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Harnessing cross-resistance – sustainable nisin production from low-value food side streams using a *Lactococcus lactis* mutant with higher nisin-resistance obtained after prolonged chlorhexidine exposure

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ABSTRACT

Nisin has a tendency to associate with the cell wall of the producing strain, which inhibits growth and lowers the ceiling for nisin production. With the premise that resistance to the cationic chlorhexidine could reduce nisin binding, variants with higher tolerance to this compound were isolated. One of the resistant isolates, AT0606, had doubled its resistance to nisin, and produced three times more free nisin, when cultured in shake flasks. Characterization revealed that AT0606 had an overall less negatively charged and thicker cell wall, and these changes appeared to be linked to a defect high-affinity phosphate uptake system, and a mutation inactivating the oleate hydratase. Subsequently, the potential of using AT0606 for cost efficient production of nisin was explored, and were able to attain a high titer of 13181 IU/mL using a fermentation substrate based on molasses and a by-product from whey protein hydrolysate production.

Keywords: Nisin production, Chlorhexidine, Adaptive laboratory evolution, Molasses, Dairy waste

1. Introduction

L. lactis is a Gram-positive bacterium that has been employed to produce fermented foods for centuries, especially dairy products (Koutsoumanis et al., 2020). Some *L. lactis* strains produce nisin, a positively-charged antimicrobial peptide, which can effectively inhibit or kill certain Gram-positive bacteria, including ones that are pathogenic and cause food spoilage (Soltani et al., 2021). Nisin acts by inhibiting cell-wall biosynthesis and by forming pores in the cytoplasmic membrane, and both of these activities are initiated by binding of nisin to lipid II (AlKhatib et al., 2014). Due to its superior antimicrobial activity and non-toxic nature, nisin has already for more than half a century served as an excellent food-grade bio-preservative, primarily in meat, beverage and dairy products(Gharsallaoui et al., 2016). Although nisin is active in nanomolar concentrations, it is still a costly food preservative, and reducing its manufacturing cost still remains relevant. Therefore, isolating strains with higher nisin producing ability and developing new low-cost media for its industrial production are of great commercial interest.

Nisin-producing *L. lactis* strains are able to protect themselves against nisin through their immunity system, which is comprised of the lipoprotein NisI and the ABC transporter NisFEG; NisI binds nisin and prevents it from attacking the cell membrane, whereas the ABC transporter expels nisin from the cytoplasmic membrane, either prior to or during pore formation (Liu et al., 2020). Despite the effectiveness of these immunity mechanisms, producing strains are not fully recalcitrant towards nisin, which limits the nisin titers achievable (Dzhavakhiya et al., 2018). Therefore, by improving nisin resistance it should be possible to enhance the nisin producing ability.

For Gram-positive bacteria, nisin resistance can be increased in different ways, e.g. by overexpressing nisin resistance mechanisms, by lowering the overall negative net charge of the cell wall, by changing the ratio between phosphatidylglycerol and diphosphatidylglycerol and by thickening the cell wall (Zhou et al., 2014). There are many reports describing the use of genetic engineering to improve nisin resistance. Şimşek et al. (2009) were able to improve nisin resistance by introducing several copies of the nisin resistance genes, *nisFEG*, in *L. lactis* LL27, and achieved a 24% increase in the nisin titer. Cao et al. (2018) introduced

a plasmid expressing genes involved in MurNAc O-acetylation and GlcNAc N-deacetylation, into *L. lactis* F44, and managed to improve the cell wall integrity and nisin production by 76%. Although genetic engineering is helpful for improving the performance of microbial cell factories, the use of genetic engineering complicates food applications; not only due to consumer acceptance issues, but also because of regulatory issues. A preferred option is thus to rely on approaches that do not involve genetic engineering, such as adaptive laboratory evolution (ALE), which has become popular in recent years for enhancing performance of microorganisms used in food fermentations (López-González et al., 2018a). During adaptive laboratory evolution, microbial populations are cultivated in the presence of a particular stressor for an extended period of time, and adapted strains with improved phenotypes are finally selected.

Besides efficient strains, in order to establish cost-efficient production of nisin, low-value fermentation substrates are needed. It has been reported that both M17 and MRS broth support excellent growth and nisin production (Liu et al., 2005), however, these media are not suitable for large-scale production of nisin as they are too costly. Alternative, cheaper substrates have been explored, including barley extract(Furuta et al., 2008), defatted rice bran (Liu et al., 2017) and whey mother liquor(Zhao et al., 2021). Unfortunately, the former two require complex pretreatment and the latter needs supplementation with a nitrogen source, e.g. yeast extract, to support growth and nisin production of *L. lactis*.

In this work, first ALE was attempted to enhance tolerance to the cationic disinfectant chlorhexidine, aiming to generate *L. lactis* mutants that have cell walls with reduced negative charge and improved nisin-producing ability. Chlorhexidine is a cationic disinfectant which acts in a similar manner to that of nisin; chlorhexidine initially binds to negatively charged phosphate groups in the cell wall, after which it is inserted into the cytoplasmic membrane, causing leakage of key metabolites, which ultimately kills the target microorganism (Bolla et al., 2020). Mutants with increased chlorhexidine resistance were successfully isolated, which was characterize in terms of growth, and nisin production, and furthermore by whole-genome sequencing. Next a low cost medium was developed for producing nisin, which is composed

of molasses and a by-product from whey protein hydrolysate production. To the best of our knowledge, this is the first report describing the use chlorhexidine to enhance the performance of a microbial cell factory.

2. Materials and methods

2.1 Microorganisms and growth condition

The nisin-producing strain *L. lactis subsp. lactis* ATCC 11454 and the nisin-sensitive indicator strain *Micrococcus luteus* ATCC 10240 were obtained from German Collection of Microorganisms and Cell Cultures (Leibniz Institutes, Germany). The strains were grown aerobically in M17 broth (Oxoid, Darmstadt, Germany) supplemented with 1% glucose (GM17) and NB broth (Oxoid, Darmstadt, Germany) at 30°C, respectively.

2.2 Adaptive Evolution Experiments

Selection of strains adapted to chlorhexidine digluconate (CHG) was performed using a serial-transfer regime and is depicted in Fig1A. A single colony of ATCC 11454 were used to inoculate 5 ml of GM17 broth prepared in test tube and cultivated at 30°C. Then the overnight culture was transferred into (1:10,vol/vol) 5 ml fresh medium containing 1 μg/ml of CHG. After the culture entered the stationary phase, the procedure was repeated until growth became less affected by CHG, after which the CHG concentration was increased. Culture from the last tubes containing 2, 4, 6, and 8 μg/mL of CHG were plated on solid GM17, and 10 colonies were randomly collected, resuspended in 1 mL of GM17 broth containing 25% glycerol, and stored at -80°C.

2.3 Nisin Activity Assay

Total nisin activity was determined using a slightly modified version of the hot acid extraction method described by (Zhang et al., 2014). Briefly, the fermentation broth was mixed with four volumes of 0.02 M HCl, boiled in water for 5 min and cooled on ice for 15 min. The supernatant was obtained by centrifugation at 10,000 g for 5 min at 4°C, and then stored at -20°C. To determine soluble nisin or extracellular nisin, the same fermentation broth was centrifuged at 10,000 g for 5 min, the pellet was discarded, and the supernatant

was treated as described above, and then kept at -20°C. Cell-bound nisin was calculated by subtracting soluble nisin from the total nisin. To calculate the percentage of nisin in soluble form, the soluble nisin activity was divided by the total nisin activity.

2.4 Initial comparison of performance of ATCC11454 and its CHG Adapted derivatives

2.4.1 Growth and nisin production

Pre-cultures of ATCC11454 and its CHG adapted derivatives were prepared in 24-well plates filled with 1 mL GM17 medium. The 24-well plates were incubated overnight at 30°C. 10 µL pre-culture was inoculated into 48-well plates filled with 1 mL GM17. Growth experiments were performed using a Biolector (M2p-labs, Germany) with 800-rpm shaking. After 12 hours of cultivation, samples were withdrawn to determine optical density and nisin production (detailed below). Strains were characterized in terms of Minimum Inhibitory Concentration (MIC) to nisin and CHG, and to determine stability of their nisin-production phenotype.

2.4.2 Minimum Inhibitory Concentration (MIC) to nisin and CHG

Susceptibility to nisin and CHG was determined using the broth microdilution method (López-González et al., 2018). Briefly, overnight cultures of single isolates prepared in 24-well plates were diluted 1:100 in GM17, of which 2 µl were used to inoculate 200 µl of GM17 with different concentration of nisin (0, 80, 160, 320, 640, 1280, 2560, 5120 IU/mL) and CHG (0, 1, 2, 4, 6, 8, 10 µg/mL) respectively. The most promising strains, having stable phenotype, were analyzed further.

2.4.3 Strain stability assessment

The stability of the observed phenotypes was assessed by successive sub-culturing. If the nisin titer and resistance to chlorhexidine and nisin were unchanged after ten rounds of successive sub-culturing, the strains were considered stable.

2.5 Characterization of growth and nisin production in Shake Flasks

Exponentially growing pre-cultures were used in all experiments. A single colony was inoculated into 5 mL of GM17 in a 20 mL test tube. This tube, together with its 10 fold serial dilution was incubated overnight

at 30°C with shaking (200 rpm). Tubes containing exponentially growing cultures with optical density around 0.5 were used to inoculate (1%) 50 mL GM17 in 300 mL shake flasks. Cultivation was performed at 30°C and 200 rpm. At different time points, samples were withdrawn, to determine cell density (OD_{600}), soluble and total nisin production as well as pH.

2.6 Analytic methods

The bacterial growth was monitored by measuring the optical density (OD_{600}) at 600 nm (UV-1600PC spectrophotometer, VWR, Denmark). Changes in pH during the growth was detected with a pH meter (Lab845, SI Analytics, Denmark). Quantitative assessment of carbohydrates, lactate and acetate in culture broth was done using an high-performance liquid chromatography (HPLC) (Dionex, Sunnyvale, USA) equipped with a ion exclusion column (Aminex HPX-87H;Bio-Rad, Hercules, USA) and a refractive index detector (Shodex RI-101;Showa Denko K.K., Tokyo, Japan) (Liu et al., 2016). Activity of nisin was assayed using the agar diffusion bioassay method described by Zhao et al.(2021).

2.7 Measurement of surface physicochemical properties

2.7.1 Cell Surface hydrophobic properties

Partitioning between two immiscible liquids is affected by both lipophilic compounds and net surface charge of bacteria. The MATS (Microbial Adhesion To Solvents) method was used to evaluate the hydrophilic/hydrophobic properties and their Lewis acid-base characteristics of the cell surface (Bellon-Fontaine et al., 1996). This partitioning method is based on comparing affinities of microbial cell for monopolar and nonpolar solvents, which exhibit similar van der Waals properties. On this basis, chloroform, a monopolar and acidic solvent (electron acceptor), and hexadecane, a nonpolar alkane, were selected as solvents using the method described in detail by Giaouris et al. (2009). The MATS values obtained with hexadecane and chloroform are considered as a measure of cell surface hydrophobicity/hydrophilicity and describe of electron donor characteristics of bacteria as well. The equation for calculating the affinity of each solvent is affinity/ % = $(A_0-A_1)/A_0 \cdot 100$, where A_0 and A_1 is the cell density value of the bacterial in aqueous phase before and after mixing, respectively.

2.7.2 Cell Surface Charge

Cell surface charge was determined by using the method described by Hao et al. (2017). Harvested cells were first suspended in 10 mM of PBS, pH 5.0 at 30°C for 15 min. Then the pellets obtained after centrifugation were resuspended at a concentration of 10^7 CFU/mL using 1.5 mM NaCl. The pH of the suspension was adjusted to pH's ranging from 2.0 to 7.0, by adding HNO_3 or KOH, respectively. Approximately 1 mL of this sample was transferred into the ZetaSizer DST1070 cuvette and then inserted into the ZetaSizer Nano instrument (Nano-ZS, Malvern, Malvern, UK). The electrophoretic mobility (EM) for biological cells was determined in the presence of a 50-V electric field at 20°C, using the Laser Doppler Velocimetry technique.

2.8 Phase-contract Microscopy and Transmission Electron Microscopy (TEM)

Approximately 10 µL of fresh overnight culture was placed on a glass slide and then examined using an Olympus BX53 Microscope (1000x). Phase-contract pictures were obtained using a digital camera.

Prior to TEM, cells in overnight cultures were fixed by adding equal parts bacterial culture with an aqueous solution containing 4% glutaraldehyde and 8% paraformaldehyde. Sample preparation and microscopy were performed at the DTU Nanolab (Kongens lyngby, Denmark). Images were acquired with Tecnai G2 T20 transmission electron microscope (FEI, USA) fitted with a TVIPS-XF416 CMOS camera (TVIPS GmbH, Gauting, DE).

2.9 Whole genome sequencing

Whole bacterial genome re-sequencing was carried out on the DNBseq platform at BGI-Tech (Denmark). The resulting raw 150-bp paired-end reads were processed and then mapped to the reference genome ATCC 11454, downloaded from American Type Culture Collection (https://www.atcc.org/), using Geneious Prime software (Auckland, New Zealand). Next, single nucleotide variations (SNVs) of mutants and parental strain was detected using the respective function in same software accepting variant frequency more than 90% and strand bias less than 75%. SNVs occurring in both the mutants and parental strain were identified as false-positive mutations and were not considered in the analysis.

2.10 Bioreactor fermentations

The waste molasses used was provided by Nordic Sugar A/S (Nakskov, Denmark), and is by-product of beet sugar production. FCH is a by-product of whey protein hydrolysate production, which has a milky white turbid appearance (see supplementary materials). FCH was provided by Arla Food Ingredients (Viby, Denmark) (Liu et al., 2021). Prior to fermentation, FCH was pre-treated as described by Sindayikengera and Xia, 2006, and then hydrolyzed using 1% food-grade Flavourzyme (Novozymes, Denmark) at 50°C, at pH 7. The supernatant collected by centrifugation termed FCHH was used as nitrogen source. In order to promote growth and nisin production, 100 mM KH_2PO_4 was added to this combined medium (De Vuyst and Vandamme, 1993).

For the fermentations, one-liter bioreactors (Sartorius, Germany) containing 450 mL medium (5% molasses, 20% FCHH, 1μg/mL hemin) were used. The bioreactors were inoculated with AT0606 at an initial OD_{600} of 0.02. The pH was maintained at 5.75±0.5 by automatic addition of 5 M NaOH, and the dissolved oxygen (DO) level was monitored with electrodes (Mettler Toledo) and controlled around 30%,by automatically adjusting stirring speed and air flow. Samples were withdrawn periodically for sucrose concentration, cell density (OD_{600}) and nisin production analysis. Additional bioreactor fermentations were carried out using commercial M17 medium supplemented with sucrose as well as using a molasses-yeast extract (2%) medium similar to the one mentioned above, just without FCHH.

3. Results and Discussion

3.1 Adaptation to CHG and preliminary characterization of resistant strains

Attempting to generate natural *L. lactis* strains with superior ability to produce nisin, an ALE was carried out in the presence of CHG, a gluconate salt of cationic chlorhexidine, believing that cross-resistance to nisin might occur, and that additional nisin resistance might favor a higher nisin production titer. The model strain used in this study was the well-characterized nisin-producing *L. lactis* ATCC 11454. In a previous study it was shown that aerated culturing was superior to static culturing (no active aeration), and gave rise to higher titers of nisin when using *L. lactis* ATCC 11454 (Jiang et al., 2015). Papiran and Hamedi, (2021)

recently found that adaptation of ATCC 11454 to increased aeration also was beneficial for nisin production. Thus, ALE was decided to carry out under aerated conditions.

MIC of CHG for ATCC 11454 was found to be 2 µg/mL. In the course of the evolution, as the strain adapted to the presence of chlorhexidine, the concentration of chlorhexidine was gradually increased. Cultures that grew in the presence of 2, 4, 6, and 8 µg/mL CHG were plated on GM17, and for each ten random colonies were selected for characterization. As shown in Fig.1B, the growth rate, in general, for the strains isolated from these cultures, declines with CHG concentration. Subsequently, the nisin-producing capacity of the strains was determined. As shown in Fig.1C, for most strains isolated from cultures with 2-6 µg/mL CHG, more nisin was produced as compared to the parent strain, and especially the isolates from the culture with 6 µg/mL CHG (AT06) stood out. The most chlorhexidine resistant isolates (AT08), however, produced less nisin than its parent. The CHG evolved strains in general was found to produced more soluble nisin, which is in line with the hypothesis that chlorhexidine adaption could lead to strains having a less negatively charged cell wall.

When MIC of nisin against the different isolates was determined, most cross-resistance was found to occurr on AT06 strains (see supplementary materials), and thus two isolates from this series: AT0606 and AT0610, which retained a stable phenotype after successive sub-culturing, were focus on further study. Whole genome sequencing revealed that these two isolates were identical, and in the following sections results are only shown for AT0606.

3.2 Characterization in shake flasks reveals that CHG adaptation reduces adsorption of nisin to the cell wall

Above, the strains isolated from the CHG ALE was characterized using a BioLector, i.e. in relatively small scale, making it difficult to monitor nisin production and degradation over time. It has been reported that the soluble nisin titer declines after entering the stationary phase, which has been suggested to be due to proteolytic degradation and adsorption of nisin onto producer cells (Pongtharangkul and Demirci, 2006). For the preliminary characterization, nisin was extracted a few hours after the cultures had entered the

stationary phase, making it difficult to conclude the extent of these phenomena. Therefore, the strains were characterized in larger scale, i.e. in shake flasks, and monitor nisin production more closely. As shown in Fig. 2A and C, AT0606 grows approximately 15% slower that the WT; the specific growth rate of the AT0606 strain is around 0.80 h^{-1}, whereas that of the WT is 0.93 h^{-1}. An interesting observation, was that the final cell density for the AT0606 culture was slightly higher than that of the WT, which perhaps could be due to a shift towards a more mixed-acid fermentation pattern for the AT0606 strain (data not shown)., as such a shift would allow formation of more ATP, and a higher biomass yield (Cesselin et al., 2018). The latter, however, was not the case and only minor changes in the fermentation pattern occurred.Nisin accumulated gradually as cells grew, however, after entering the stationary phase, the nisin titer began to drop for both strains at similar rates. For both WT and the CHG adapted strain, the nisin activity reached its peak at 12 hours, where the WT had accumulated 1100 IU/mL soluble and 2000 IU/mL total nisin, and the adapted strain 3400 IU/mL soluble and 4000 IU/mL total nisin, which constituted an increase of 209% and 100%, respectively (Fig.2D and E). Another obvious difference between the WT and AT0606, was that AT0606 produced far more soluble nisin than the WT, especially after 8h when the pH for both cultures were below 5 (Fig.2B and F). These results clearly demonstrated that the CHG adapted mutant had less nisin adsorbed to its cell wall, strongly suggesting that cell surface modifications had taken place. Bacteriocin production by LAB has been reported to follow primary metabolite growth-associated kinetics, i.e. take place in the growth phase and cease once stationary phase is reached (Abbasiliasi et al., 2017), which is indeed what was observed for nisin.

3.3 Surface properties of AT0606

For Gram-positive bacteria, a high resistance to nisin often coincides with altered cell wall properties (López-González et al., 2018). This is also highly likely in this study, as the CHG adapted strain AT0606 clearly binds less of the total nisin. For this reason, the overall cell surface properties was investigated, as this might provide some hints that could help explain the observed phenomenon. The surface hydrophobicity of lactic acid bacteria can be determined in different ways, typically by measuring

241 partitioning of cells between an aqueous and a nonpolar hydrocarbon phase. Lewis acid-base properties of
242 surfaces of microorganisms have an impact on how these interact with each other and with compounds in
243 their surroundings. To investigate these properties, MATS method was developed by Bellon-Fontaine et
244 al.(1996), which is based on a comparison of microbial cell affinity to a nonpolar solvent and a monopolar
245 solvent. Both the WT and AT06060 had relatively hydrophobic cell surfaces, with affinities for the nonpolar
246 hexadecane above 40% (Fig.3A), which is common for *Lactococcus* spp isolated from a dairy environments
247 (Floury et al., 2015) . However, when compared with the WT, AT0606 had a less hydrophobic surface.
248 The latter could be due to a decrease in the concentration of the overall nitrogen and hydrocarbon-like
249 compounds or an increase the concentration of oxygen in the outer cell wall (Boonaert and Rouxhet, 2000).
250 Similar results were reported for nisin-resistant *Staphylococcus aureus* (Martínez et al., 2008) and *Listeria*
251 *monocytogenes* strains (Maisnier-Patin and Richard, 1996). The MATS test revealed that the surface of
252 both of WT and AT0606 strains served as electron donors (Lewis-base), since their affinity to the Lewis-
253 acid chloroform was much higher than towards hexadecane (Fig.3A). Remarkably, the affinity of the
254 AT0606 strain towards chloroform was lower than for the WT.
255 The electrophoretic mobilities (EM) of the two *L. lactis* strains at pH values ranging of 2-7 was also
256 measured, as it previously has been shown there is a correlation between the electrophoretic mobility and
257 the N/P (protein/phosphate) ratio in bacterial surfaces(Mozes et al., 1988). As shown in Fig.3B, EM of the
258 WT and AT0606 strains at different pH values indicated that their isoelectric points were around pH 2.5
259 and 4.5, respectively. Overall, these findings indicated that the cell surface of the CHG adapted strain
260 AT0606 had an overall less negative charge. This difference in the overall surface electrical properties is
261 mainly linked to variations of three types of ionized groups in the cell envelope, which are protonated
262 carboxylate and amino groups in proteins, and phosphate groups present in teichoic acids (Boonaert and
263 Rouxhet, 2000). The observed lower negative surface charge of AT0606 could be due to either i) a lower
264 content of negatively charged groups, ii) that the negatively charged groups are embedded deeper into the
265 peptidoglycan (Giaouris et al., 2009). As mentioned previously, nisin carries a global positive net charge,

and the decrease in surface negative charge could impede nisin to reach membrane-anchored lipid II by electrostatic repulsion, and this could account for the higher nisin resistance and less cell wall-bound nisin.

3.4 AT0606 has a thicker cell wall

It has been reported that that the thickness of the peptidoglycan layer is important for nisin resistance (Kramer et al., 2008), therefore, variation of cell wall thickness in AT0606 was investigated using Transmission Electron Microscopy (TEM). Indeed, the results showed that AT0606 possesses a clearly thicker cell wall (see supplementary materials), more than 9 nm thicker than that of the WT (Fig.4). It has been reported that the outer thick peptidoglycan cell wall of Gram-positive bacteria has a decreased cross-linked structure, which performances like a molecular sieve, preventing cationic antibacterial peptides from gaining access to the membrane(Omardien et al., 2016). Thus, it is reasonable to conclude that the higher tolerance of AT0606 towards nisin, as well as its ability to produce more soluble nisin, can be attributed to a less negatively-charged thicker cell wall, that reduces binding of nisin to the cell wall and prevents it from binding to its receptor.

3.5 Scrutinizing the Genomes of the CHG-Adapted Strains.

In order to find the underlying explanation for the enhanced nisin resistance and production properties of the adapted strain AT0606, its genome as well as that of the parent strain were sequenced. The genome of AT0408 was also sequenced, an interim isolate in the adaptive evolution between WT and AT0606, which has a similar MIC to nisin as AT0606, but which does not produce more nisin (see supplementary materials). By comparing to the released ATCC11454 sequence (downloaded from https://www.atcc.org/), it was possible to determine all the mutations in AT0408 and AT0606, which are listed in Table 1. A total of 8 single-nucleotide variations (SNVs) were identified in the genome of AT0606, three of which were present in AT0408. The latter three SNVs are found in genes encoding Glutamine-fructose-6-phosphate aminotransferase, a putative ABC transporter ATP-binding protein, and a PTS system mannitol-specific EIICB component, respectively. It was speculated that the mutation in the gene encoding the putative ABC transporter ATP-binding protein could affect nisin resistance, as its N-terminal involved ATP-binding is

291 similar to *ysaB* in *L.lactis* IL1403, which is involved in nisin resistance (Kramer et al., 2006). However, it
292 should be noted that AT0408 was genetically unstable, and lost nisin resistance and CHG tolerance after
293 few passages without CHG. In contrast, AT0606 had a stable phenotype and maintained a high resistance
294 to both nisin and CHG, and in addition possessed an enhanced nisin producing ability. Thus, it is likely that
295 the properties of AT0606 can be ascribed to the remaining five SNVs. All of the latter mutations, except
296 one, occurred in protein coding regions. The intergenic mutation was located in the promoter region, 71bp
297 away from the transcriptional start site of *sugE* (or namely qacE), encoding a protein putatively involved in
298 multidrug resistance (van der Meulen et al., 2017). It is tempting to speculate that this C-71A SNV could
299 lead to altered expression of *sugE*, and lower susceptibility of AT0606 to CHG (Abuzaid et al., 2012). One
300 mutation was found in *nagZ* encoding a putative N-acetylglucosaminidase, which potentially could result
301 in an altered cell envelope. However, the mutation resulted in an alanine to valine exchange, and it is
302 tempting to speculate that this minor change, most likely, does not affect protein function. Another SNV
303 was observed in a gene encoding a WxL domain-containing protein, in its N-terminal. The C-terminal of
304 this WxL protein has experimentally been shown to bind non-covalently to PG *in E. faecalis* (Chapot-
305 Chartier and Kulakauskas, 2014). However, its function has not yet been determined. In addition to these
306 mutations, two with anticipated more drastic effects were identified; amber mutations had been introduced
307 in the early half of *pstC* and *sph*, encoding a phosphate ABC transporter permease subunit and oleate
308 hydratase, respectively. Hampered phosphate uptake has previously been shown to increase oxidative stress
309 resistance in *L. lactis* (Cesselin et al., 2009), which might save ATP which could be used to synthesize
310 nisin. It is also plausible that reduced phosphate uptake could help reduce occurrence of negatively charged
311 phosphate in the cell wall, thus reducing the negative charge of the cell wall. This hypotheses that needs to
312 be substantiated. The amber mutation in *sph*, eliminates the FAD-binding domain, and is expected to
313 completely inactivate oleate hydratase, which are involved in formation of hydroxyl fatty acids, which are
314 known to change bacterial cell surface hydrophobicity and cell morphology (Radka et al., 2021) . In this
315 study, the AT0606 was found to have lower hydrophobicity and a thicker cell wall, and most likely the
316 underlying cause in the mutation in *sph*.

3.6 Nisin from Low Value Food Industrial Side Streams

Cost-effective and sustainable production of green compounds requires renewable inexpensive feedstocks. Various efforts have been made to use carbohydrates derived from nonedible plant biomasses as feedstocks, however, there are still significant challenges ahead (Liu et al., 2021). Molasses, a leftover from the sugar refining process, contains on average 50% sugars, mostly sucrose, and also has a high content of vitamins and minerals (Yan et al., 2011). FCH, a by-product of Arla Foods Ingredients whey protein hydrolysate production, is rich in proteins/peptides and has a solids content of 11% (J.-M. Liu et al., 2020). Together, Molasses and FCH could serve as a complete medium for *L. lactis*. Therefore, such a medium was explored whether would be suitable for producing nisin using the CHG-adapted *L. lactis* strain AT0606. Unfortunately, AT0606 grew poorly in this medium, probably because AT0606 is missing a protease plasmid, and thus cannot utilize the nitrogen source (proteins) in FCH efficiently. Flavorzyme, a commercially available protease, was attempted to be used to degrade the protein. After 12 hours of hydrolysis with 1% Flavorzyme, the milky appearance of FCH nearly disappeared, indicating that proteins has been degraded (see supplementary material). The supernatant obtained after centrifugation, termed FCHH (FCH Hydrolyzed) served as an excellent nitrogen source for AT0606. It has been reported that whey protein hydrolysates are able to chelate calcium (Xixi et al., 2015), the presence of which was previously found to be beneficial for nisin production; Ca^{2+} can bind to the cell wall, and help displace nisin, which results in higher nisin titers(Zhao et al., 2021). For this reason, experiments were carried out to optimize the FCCH content of the composite Molasses-FCHH medium. As shown in Fig. 5A, a high FCHH content greatly stimulated cell growth, but at the cost of the soluble nisin being produced. The highest total and soluble nisin production was observed when 20% FCCH was used.

Nisin production can be stimulated by aeration, however, aerobic conditions can prompt formation of reactive oxygen species (ROS), which can damage various cell components and hamper growth (Kaneko et al., 1990). It is possible to alleviate oxidative stress, e.g. by adding hemin which helps eliminate ROS (Kaneko et al., 1990). Furthermore, hemin activates respiration, which boosts the ATP supply and

342 stimulates nisin production (Zhao et al., 2021). An excess of hemin can have adverse effects on growth,
343 which is why different concentrations (1-4 μg/mL) were tested to determine the optimal amount for AT0606.
344 As shown in Fig.5B, indeed, the biomass and the total nisin titer both increased after introduction of 1 to 2
345 μg/mL hemin. However, the soluble nisin percentage decreased at higher concentrations. Use of 1 μg/mL
346 hemin resulted in the highest amount of total and soluble nisin. It is worth noting that, in these experiments,
347 all the sugar in these fermentations was not fully consumed, due to the low buffer capacity, which resulted
348 in a rapid drop in pH to 4.1-4.3, at which point sugar metabolism and growth ceased.

349 Being a lactic acid bacterium, *L. lactis* generates lactic acid, and the acidic environment will lead to arrest
350 of its growth, which eventually affects nisin production, as nisin biosynthesis is growth-associated (Zhao
351 et al., 2021). Therefore, controlling the pH is necessary for achieving high cell density and efficient nisin
352 production. Liu et al.(2010) examined the pH range from 5-6.5, and found pH 5.75 to be best for nisin
353 production when using *L. lactis* ATCC11454, and this particular pH also supported the highest biomass
354 yield. At other pHs, growth and nisin production were affected negatively. Therefore, for AT0606, pH 5.75
355 was chosen to carry out the fermentation. Three different fermentation media were used; 1) the molasses-
356 FCHH medium, 2) a molasses-yeast extract (YE) medium, otherwise similar in composition to the
357 molasses-FCHH medium, and finally 3) commercial M17 medium supplemented with sucrose (SM17). As
358 shown in Fig 6, compared to SM17, the molasses-based media were superior to SM17, supporting both
359 higher biomass yields and higher nisin titers. The maximum nisin titer was reached, for all three cultures,
360 after 10 hours; 9981 IU/mL using SM17, 13181 IU/mL using the molasses-FCHH medium and 14069
361 IU/mL total nisin using the molasses-YE medium. Thus, using a medium composed solely of low-value
362 by-products from the food industry resulted in a 32% higher yield of nisin, that when using expensive M17.
363 Replacing FCHH with YE only had a modest effect, which was surprising, as YE has been shown to be a
364 superior nitrogen source for LAB (Aeschlimann and von Stockar, 1990). Remarkably, most of the nisin
365 produced by AT0606 was on soluble form (9420 IU/mL) at pH 5.75.

Currently, it is estimated that the worldwide production of molasses is around 70 million tons/year and the price is maintained at around 120-160 USD/ton for the Europe market (https://ihsmarkit.com). However, most molasses ends up as waste, and is of great environmental concern due to its high biochemical oxygen demand (BOD) and chemical oxygen demand (COD) (Yan et al., 2011). Recently, molasses was investigated as a microbial substrate for producing different value-added products (Zheng et al., 2021). There is great potential in using microorganisms to convert these waste residues into useful materials. In this study, molasses was found to contain insufficient nitrogen to support efficient growth of *L. lactis* (data not shown), but that supplementation with other cheap nitrogen sources is possible. FCHH was found to be nearly as efficient as YE, an efficient but costly nitrogen source (approx. $4/kg, Angel Yeast). Nisin can be used to reduce food losses, and this study has demonstrated that nisin can be produced using resources that would otherwise be wasted. The work is thus directly in line with current trends, supporting sustainability in a world with limited resources.

4. Conclusion

For the first time ever, it has been shown that chlorhexidine adaptation is useful for improving production of nisin. Fermentation media constitute a major cost in industrial fermentations. This study has demonstrated that a medium composed of only low value byproducts from the food industry is excellent for producing nisin with the adapted strain. Finally, it is important to stress that the improved nisin strain described in this communication is 100% naturally derived from a natural nisin-producing *L. lactis* strain of dairy origin. The latter is in line with consumers demanding natural solutions for producing foods and food ingredients.

E-supplementary data for this work can be found in e-version of this paper online.

Acknowledgement

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Availability of data and materials

Raw sequencing data of WT, AT0408 and AT0606 was deposited in NCBI (https://www.ncbi.nlm.nih.gov/) with SRX accession no. SRX10046314, SRX13150331 and SRX13150332 , respectively.

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553 **Figure Captions**

554 **Fig.1.** Outline of the ALE experiment used to isolate CHG mutants, and results obtained from preliminary
555 characterization of cultures grown in a Biolector. (A) For the adaptation, ATCC 11454 wild-type (WT) was
556 sub-cultured in GM17 containing CHG, the concentration of which increased gradually over time. From
557 cultures adapted to 2, 4, 6, and 8 µg/ml CHG, cells were plated on GM17 agar, and ten colonies randomly
558 selected from each plate, giving rise to four sets of ten isolates. These sets were designated AT02, AT04,
559 AT06 and AT08. (B) Growth characterization using a BioLector microscale cultivation device.
560 Samples were incubated at 30°C with shaking at 800 rpm and the cell density was recorded automatically.
561 Wild type and isolates are colored in cyan and purple, respectively. (C) Nisin production performance of
562 all the strains contained in the sets AT02, AT04, AT06 and AT08, grown in the BioLector. Left, total nisin
563 activity relative to wild-type; middle, soluble nisin relative to wild-type; right, the fraction of the nisin,
564 which is soluble. (D). MIC to CHG and nisin, and nisin production for the two best performers from the
565 AT06 set as compared to the WT.

566 **Fig. 2.** Time course of cell density (A), pH (B), growth rates (C),total (D) and soluble (E) nisin production
567 as well as soluble nisin percentage (E) of *L. lactis* ATCC11454 wild type (WT) and its CHG adapted
568 derivative AT0606 grown in aerated cultures. All the fermentations were carried out in 300-mL shake flasks
569 filled with 50 mL GM17 medium. The experiments were performed in duplicates and standard deviations
570 was indicated by error bars.

571 **Fig.3.** Surface physicochemical properties of WT and AT0606. (A) Affinities of WT and AT0606 cells
572 for the chloroform and hexadecane solvents used in the MATS analysis. (B) Electrophoretic mobilities of
573 WT and AT0606 cells suspended in 1.5 mM NaCl at various pHs.

574 **Fig.4.** Comparison of cell-wall thickness of WT and AT0606. Thickness was morphometrically evaluated
575 using transmission electron microscopy. At least 10 cells of each strain with almost equatorial cut surfaces
576 were determined and data are presented as mean ± SD. (**), $p < 0.01$.

577 **Fig.5**. Nisin production from sugar and dairy by-product. (A) Effect of FCCH concentration on the
578 performance of AT0606 using 1% molasses. (B) Effect of hemin concentration on the performance of
579 AT0606 using 1% molasses. All the fermentations were carried out in 100-mL shake flasks filled with 20
580 mL medium and samples were taken in 12 h. The experiments were performed in duplicates and standard
581 deviations was indicated by error bars.

582 **Fig. 6.** Batch fermentation of the strain AT0606 in 5% molasses 20% FCHH, 5% molasses 2% YE and
583 M17 with sucrose (SM17), respecitively at controlled pH. (A) Relation between sucrose consumption and
584 biomass concentration. Sucrose concentration and OD_{600} demonstrated by solid and dashed lines
585 respectively.(B)Total and soluble nisin production over time. Histograms represent total nisin (with the
586 error bar at the top), which can be divided into two parts: white diagonal stripes, soluble nisin (with the
587 error bar in the middle) and non-white diagonal stripes, cell-bound nisin that calculated as total nisin minus
588 soluble nisin.

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599 **Graphic for table of contents**

600 **Table 1.Mutations identified in the CHG-adapted mutant AT0404 and AT0606.**

Strains	RP[a]	Variation[a]	Region	NC[b]	AAC[c]	product or (distance from CDS) neared product
AT0408	901907	SNV	gene CDS	G -> T	H -> N	Glutamine--fructose-6-phosphate aminotransferase **Glms**
	1670503	SNV	gene CDS	T -> A	L -> M	putative ABC transporter ATP-binding protein
	1890765	SNV	gene CDS	G -> A	A -> V	PTS system mannitol-specific EIICB component **MtlA**
AT0606	219156	SNV	gene CDS	G -> T	Truncation	phosphate ABC transporter permease subunit **PstC**
	544506	SNV	gene CDS	G -> T	S -> I	WxL domain-containing protein
	901907	SNV	gene CDS	G -> T	H -> N	Glutamine--fructose-6-phosphate aminotransferase **Glms**
	956229	SNV	gene CDS	C -> A	Truncation	Oleate hydratase **Sph**
	1471694	SNV	gene CDS	G -> A	A -> V	putative beta-N-acetylglucosaminidase **NagZ**
	1670503	SNV	gene CDS	T -> A	L -> M	putative ABC transporter ATP-binding protein
	1808874	SNV	intergenic	C -> A	None	(-71bp)Quaternary ammonium compound-resistance protein **SugE**
	1890765	SNV	gene CDS	G -> A	A -> V	PTS system mannitol-specific EIICB component **MtlA**

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602 [a]Reference position refers to the genome sequence of L. lactis ATCC11454. [b]SNP, single nucleotide polymorphism;
603 [c]Amino acid change.
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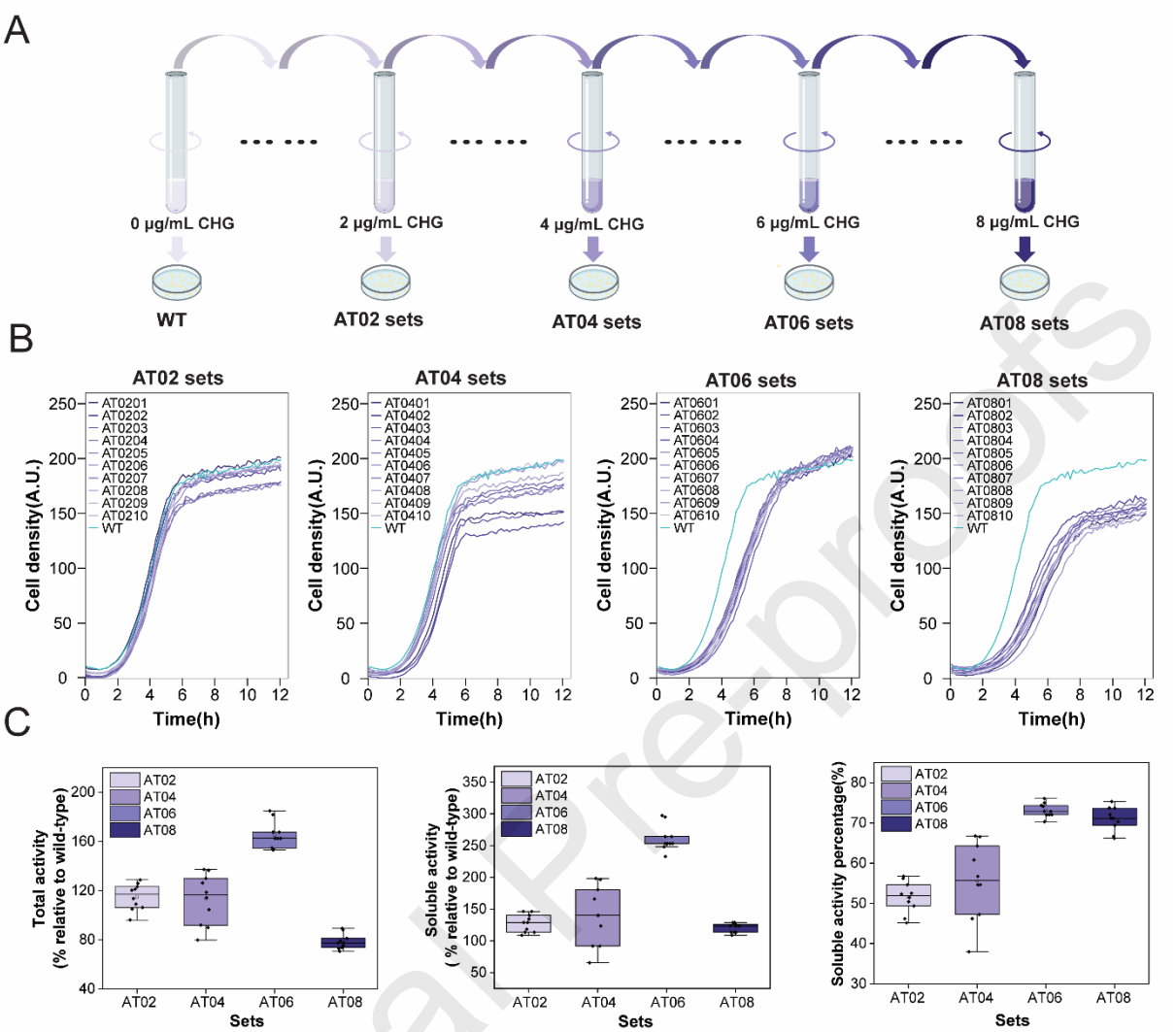
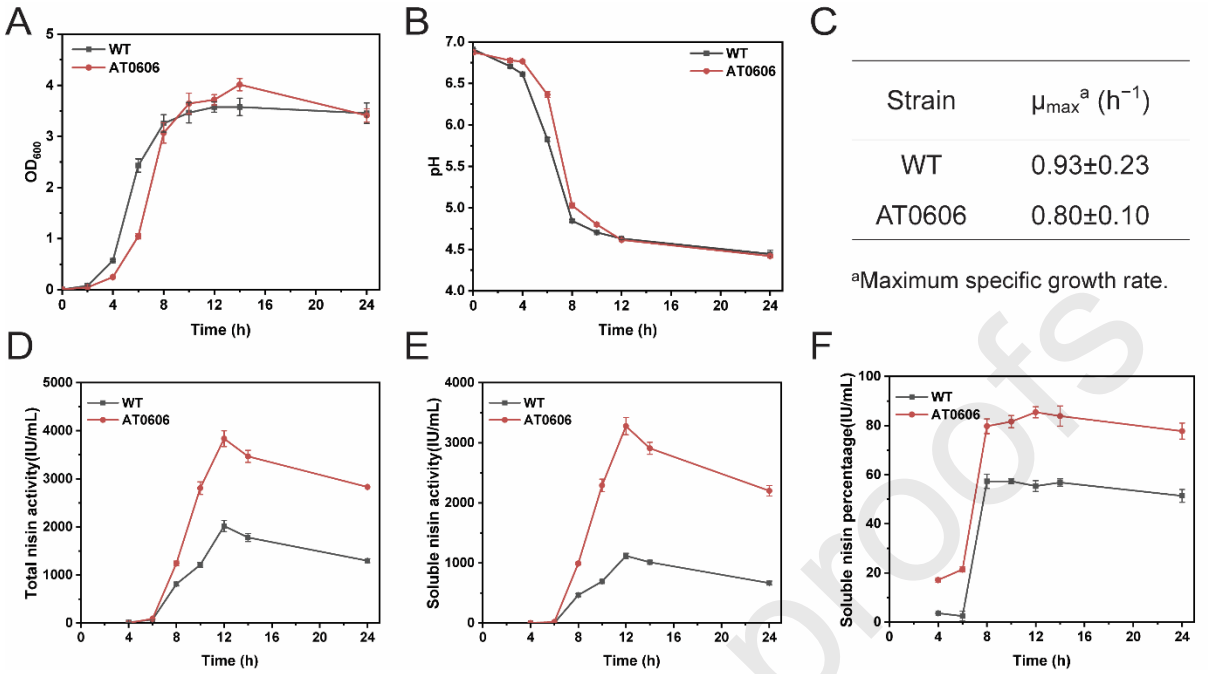


D

	MIC of CHG(μg/mL)	MIC of nisin(μg/mL)	Total activity(% relative to wild-type)	Soluble activity(% relative to wild-type)	Soluble activity percentage(%)
WT	2	128	100	100	46
AT0606	6	256	185	298	74

Fig. 1.



Strain	μ_{max}[a] (h^{-1})
WT	0.93±0.23
AT0606	0.80±0.10

[a]Maximum specific growth rate.

Fig. 2.

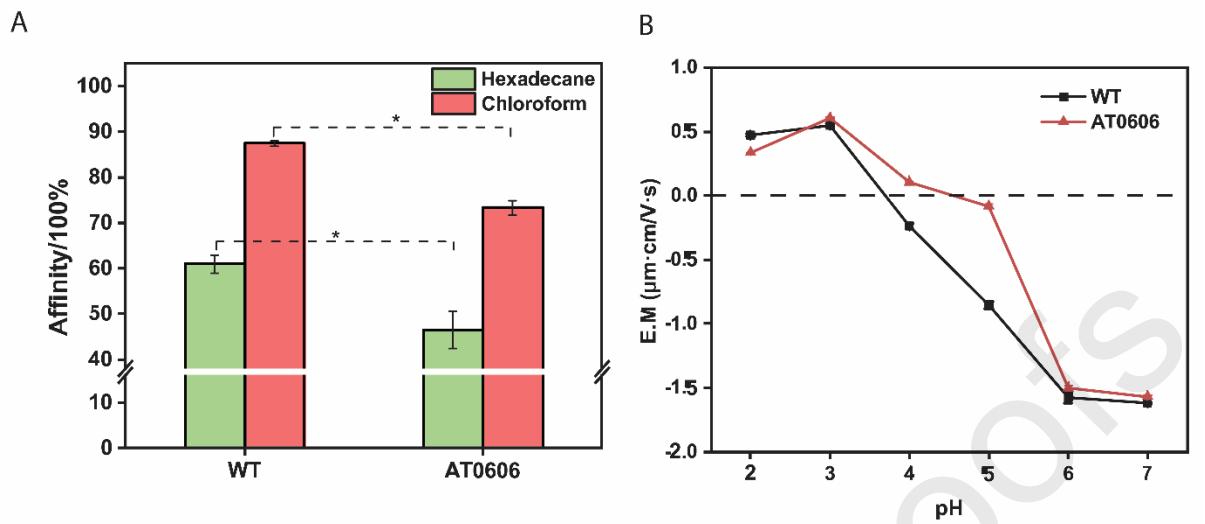


Fig.3.

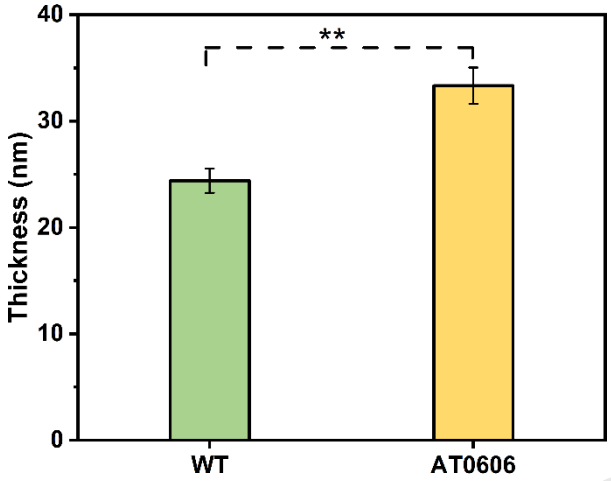


Fig.4.

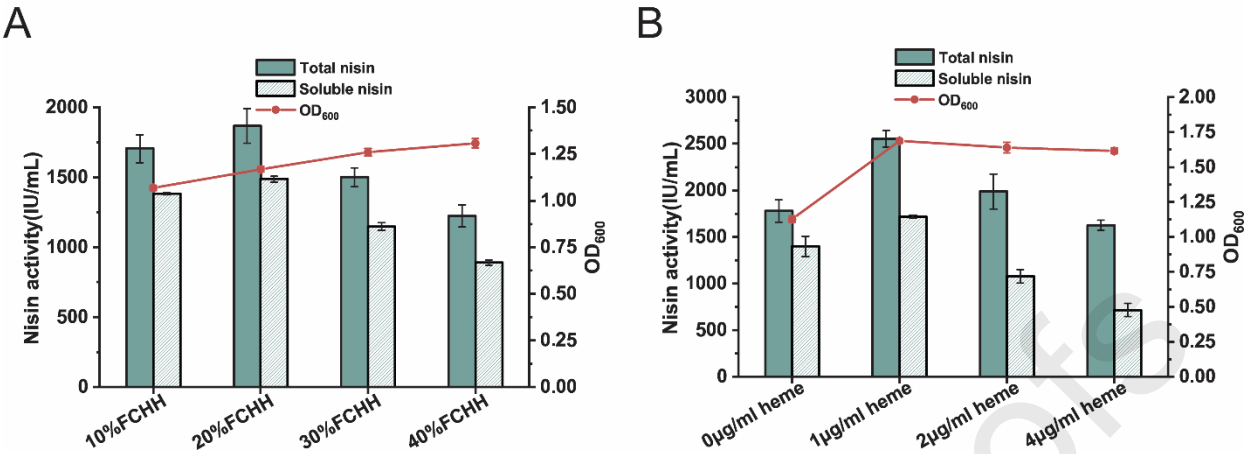


Fig.5

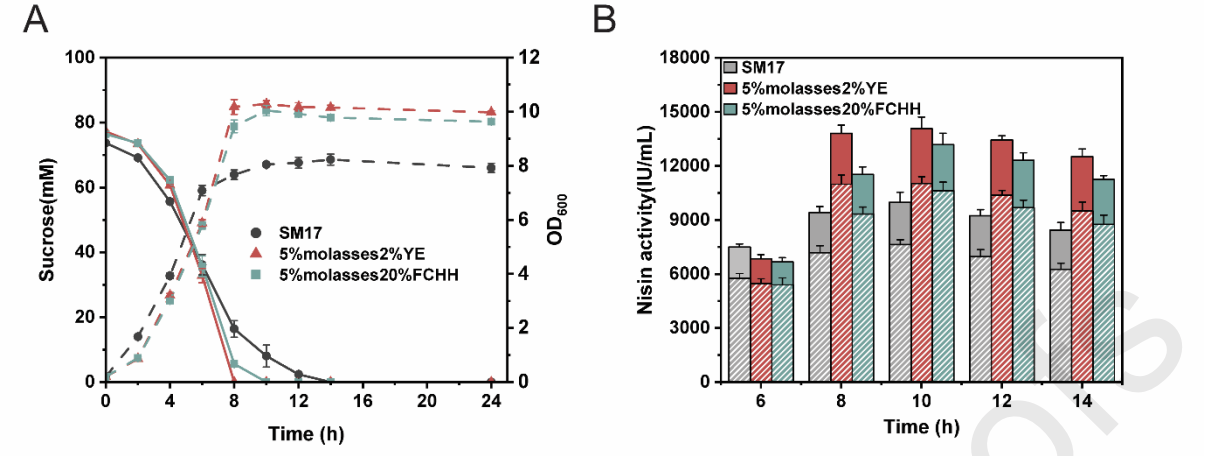


Fig. 6

CRediT authorship contribution statement

Ge Zhao: Methodology, Formal analysis, Investigation, Writing - original draft. **Paul J. Kempen:** Investigation, Data curation, Methodology. **Radhakrishna Shetty:** Methodology, Software, Formal analysis. **Liuyan Gu:** Software, Data curation. **Shuangqing Zhao:** Investigation. **Peter Ruhdal Jensen**: Conceptualization, Investigation, Supervision, Project administration. **Christian Solem:** Conceptualization, Visualization, Supervision,Writing - review & editing.

686 The main highlights are as follows:

687 • It is first time to use CHG adaptive evolution to improve nisin production.
688 • Best adaptive strain has 3.1- and 2.0-folds more soluble and total nisin.
689 • Less negative-charged and much thicker cell walls may cause more nisin production.
690 • A combination of molasses and dairy waste was used for nisin production.
691 • Molasses-FCHH medium supported higher nisin yield than M17 medium.
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