTGAA
KO116         181 AATCCCCTGGCTTAAGATCGCCTCGTATTAGGGCGCTTTTAATACGCTCTAGCAGCTGCA
R57-5         176 AATCCCCTGGTTTGAGGTCTCCACGTATCAAGGCGCTTTTAATGCGCTCTAGCAGTTGCA

huangheensis  241 GGGCAACGGAACTACGGTCAATCGAACTCCCGAATGACTCCATGGATGACGTCCTTGGCT
KO116         241 GCGCAACTGAACTGCGATCCATCGAATCTCCAAAAGAGGTTGTACTGGTCGAGTTAATCT
R57-5         236 GCGCAACGGAGCTGCGCTCCATCGAATCCCCAAAAGACGTTGTAGTGGTCGAGGTAATCT

huangheensis  301 GGCGTTTTACTGGCATGGTCCGGTCTTGCTCGACTGCCGTCAAG-----------ACTCT
KO116         301 CGGGCTGGCGTTTAAAACTCATGTGGGCCTCGCGTGGCTCATCGGTGTTTTCTGCATTTT
R57-5         296 CGGGCTGGCGTTTAAAACTCATGGGGGCCTCGCGTGGCTCATCGGTGTTTTTTGCATCCT

huangheensis  350 GGAAAGCCGCC-GGAAATCAGGCTTCCACTCTATCCATGATAACGATTCTCCAGCCGACC
KO116         361 ATCAATGTCCTAAGCCTAGCGGCTTAAG-CCATTCGATTGCATATA-TTTAAAAGAAATA
R57-5         356 ATCAATGCCATAAGCCTAGCGGCTTAAG-ACATCTGATGGCATATA-TTTAAAAGTAATT

huangheensis  409 TTGTTGTGCTGTTTCAACTGGGACGC-----CTGGCGCTTCTGCAAAATCGAGCCTAGCA
KO116         419 TAGTTATGTTTCATTGGTTGGTGAGTCTGGCTTGCTAACAGGTTGTTCTTTAGTCTAGTC
R57-5         414 TGTTTATTTTGTTTCGGTTTATATGCCCGACTTGCTAATGGTTTGTGCTTTAGTCTAGTC

huangheensis  464 TGTGACAGGACATCAGACATCAGATGTCTGATTAC---------AAGAATAAGGACCATG
KO116         479 TGAAATCAGACATCTGATGTCTGTTGTTTGTT-CAGAAAGATATAAAACACGAGAACCTG
R57-5         474 TGAAATCAGACATCTGATGTCTGGTGTTTGTATTAGCTAGACATAAAACAAGAGAATCTG

huangheensis  515 AATGTCAGACTCCAGAATCATTGTCATGACCACCGGTGGAACCATAGCCAGCGAACTAGA
KO116         538 AATGACCAAAAAGCACATCGTGGTATTGACCACGGGCGGCACGATTGCCAGCAAGCCCAG
R57-5         534 CATGAGCCAAAAGCACATCGTGGTGTTGACCACGGGCGGCACTATTGCCAGCAAGCCCAG

huangheensis  575 TATTTCTGGCCGCAGTCTTTCCGGCGCGCTGTCCGGCGCCGACCTGCTGTCTCGTGCCAA
KO116         598 TGACTCAGGGCGAAGCCAGTCAGGGGCTTTAAGCGGCGAGCAGTTGCTGGATCAGGTGGC
R57-5         594 TGATTCAGGGCGTAGCCAGTCAGGGGCATTAAGCGGGGAGCAGCTACTTGATCGGGTGGC

huangheensis  635 CGTCCCTGAACGTTTTCGGTCACGTGTGGAGGTACATTCGGTACTGCAGAAACCCAGCAA
KO116         658 ACTGCCGAAGGGCGTCGACGTTACGTTAGAAGTGATATCGATCCTGCAGAAACCCAGCAA
R57-5         654 GCTGCCCCAGGGCGTCGACGTTACGTTAGAGGTGATGTCGATTCTGCAGAAACCCAGTAA
                                                                     ⇦ ANSR

huangheensis  695 CGCCATCACTCTCGATGACTTGTTGACAGTGCGC-
KO116         718 TGCCGTCACCCTCGCCGATCTTGCTGAACTCCAT-
R57-5         714 TGCCGTCTCCCTCGCCGATCTTGCTGAGCTCCATC
```

Figure S1. Multiple sequence alignment of the L-asparaginase gene promoter region of entries NZ_CP013106.1 (*H. huangheensis*), NZ_CP011052.1 (*Halomonas.* sp. KO116) and LN813019.1 (*Halomonas.* sp. R57-5). The positions of the primers are underlined and conserved bases in these regions are highlighted.

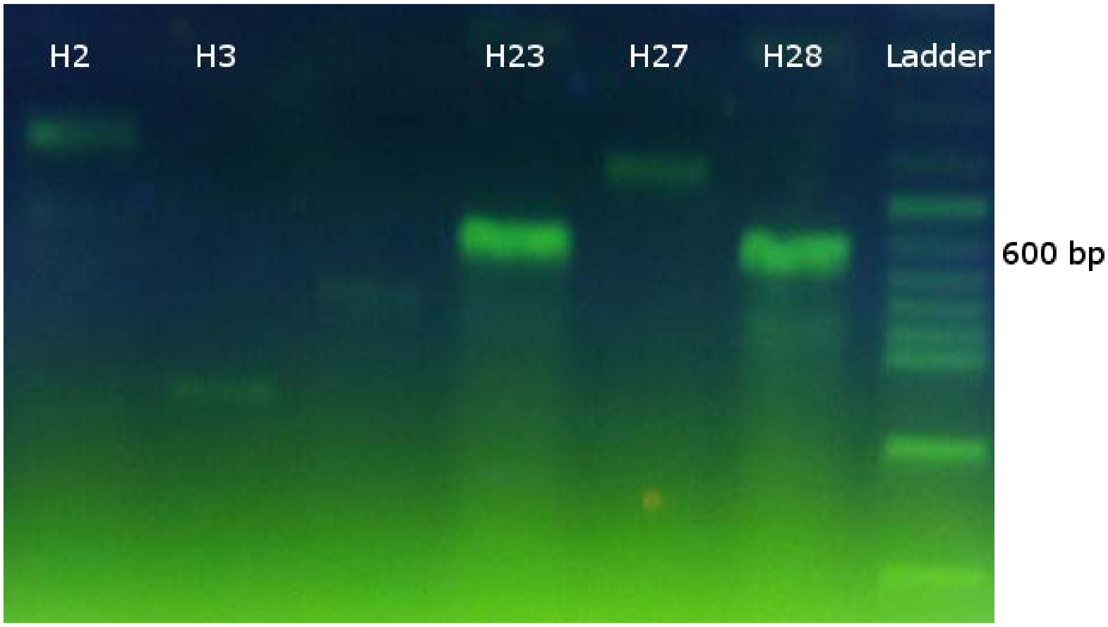


Figure S2. Amplification of genomic DNA from isolates H2, H3, H23, H27 and H28 using primers ANSF and ANSR. A band of the expected size (600 bp) can be seen with H23 and H28.

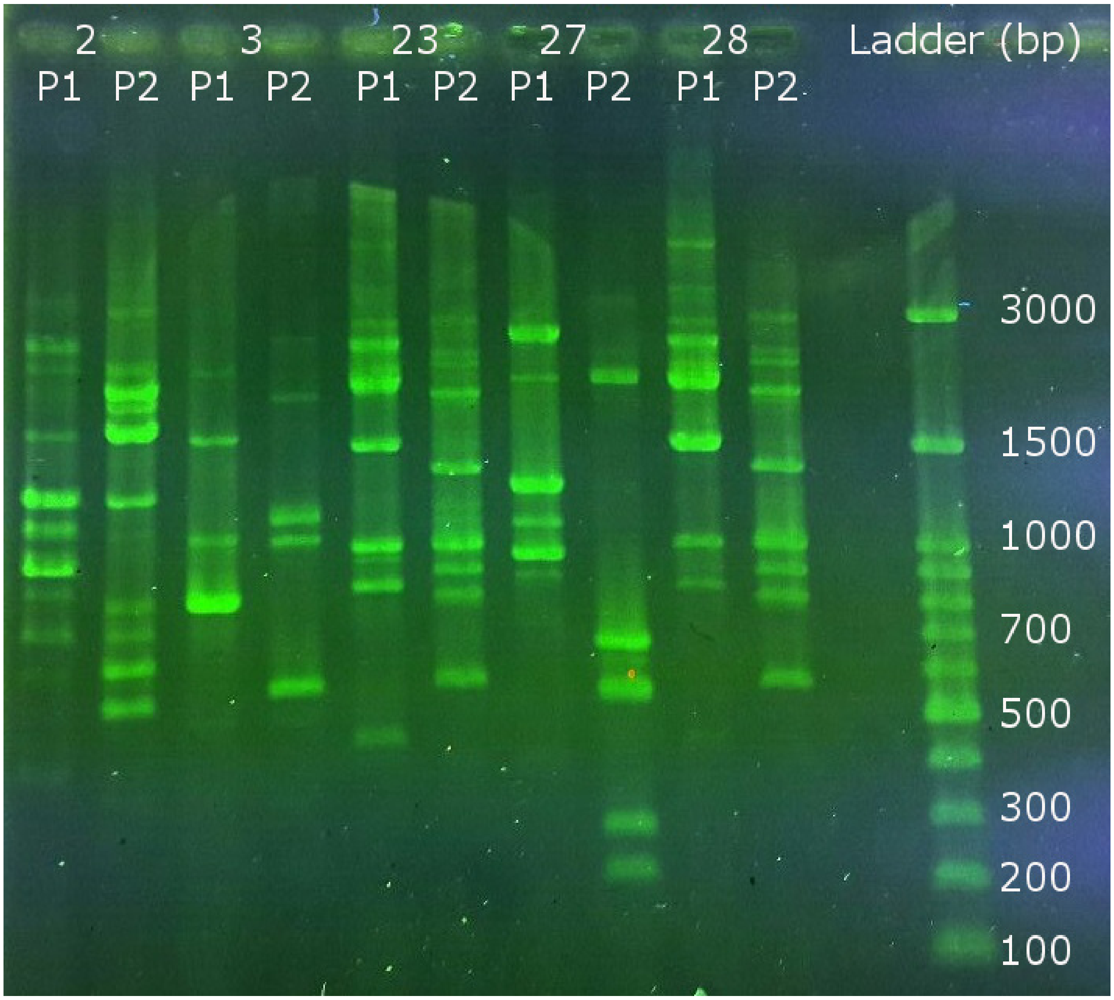


Figure S3. RAPD-PCR profiles of isolates H2, H3, H23, H27 and H28. Genomic DNA samples from each isolate were amplified using primers 5´-CCGCAGCCAA-3´ (P1) and 5´-GATGACCGCC-3´ (P2).

```
Length: 1357
Identity:    1271/1357 (93.7%)
Similarity:  1271/1357 (93.7%)
Gaps:          35/1357 ( 2.6%)
Score: 5981.5

H23 17 GGTAGCTTGCTACCCGCTGACGAGCGGCGGACGGGTGAGTAATGCATAGG
       ||.||||||||.||.|..|.||||||||||||||||||||||||||||||
H28  5 GGAAGCTTGCTTCCAGGCGTCGAGCGGCGGACGGGTGAGTAATGCATAGG

H23 67 AATCTGCCCGGTAGTGGGGGATAACCTGGGGAAACCCAGGCTAATACCGC
       ||||||||||.||||||||||||||||||||||||.||||||||||||||
H28 55 AATCTGCCCGATAGTGGGGGATAACCTGGGGAAACTCAGGCTAATACCGC

H23 117 ATACGTCCTACGGGAGAAAG--GGGG--CTCCGG--CTCCCGCTATTGGA
        ||||||||||||||||||||  ||||  ||.|||  ||..||||||.|||
H28 105 ATACGTCCTACGGGAGAAAGCAGGGGATCTTCGGACCTTGCGCTATCGGA

H23 161 TGAGCCTATGTCGGATTAGCTAGTTGGTAAGGTAATGGCTTACCAAGGCA
        ||||||||||||||||||||||||||||.||||||.||||.||||||||.
H28 155 TGAGCCTATGTCGGATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCG

H23 211 ACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACATCGGGACTGAGAC
        |||||||||||||||||||||||||||||||||||||..|||||||||||
H28 205 ACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGAC

H23 261 ACGGCCCGAACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGG
        |||||||..|||||||||||||||||||||||||||||||||||||||||
H28 255 ACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGG

H23 311 GGGAACCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCCTCGGGTTG
        ||.||||||||||||||||||||||||||||||||||||||.||||||||
H28 305 GGAAACCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTG

H23 361 TAAAGCACTTTCAGCGAGGAAGAACGCCT-AGTGGTTAATACCCATTAGG
        ||||||||||||||||||||||||.|||| || ||.||||||||.|.|||
H28 355 TAAAGCACTTTCAGCGAGGAAGAAGGCCTGAG-GGCTAATACCCTTCAGG

H23 410 AAAGACATCACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCG
        ||.|||||||||||||||||||||||||||||||||||||||||||||||
H28 404 AAGGACATCACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCG

H23 460 CGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCG
        ||||||||||||||||||.|||||||||||||||||||||||||||||||
H28 454 CGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCG

H23 510 CGCGTAGGTGGCTTGATAAGCCGGTTGTGAAAGCCCCGGGCTCAACCTGG
        ||||||||||||||||||||||||||||||||||||||||||||||||||
H28 504 CGCGTAGGTGGCTTGATAAGCCGGTTGTGAAAGCCCCGGGCTCAACCTGG

H23 560 GAACGGCATCCGGAACTGTCAGGCTAGAGTGCAGGAGAGGAAGGTAGAAT
        ||||||||||||||||||||||||||||||||||||||||||||||||||
H28 554 GAACGGCATCCGGAACTGTCAGGCTAGAGTGCAGGAGAGGAAGGTAGAAT

H23 610 TCCCGGTGTAGCGGTGAAATGCGTAGAGATCGGGAGGAATACCAGTGGCG
        ||||||||||||||||||||||||||||||||||||||||||||||||||
H28 604 TCCCGGTGTAGCGGTGAAATGCGTAGAGATCGGGAGGAATACCAGTGGCG

H23 660 AAGGCGGCCTTCTGGACTGACACTGACACTGAGGTGCGAAAGCGTGGGTA
        ||||||||||||||||||||||||||||||||||||||||||||||||||
H28 654 AAGGCGGCCTTCTGGACTGACACTGACACTGAGGTGCGAAAGCGTGGGTA

H23 710 GCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACC
        |||||||||||||||||||||||||||||||||||||||||||||||||.
H28 704 GCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACT

H23 760 AGCCGTTGGGTGCCTAGAGCACTTTGTGGCGAAGTTAACGCGATAAGTCG
        ||||||||||..|||.|||..||||||||||.||||||||||||||||||
H28 754 AGCCGTTGGGGTCCTTGAGACCTTTGTGGCGCAGTTAACGCGATAAGTCG

H23 810 ACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGG
        ||||||||||||||||||||||||||||||||||||||||||||||||||
H28 804 ACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGG

H23 860 GGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGA
        ||||||||||||||||||||||||||||||||||||||||||||||||||
H28 854 GGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGA

H23 910 ACCTTACCTACCCTTGACATC----------TACAGAAGCCGGAAGAGAT
        |||||||||||||||||||||          |.||||     ||.| |||
H28 904 ACCTTACCTACCCTTGACATCGTGCGAACTTTCCAGA-----GATG-GAT

H23 950 TCTGGTGTGCCTTCGGGAACTGTA-AGACAGGTGCTGCATGGCTGTCGTC
        |    .|||||||||||||| |.| |||||||||||||||||||||||||
H28 948 T----GGTGCCTTCGGGAAC-GCACAGACAGGTGCTGCATGGCTGTCGTC

H23 999 AGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTT
        ||||||||||||||||||||||||||||||||||||||||||||||||||
H28 993 AGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTT

H23 1049 GTCCTTATTTGCCAGCGAGTAATGTCGGGAACTCTAAGGAGACTGCCGGT
         ||||.||||||||||||| |...|||||||||||||.|||||||||||||
H28 1043 GTCCCTATTTGCCAGCGA-TTCGGTCGGGAACTCTAGGGAGACTGCCGGT

H23 1099 GACAAACCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGGCCCTTACG
         ||||||||||||||||||||||||||||||||||||||||||||||||||
H28 1092 GACAAACCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGGCCCTTACG

H23 1149 GGTAGGGCTACACACGTGCTACAATGGCCGGTACAAAGGGCGGCGA-GCT
         |||||||||||||||||||||||||||.||||||||||||..||.| || 
H28 1142 GGTAGGGCTACACACGTGCTACAATGGTCGGTACAAAGGGTTGCAATGC-

H23 1198 CGCGAGAGT-CAGCGAATCCCTTAAAGCCGGTCTCAGTCCGGATCGGAGT
         |||||| || .|||.||||||.||||||||||||||||||||||||||||
H28 1191 CGCGAG-GTGGAGCTAATCCCATAAAGCCGGTCTCAGTCCGGATCGGAGT

H23 1247 CTGCAACTCGACTCCGTGAAGTCGGAATCGCTAGTAATCGTGAATCAGAA
         ||||||||||||||||||||||||||||||||||||||||||||||||||
H28 1240 CTGCAACTCGACTCCGTGAAGTCGGAATCGCTAGTAATCGTGAATCAGAA

H23 1297 TGTCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCA
         ||||||||||||||||||||||||||||||||||||||||||||||||||
H28 1290 TGTCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCA

H23 1347 TGGGAGT
         |||||||
H28 1340 TGGGAGT
```

Figure S4. Pairwise alignment of 16S rDNA sequences of isolates H23 and H28. The two sequences were aligned using EMBOSS Water (http://www.ebi.ac.uk/Tools/emboss/) with gap opening and extension penalties of 10 and 0.5 respectively and the DNAfull scoring matrix.

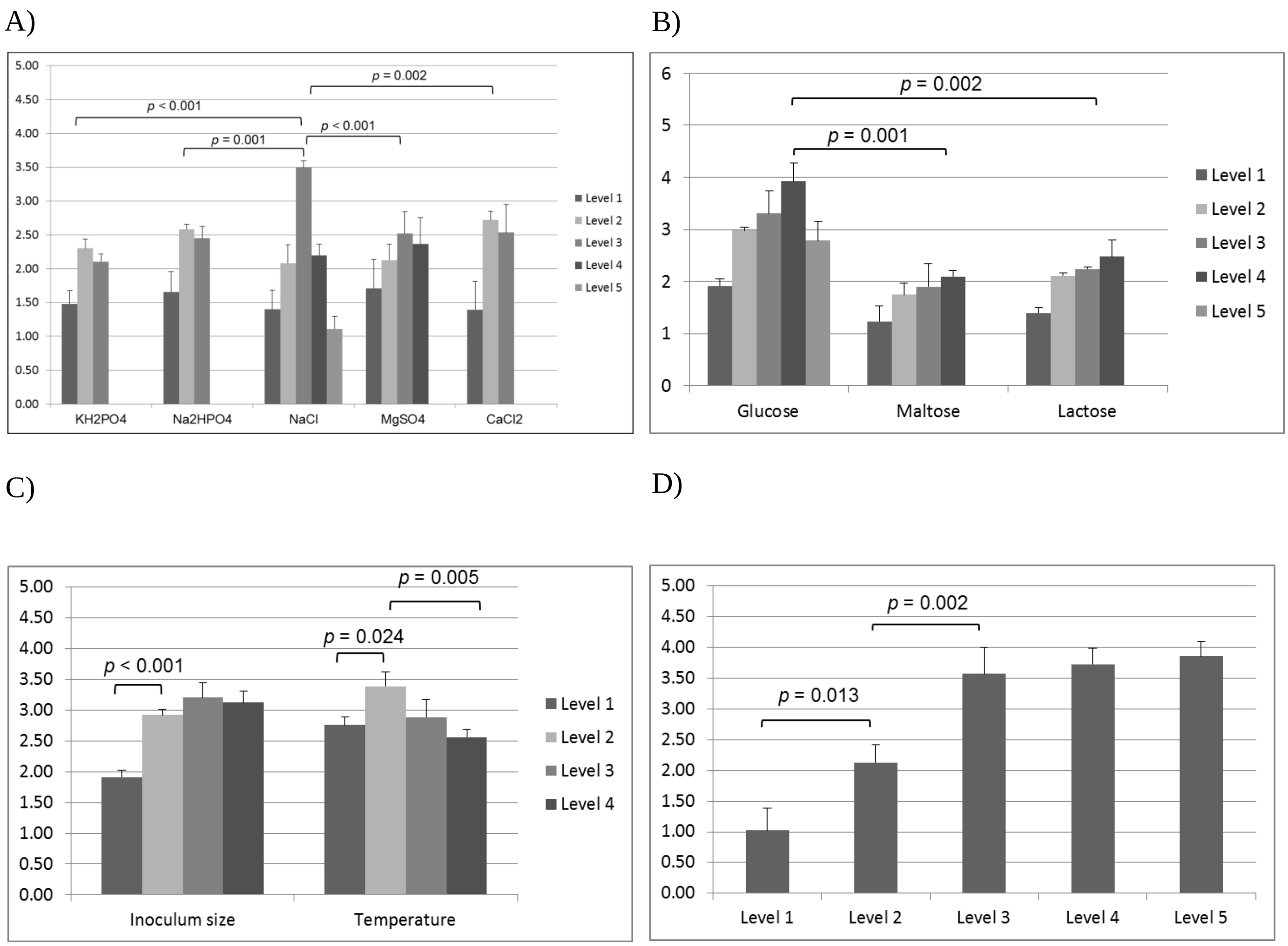
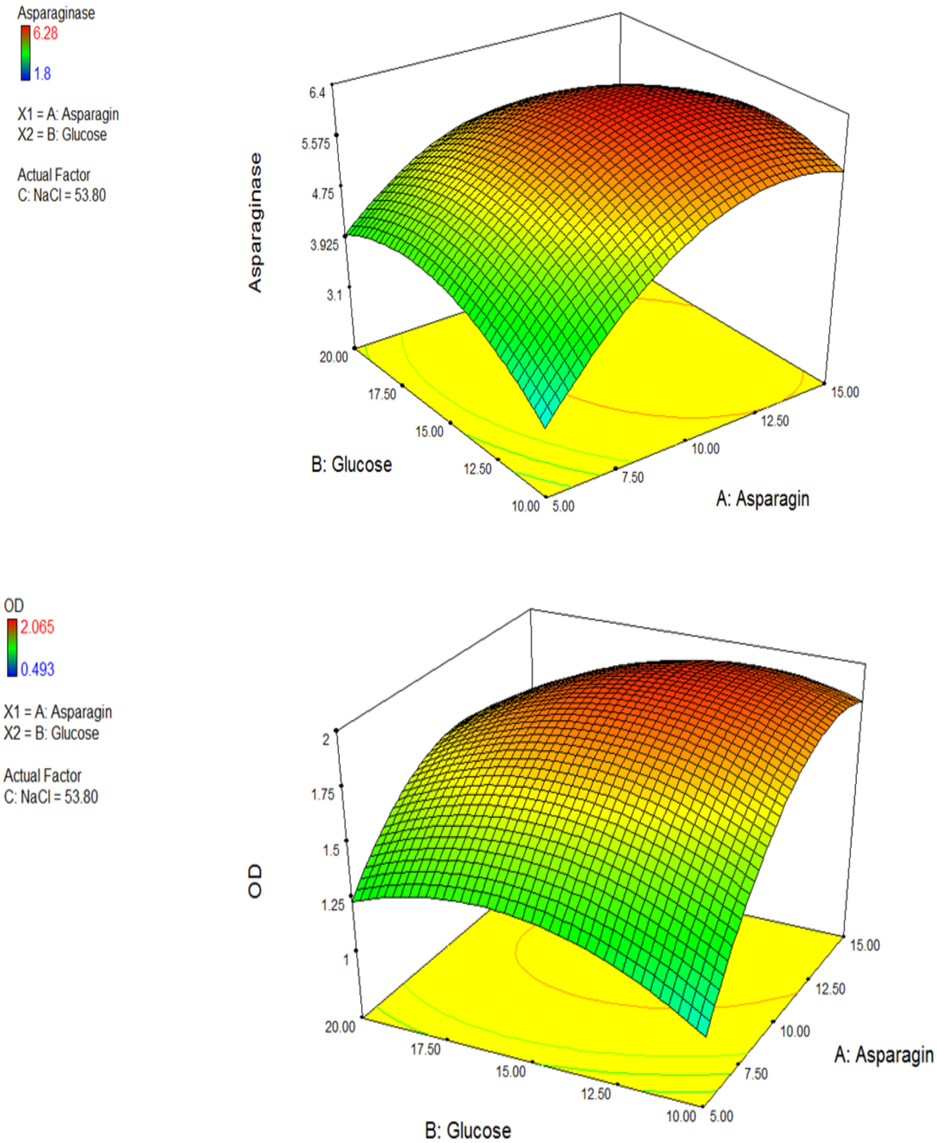


Figure S5. Effect of different salts (A), carbohydrates (B), temperatures and inoculum sizes (C) and L-asparagine concentrations (D) on L-asparaginase production by isolate H28. Vertical axes show enzyme activity in U/mL. In each case the effect was evaluated at least at 3 levels as indicated in Table S1. All *p*-values were calculated using ANOVA and Tukey's HSD test. (A) The concentration of each salt was increased until either no significant increase or a significant decrease in enzyme activity was observed (*p*-values for this stage are not shown). Subsequently, the salt resulting in the highest enzyme activity was chosen for further study (shown *p*-values refer to this stage). (B) The concentration of each carbohydrate was increased until either no significant increase or a significant decrease in enzyme activity was observed (*p*-values for this stage are not shown). Subsequently, the carbohydrate resulting in the highest enzyme activity was chosen for further study (shown *p*-values refer to this stage). (C) Inoculum size (%) and temperature were increased until either no significant increase or a significant decrease in enzyme activity was observed (*p*-values for significant differences are shown) and the levels resulting in highest enzyme activity were used throughout the rest of the study. (D) L-asparagine concentration was increased until no significant increase in enzyme activity was observed (*p*-values for significant differences are shown) and results were used to choose the range of L-asparagine concentration for the next step of optimization.



A)

B)

Figure S6. Representative response surface plots showing the effect of L-asparagine and glucose concentrations on (A) enzyme activity and (B) cell density at an optimal NaCl concentration of 54 g/L. Details of the design are provided in Table S2.

Table S1. Different levels of factors evaluated in the first step of medium optimization.

Factor	**Unit**	**Level**				
		1	**2**	**3**	**4**	**5**
KH_2PO_4	g /L	2.5	5	7.5	-	-
Na_2HPO_4	g /L	2.5	5	7.5	-	-
NaCl	g /L	5	20	50	100	150
$MgSO_4.7H_2O$ (1M)	mL	0.2	0.4	0.6	0.8	-
$CaCl_2$ (0.1M)	mL	0.1	0.2	0.4	-	-
Glucose	g /L	2.5	5	10	15	20
Maltose	g /L	2.5	5	10	15	--
Lactose	g /L	2.5	5	10	15	--
L-asparagine	g /L	2.5	5	10	15	20
Inoculum Size	%	1	5	10	15	
Temperature	°C	27	30	33	36	-

Table S2. Central composite design and corresponding results for enzyme activity and cell density.

Run	Variable levels (g/L)			Activity (U/mL)*		Cell density (OD_{600})**	
	A: L-asparagine	B: Glucose	C: NaCl	Predicted	Actual	Predicted	Actual
1	5	10	25	2.939320	3.0	0.924556	0.97
2	15	10	25	5.260343	5.1	1.671235	1.56
3	5	20	25	3.578076	3.6	1.154658	1.14
4	15	20	25	4.169099	4.3	1.307837	1.40
5	5	10	75	2.963051	2.9	0.894688	0.89
6	15	10	75	5.434074	5.5	1.783868	1.84
7	5	20	75	3.671808	3.9	1.132290	1.28
8	15	20	75	4.412830	4.4	1.427970	1.43
9	1.591036	15	50	1.920546	1.8	0.575276	0.49
10	18.40896	15	50	4.495408	4.5	1.451792	1.47
11	10	6.591013	50	4.203793	4.3	1.352425	1.39
12	10	23.40896	50	3.882161	3.7	1.246643	1.15
13	10	15	7.955179	5.140523	5.1	1.621082	1.66
14	10	15	92.04482	5.365431	5.3	1.696986	1.60
15	10	15	50	6.053923	6.2	1.932282	1.83
16	10	15	50	6.053923	5.8	1.932282	1.91
17	10	15	50	6.053923	5.7	1.932282	1.89
18	10	15	50	6.053923	6.2	1.932282	1.93
19	10	15	50	6.053923	6.2	1.932282	1.98
20	10	15	50	6.053923	6.3	1.932282	2.06

* For enzyme activity the following model was obtained:

Enzyme activity = $6.05 + 0.77A - 0.43AB - 1.01A^2 - 0.71B^2 - 0.28C^2$.

** For cell density the following model was obtained:

OD = $1.93 + 0.26A - 0.15AB - 0.32A^2 - 0.22B^2 - 0.097C^2$.