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Review

Regulation of Surfactin Biosynthesis Pathways in *Bacillus* sp.: Regulatory Network Reconstruction and Metabolic Engineering Perspectives

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Abstract

Surfactin is an amphiphilic lipopeptide, composed of a cyclic heptapeptide linked to a β-hydroxy fatty acid. This structural configuration gives rise to its remarkable biosurfactant properties. The synthesis is performed by a non-ribosomal peptide synthesis mechanism that involves surfactin synthetase, a multi-enzyme complex encoded by the *srfA* operon. The expression of this operon and surfactin synthetase activity are regulated by quorum sensing pathways such as Rap-Phr and ComXQPA controlling natural competence, YcxA and YerP efflux pumps, sporulation phosphorelay and degradative enzymes as well as surfactin-regulated pathways. The objective of this manuscript is to review well-studied regulatory metabolic pathways of *Bacillus subtilis* that control surfactin biosynthesis and to construct a global metabolic regulatory network in order to design the most efficient metabolic engineering strategies to improve surfactin production while maintaining strain stability. The surfactin synthesis regulatory network was built using KEGG pathway maps for two-component systems, quorum sensing, bacterial secretion, and ABC transporters. Most genes and interactions were based on *Bacillus subtilis* subsp. *subtilis* str. 168. Gene details were obtained from the NCBI database. The network map was created manually using Canva's flowchart tool. Modifications in amino acid and fatty acid precursor pathways did not have a significant impact on surfactin yield despite an increase in amino acid availability. The modification of genes involved in quorum sensing pathways has been associated with the regulation of natural competence development and cell cycle progression. For example, *srfA* promoter replacement and *comA* overexpression resulted in a significant enhancement of *srfA* operon expression and surfactin production. The overexpression of efflux pumps *ycxA* and *yerP* resulted in enhanced surfactin secretion and increased self-resistance to surfactin. In contrast the deletion of sporulation genes resulted in an increase in surfactin yield per biomass; however, it caused inhibition of growth. Knockout of biofilm-related genes and competing lipopeptide pathways resulted in an increase in surfactin yield. A hypothesis of triple-mutant strain combining *comA* overexpression, *ycxA* and *yerP* enhanced efflux transport, and *kinC* deletion was proposed based on the constructed regulatory metabolic network that theoretically can improve surfactin yield and strain stability; however, further validation is required to assess potential unintended effects.

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1. Introduction

Surfactin, first isolated in 1968 from *Bacillus*, is an amphiphilic lipopeptide composed of a cyclic heptapeptide linked to a β-hydroxy fatty acid, which endows it with strong biosurfactant activity [1]. Surfactins are known as a family of structurally related molecules. Their observed variability arises from differences in amino acid composition and fatty acid chain characteristics. This diversity is a key driver of research into its biosynthesis, structural variation, functional properties, and production optimisation [1,2]. Surfactin is a metabolically active compound that has been demonstrated to possess antibacterial, antifungal, and hemolytic properties [3]. Surfactin is synthesised via a non-ribosomal peptide synthesis mechanism involving surfactin synthetase, a multi-enzyme complex coded by the *srfA* operon [4,5]. Surfactin biosynthesis is principally subject to regulation by quorum sensing systems such as ComX, QPA and Rap-Phr, in conjunction with multiple global regulators (e.g., *codY*, *spo0A*, *degU*). Its progression is contingent upon the availability of key precursors, including branched-chain fatty acids and amino acids, and the efficiency of secretion systems (e.g., YerP, YcxA, KrsE) [6]. The *srfA* operon constitutes a component of the regulatory network that governs natural competence in *Bacillus subtilis*. Its regulation is dependent upon the attainment of a threshold level of the transcription factor ComK, which activates genes essential for DNA uptake, recombination, and its own expression through a positive feedback loop [7]. Surfactin itself has been shown to be a signalling molecule. It has been demonstrated to cause cell membrane depolarisation and potassium leakage, and it is detected by KinC [8,9]. KinC activates a range of processes including sporulation, biofilm formation, motility inhibition and cannibalism, thereby enabling cells to adapt to stress conditions and initiate cell differentiation. This is achieved by activating different metabolic pathways in distinct subpopulations of cells [10–12]. Metabolic engineering approaches are required to redirect metabolic flow toward surfactin biosynthesis in order to enhance its production.

2. Materials and Methods

2.1. Data Collection and Network Reconstruction Methodology

A systematic approach was applied for the reconstruction and analysis of *Bacillus* sp. regulatory metabolic networks. The KEGG database http://www.genome.jp/kegg/ (accessed on 3 March 2026) was used as the primary source for metabolic and signalling pathway information, while gene annotations and functional validation were performed using the NCBI Gene database https://www.ncbi.nlm.nih.gov/gene (accessed on 20 March 2026).

Network reconstruction was based on a reference-organism strategy using well-annotated bacterial genomes, enabling identification of conserved regulatory modules, including two-component systems, quorum sensing pathways, and ATP-binding cassette transporters. Genes were functionally categorised into metabolic and regulatory units through manual curation and pathway integration.

To ensure biological relevance, reconstructed networks were validated using published systems biology and genome-scale metabolic modelling studies.

In this review, the genes were arranged according to their metabolic flux direction in order to demonstrate their impact level. Glucose was directed towards surfactin for amino acid metabolism, and membrane receptors were channelled towards the *srfA* operon for

the regulatory metabolic network. Genes from parallel pathways were shown in order of their importance for surfactin biosynthesis.

2.2. Construction of Surfactin Regulation Pathway Maps Using KEGG Database

The regulatory network associated with surfactin biosynthesis was reconstructed using pathway information from the KEGG database, including the pathways Two-Component System, Quorum Sensing, Bacterial Secretion System, and ABC Transporters. The model organism used for pathway reconstruction was *Bacillus subtilis* subsp. *subtilis* str. 168 because it has the most characterised genetic and regulatory systems related to non-ribosomal lipopeptide biosynthesis. Gene annotations, locus tags, protein functions, and regulatory relationships were verified using the NCBI Gene database.

The pathway reconstruction process involved manual curation and integration of genes participating in surfactin synthesis, transport, quorum sensing, and signal transduction using Canva's https://www.canva.com/ (accessed on 3 April 2026) flowchart tool.

Regulatory elements including the *srfA* operon, comQXPA quorum sensing system, DegU, DegQ, Spo0A, and associated ABC transporters were identified and mapped according to KEGG orthology assignments and published transcriptomic studies. The reconstructed network incorporated interactions between metabolic and regulatory modules involved in amino acid biosynthesis, fatty acid metabolism, non-ribosomal peptide synthesis, secretion systems, and cellular signalling.

To improve the biological relevance of the model, information from published systems biology and transcriptomic analyses of surfactin-producing *Bacillus* strains was incorporated into the pathway curation workflow.

The resulting regulatory map was visualised as an integrated metabolic-signalling network illustrating the interactions between environmental sensing pathways, transcriptional regulators, transport proteins, and surfactin biosynthetic genes. The network was subsequently used for comparative analysis of regulatory mechanisms controlling surfactin production in *Bacillus* species.

Additional pathway validation and gene interaction analysis were performed using published transcriptomic and systems biology studies focused on surfactin biosynthesis in *Bacillus subtilis* and *Bacillus amyloliquefaciens*. Comparative analysis of regulatory genes, including *spo0A*, *degU*, *comA*, *abrB*, and the *srfA* operon, enabled reconstruction of an integrated regulatory network linking quorum sensing, two-component systems, amino acid metabolism, and non-ribosomal peptide synthesis pathways. Functional classification of genes and metabolic modules was carried out according to KEGG Orthology annotations and previously published metabolic engineering models [13].

3. Regulation of Amino Acid and Fatty Acid Precursor Biosynthesis

It has been observed that BkdAA and BkdAB (2-oxoisovalerate dehydrogenase, which is involved in valine, leucine and isoleucine degradation) have been shown to repress the synthesis of iso-C13 and iso-C15 fatty acids through the use of CRISPRi, thereby increasing precursor availability and favouring C14 surfactin [14]. Downregulation of branched-chain amino acid degradation genes such as *bkdAA* and *bkdAB* has been demonstrated to increase C15 surfactin proportion [15]. The deletion of the *srfA* operon has been shown to result in a reduction in *bkdAA* expression [16].

YrpC, or glutamate racemase, is an enzyme that catalyses the conversion of L-glutamate, the precursor of surfactin, to D-glutamate. In the absence of YrpC, there was a reduction in L-glutamate consumption and an enhancement in surfactin synthesis when the process was combined with active *sfpAc* [17]. The functional *sfpAc* gene demonstrated moderate *yrpC* repression, thereby facilitating surfactin production of 450 mg/L following

24 h of fermentation. This is in comparison to the 166.88 mg/L produced by the control BS168NU-Sd strain [14].

RacE, otherwise known as glutamate racemase, and MurC, also referred to as UDP-N-acetylmuramate-L-alanine ligase, have been identified as key enzymes in the consumption of L-glutamate, a vital precursor in the assembly of surfactin. The employment of CRISPRi repression in a systematic manner by RacE resulted in an augmentation of cell growth, a phenomenon that is presumably attributable to a diminution in the inhibition of DNA gyrase activity. Systematic CRISPRi repression of *MurC* caused a slight decrease in growth rate; however, it resulted in higher volumetric productivity and surfactin production per unit of biomass and concentration (420 mg/L) in comparison to the control (166.88 mg/L) BS168NU-Sd strain [14].

YvkC, otherwise referred to as phosphotransferase, utilises ATP through gluconeogenesis and flavonoid phosphate synthesis. However, the knockout of this gene did not result in a significant enhancement of surfactin biosynthesis, with a production of approximately 760 mg/L in the *yvkC* knockout strain, in comparison to 790 mg/L in the control strain [17].

PyrB, otherwise referred to as aspartate carbamoyltransferase, and PyrC, also known as dihydroorotase, play a vital role in pyrimidine metabolism. The experimental data on the concentrations of surfactin were used to repress the production of surfactin, and this was found to be completely inhibitive. It was demonstrated that excessive inhibition is detrimental, and that the reduction in repression strength restored surfactin production to 340 mg/L for *pyrB* and 360 mg/L for *pyrC*, in comparison to 166.88 mg/L for the control BS168NU-Sd strain [14]. The knockout of *lpdV*, or dihydrolipoyl dehydrogenase, which is used in fatty and amino-acid metabolism, yielded a 1.6-fold increase in surfactin production and a decrease in the Val^7 surfactin proportion from 25.2% to 21.8%, and an increase in the C14 surfactin content from 21.2% to 52.7% [18]. The deletion of *lpdV* has been demonstrated to result in alterations to the composition of surfactin isoforms, with a concomitant increase in the proportion of straight-chain surfactin variants [19].

The impact of AnsZ or asparaginase expression on L-aspartate availability was investigated, demonstrating that the expression of *ansZ* or asparaginase enhances L-aspartate availability, exhibiting a 7.8% reduction in surfactin titer and an 11.0% decrease in yield per OD_{600}, without affecting growth. It has been shown that the enhancement of a solitary precursor pool has the potential to be counterproductive, a phenomenon that can be attributed to metabolic imbalance and regulatory constraints [20].

The deletion of *bcaP*, otherwise referred to as a branched-chain amino acid transporter, has been shown to enhance the availability of branched-chain amino acids and augment surfactin production by 6.2% [20].

Fatty acids are activated by external fatty acyl-CoA ligases, including LcfA and YhfL. The knockout of these genes resulted in a 38–55% decrease in surfactin production, as evidenced by [21]. The deletion of *lcfA* and *lcfB* yielded an 84% decrease in surfactin production [19].

4. Modifications of Quorum Sensing Pathways and Global Regulators

The knockout of the *srfA* operon resulted in the inhibition of hemolytic activity in the bacterial culture. The formation of flat, unwrinkled colonies and a thin, fragile biofilm was observed, as confirmed by colony morphology and pellicle assays [22]. The strain demonstrated normal growth patterns; however, it exhibited accelerated cell death after 18 h of fermentation. It was unable to sustain a stationary phase or sporulate. The Embden–Meyerhof–Parnas pathway exhibited significant upregulation, leading to enhanced glycolysis. Concurrently, the genes responsible for converting pyruvate to acetyl-CoA, as well as those involved in oxidative phosphorylation, were downregulated.

This indicated reduced ATP production efficiency [22]. The measurements of dissolved oxygen revealed no reduction in oxygen availability. Consequently, it was determined that metabolic defects were not caused by oxygen limitation. The deletion of *srfA* has been demonstrated to result in significant upregulation of genes associated with chemotaxis, flagellar assembly, motility and swarming [22]. A study of the *srfA* knockout strain has revealed substantial metabolic changes, including alterations in carbon catabolite regulation, enhanced glycolysis, a weakened TCA cycle, impaired respiration, utilisation of alternative carbon sources, reduced amino acid metabolism and disrupted PTS sugar transport [16]. *srfAA* knockout has been demonstrated to exert a positive effect on the volatile metabolome, with a consequent increase in the synthesis of benzaldehyde by 90.88%, 2,4-di-tert-butylphenol by 412.93%, acetylacetone by 438.40% and 2,5-dimethylpyrazine by 509.91%, as compared with the control strain [23].

The replacement of the native *srfA* promoter with the constitutive promoters P43, PHpaII and PSB resulted in a significant decrease (98–99%) in *srfAA* and *srfAB* transcription and surfactin production [17]. The substitution of the native *srfA* promoter in *PRsuc-srfA* and *PRtpxi-srfA* resulted in a 311.35 mg/L surfactin yield, representing a ~9.5-fold increase in comparison with the native promoter. This was accompanied by a 678-fold increase in *srfA* transcription [24]. In order to enhance surfactin biosynthesis, the native *srfA* promoter was replaced with the strong constitutive promoter P43. In comparison with the parental strain, engineered strains yielded 2280 $\pm$ 8 mg/L and 1500 $\pm$ 0.11 mg/L of surfactin, exhibiting higher proportions of C13 and iso-C14 surfactins, while concomitantly reducing C14 and C15 fractions [25].

The *srfA* operon is known to be activated by quorum sensing signals. The effects of ComX pheromone and CSF (competence-stimulating factor) have been investigated, demonstrating that the expression of *comX* leads to an increase in the synthesis of surfactin [19,26]. ComX, designated as the primary competence pheromone, has been demonstrated to induce *srfA* expression in wild-type cells when cultivated in a suitable medium. Conversely, this induction is not observed in cultures derived from *comX* knockout mutants [27].

The *srfA* expression and competence development are subject to regulation by the ComX, ComP, ComA pathway [28]. The deletion of either *comP* or *comA* led to a significant decrease in *srfA* expression [26].

The deletion of *comA* resulted in growth defects, impaired biofilm formation, and abnormal colony morphology, all of which were identical to the effects of the deletion of *srfA* itself. The restoration of growth upon the addition of surfactin confirmed that the observed phenotypes were caused specifically by the loss of surfactin production, rather than by secondary effects of the *srfA* mutation [19,22,26]. Furthermore, the additional insertion of *PsrfA* upstream of *comA* enhanced *comA* expression and increased surfactin production by 1.2–1.4 times [29]. It has been demonstrated that *comA* binds specifically to the *srfA* promoter in two distinct *comA* binding sites located upstream of the promoter [30,31].

comK has been identified as the primary transcriptional regulator of competence, governing the genes essential for DNA uptake and integration [28,32]. The introduction of the plasmid-borne *comK* has been shown to reduce the activation threshold for *comK* autoinduction. Furthermore, *comK* has been demonstrated to enhance its own promoter expression and transformation efficiency, although *srfA* expression is reported to be 200-fold lower [28,32].

The results of these studies highlighted that the overexpression of *rapG* protein of response regulator aspartate phosphatase almost completely inhibited the expression of *srfA*. However, the opposite was observed in the absence of *rapG*, with *srfA* expression increasing. Multicopy *rapH* has been demonstrated to exert a marked effect on the expression

of the *srfA* gene, resulting in a significant repression of *rapF* expression. In addition, it has been observed that RapH functions as an inhibitor of DegU-regulated genes [33].

The oligopeptide uptake, signal transduction, activation of DegU and activation of *srfA* expression are all affected by the Opp transporter system [34].

Furthermore, the results of this study indicated that the expression of *phrC*, known as a secreted regulator of the activity of phosphatase Rap, results in an increase in surfactin biosynthesis [19]. The addition of PhrG has been demonstrated to restore *srfA* expression in a mutant with multicopy *rapG* [33].

DegU, a transcriptional regulatory protein, has been demonstrated to regulate the expression of degradative enzymes, as well as the biosynthesis of surfactin. In addition, it has been shown to facilitate the transport of phosphate and amino acids, as well as metal ions [35].

Overexpression of the *sfpAc* (4′-phosphopantetheinyl transferase) gene has been demonstrated to be essential for the conversion of the peptidyl carrier protein (PCP) from the apo- to holo-form. This process is a vital component of pantothenate and CoA biosynthesis. The *sfpAc* gene is known for its role in the activation of surfactin synthetase [17]. The *sfpAc* gene has been found to be inactive as a pseudogene due to an additional T base at its position within *Bacillus subtilis* subsp. *subtilis* str. 168 strain. To address this, a copy of *sfpAc* has been integrated into the *yrpC* or glutamate racemase locus. This integration has resulted in increased transcription of *sfpA* by 17.8 times compared to the control strain, along with a concomitant increase in surfactin production to 747.5 $\pm$ 6.5 mg/L; the incubation period was eight hours [17]. The *yerP* strain 802 was engineered to produce surfactin by introducing a functional *sfp* gene. Following a 72 h incubation period, this strain produced 224 mg/L of surfactin, whereas the original *yerP* strain 802 did not produce any surfactin [36]. It is imperative to note that strains devoid of a functional *Sfp* gene are incapable of producing surfactin [1]. The integration of a functional *sfp* gene into the ydeO locus in the *yde* operon that encodes integral inner membrane proteins resulted in the production of surfactin at a concentration of 450 mg/L after a 24 h fermentation period [14]. The substitution of the native *sfp* gene in B. subtilis 168 for a functional *sfp* gene from B. velezensis BS-37 led to an increase in growth by 23.7%, but a decrease in surfactin production to 280 mg/L [37]. In the present study, B. subtilis 168 and B. subtilis strain D6, which were found to be non-surfactin producers, were subjected to a genetic modification. The sfp gene was replaced with a functional *sfp* gene, causing the creation of a genome-reduced strain that demonstrated a surfactin production capacity of 1000 mg/L [29].

The gene *codY*, which is known as a transcriptional regulator, has been observed to repress *srfA* transcription in response to GTP and branched-chain amino acids. The knockout of *codY* resulted in a 51% increase in surfactin concentration in comparison with the control strain. Furthermore, *srfAA* and *srfAB* transcription increased by 1.31-fold and 0.97-fold, respectively [17,38]. Mutants affecting *codY*-related modifications have been observed to demonstrate increased surfactin yield [39]. The Δ*codY* strain exhibited no substantial discrepancies in growth when compared to the control group. The augmentation in surfactin production was not attributable to growth effects; rather, it was a consequence of metabolic and regulatory alterations, in addition to heightened intracellular valine availability. This, in turn, led to enhanced Val^7 surfactin incorporation in the *BBG255* strain [18]. The deletion of the *codY* gene resulted in a 2-fold decrease in surfactin yield, an increase in biomass formation, and enhanced *comA* expression for the *D6S06* strain [29]. The inactivation of the global repressor *codY* resulted in a 10.3% increase in biomass production and an 11.3% increase in surfactin concentration for the *BBG260* strain [19,20]. In the case of the *codY* knockout mutant, there is an increase in the expression of amino acid biosynthesis, including branched-chain amino acid synthesis, aromatic amino acid synthesis, histidine

and lysine metabolism, *B. amyloliquefaciens FMBJ* strain [35]. In the presence of Tris-HCl at low pH, the *codY* mutant exhibited a marginal decline in *srfA* expression in the *LAB2694* strain in comparison to the wild-type *LAB452* strain [26].

The deletion of *abrB*, known as a transition state regulatory protein, resulted in a significant fivefold increase in mycosubtilin expression. This finding identifies *abrB* as the primary repressor of the *myc* operon. However, no effect on the *srfA* operon was observed, which clearly demonstrates that *srfA* and *myc* are differentially regulated despite both encoding lipopeptide synthetases [32]. The results of the experiment demonstrated that the growth rate of the *KM1019 abrB* knockout strain was found to be 0.08 h^{-1}, which is significantly lower than the 0.176 h^{-1} recorded for the control *KM1016* strain. Furthermore, the experiment revealed no alterations in the biosynthesis of surfactin [40]. *abrB* participates in the regulatory network controlling surfactin gene expression, particularly when sporulation regulators are inactive [41].

5. Surfactin Secretion and Sporulation Regulation

The overexpression of *yerP*, or the hydrophobic and amphiphilic efflux exporter under the promoter P43 in the *yvkC* locus, resulted in a 4.9% increase in surfactin concentration in comparison to the control strain [1,17]. *yerP* knockout exhibited growth inhibition in the presence of surfactin [36]. It was demonstrated that the expression of *yerP* resulted in a 1.45-fold increase in surfactin production, with no significant impact on growth and surfactin isoform profile [42]. The replacement of the native *yerP* promoter with the standard P43 promoter has been shown to increase *srfA* transcription or surfactin production. Extended promoters P43L and P43LN have been shown to increase biomass by 45.9% and surfactin production by 31.9% [20].

The overexpression of the *ycxA* gene, which is associated with the *yvkC* locus and the P43 promoter, resulted in a 3.5% increase in surfactin concentration [17]. Furthermore, it was demonstrated that no changes in the surfactin isoform profile were observed following *ycxA* expression [42]. However, overexpression of *ycxA* led to an 89% increase in surfactin secretion [19].

KrsE, encoding an MFS transporter associated with kurstakin export, was cloned and overexpressed, resulting in a 52% increase in surfactin production [39]. It has been demonstrated that *krsE* has the capacity to enhance surfactin secretion by 52% [19].

The overexpression of the putative efflux transporter YfiS resulted in a 37.4% increase in surfactin concentration [17].

Expression of *srfA* was found to decrease by approximately twofold in *spo0B* and *spo0H* mutant strains, yet no discernible effect was observed on surfactin production [41]. The *spo0H* null mutation has been demonstrated to result in a significant decrease in *srfA* expression, particularly during the early stationary phase of the cell cycle. Following entry into the stationary phase, *srfA* expression exhibited a partial recovery, reaching 30% of the control levels. This reduction in expression has been shown to lead to a decrease in the expression of *comG* [28].

The knockout of *spo0A* was associated with a growth rate of 0.28 h^{-1}, a significant reduction in *PsrfA* promoter activity, and the production of 200 mg/L of surfactin. Furthermore, a rapid decline in surfactin has been observed after 12 h of fermentation [40]. The deletion of *spo0A* resulted in a surfactin concentration of 1.7 $\pm$ 0.5 mg/L [35].

6. Regulation of Surfactin-Affected and Competitive Pathways

The knockout of biofilm synthesis pathways such as *eps* and *tasA-sipW-yqxM* operons resulted in a 4.1% increase in surfactin concentration and an 11.4% increase in biomass for the eps knockout mutant strain; a 15.8% increase in surfactin and 24.3% increase in biomass

for the eps and *tasA-sipW-yqxM* double knockout mutant strains; and no effect on growth in both strains [20].

In order to redirect metabolic flux towards surfactin, deletion of other non-ribosomal peptide metabolic pathways, such as iturin A or *itu* operon and fengycin or *fen* operon, was achieved in combination. This resulted in a surfactin concentration of 32.88 mg/L, representing a 10% increase in concentration relative to the control strain [24]. Δ*ituB* and Δ*fenA* mutants have been demonstrated to exhibit colony morphology and biofilm formation that were analogous to those of the wild type. The production of surfactin by the fengycin-deficient strain was found to be 13.5% higher in terms of concentration and 41% higher in terms of biomass when compared to the control strain. Similarly, the bacillaene or *pks* operon knockout strain demonstrated a 33.3% increase in surfactin concentration and a 41% increase in biomass compared to the control strain. The bacilysin or *bac* operon deficient strain also exhibited a 33.3% increase in surfactin concentration and a 41% increase in biomass compared to the control strain. Notably, the Δ*fenA* Δ*bac* double mutant strain produced 30.2% more surfactin, while the Δ*fenA* Δ*bac* Δ*pks* triple mutant strain resulted in a 44.8% increase in surfactin compared to the control strain. The final surfactin concentration was recorded at 2780 mg/L [20]. The double mutant strain, designated as Δ*fenA* Δ*pks*, exhibited an 11.4% increase in surfactin production, with a yield of 3320 ± 80 mg/L [43].

7. Surfactin Biosynthesis Metabolic Regulatory Network

As illustrated in Figure 1, the surfactin biosynthesis metabolic network demonstrates the relationship between surfactin biosynthesis and other regulatory pathways. These include two quorum-sensing pathways, a competence development pathway, a sporulation pathway and a degradation enzymes pathway. In *Bacillus* species, these pathways are interconnected to form a unified, coordinated system.

The process is initiated by the signalling peptide ComX. The term "ComX" refers to the "competence pheromone X", which is a key signalling molecule that plays a regulatory role in the transition from the exponential log growth phase to the stationary phase. In addition, it has been observed to activate the synthesis of secondary metabolites. As the concentration of ComX increases in the media beyond the threshold level, this is indicative of high cell density. ComX has been demonstrated to bind to the cell membrane-bound histidine kinase receptor ComP. The process of activation of ComP results in the phosphorylation of the response regulator ComA, thereby converting it into its active form, ComA-P. The ComA-P has been shown to directly activate the expression of the *srfA* operon. The *srfA* operon comprises the surfactin synthase complex and a short 141-nucleotide sequence, designated ComS. The surfactin synthase complex has been identified as the key mediator of surfactin biosynthesis, whereas the ComS small peptide has been implicated in the activation of late competence gene expression. Furthermore, a correlation has been demonstrated between surfactin production and competence development. The complex of surfactin synthase comprises three surfactin synthase subunits: SrfAA, SrfAB, and SrfAC. These subunits synthesise a cyclic peptide consisting of seven amino acids. In addition, the complex contains the thioesterase SrfAD, which binds to a fatty acid residue, forming a lipopeptide molecule of surfactin. Surfactin is exported outside the cell by means of efflux pumps, i.e., hydrophilic and amphiphilic exporters YerP, YcxA and KrsE. YerP provides resistance to surfactin by creating a gradient of surfactin in the medium, which is negative in the intracellular space. Surfactin has been proved to possess the capacity to compromise the integrity of cell membranes.

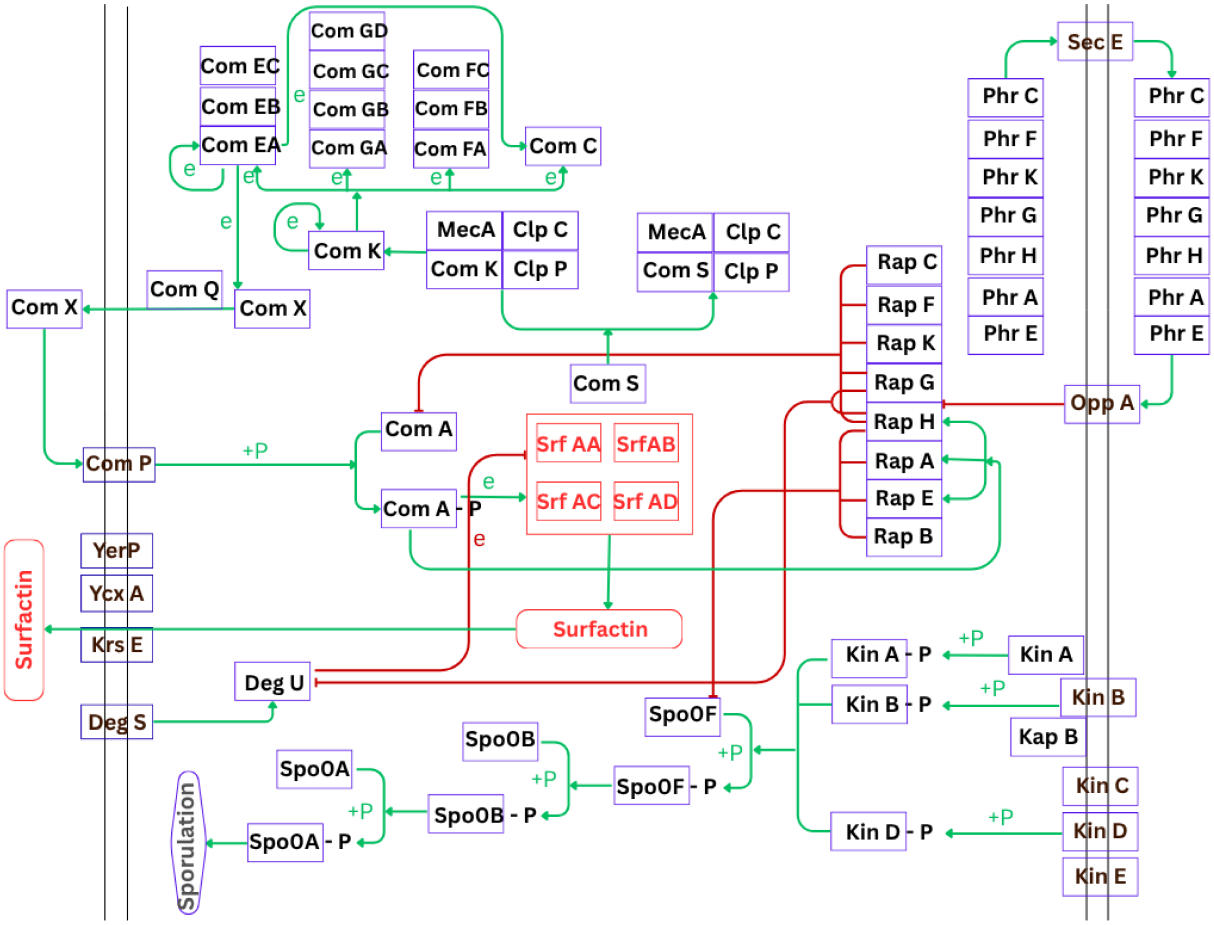

Figure 1. Schematic model for surfactin biosynthesis metabolic network that also is responsible for competence and interaction between other regulatory pathways including Phr-Rap, sporulation, degradative enzymes and surfactin secretion. Double line represents cell membrane; green arrow shows activation; red dead-end line shows inhibition; 'e' represents gene expression regulation; and 'P' represents phosphorylation.

It has been established that the ComS binds to the stress response proteolytic complex, which consists of the proteases MecA, ClpC and ClpP. The ComS releases the competence transcription factor ComK and inhibits its degradation by replacing it in the MecA-ClpC-ClpP complex. It has been demonstrated that free ComK has the capacity to enhance its own expression in a direct manner. ComK activates late competence operons, including *comG*, *comE*, *comF*, *and comC*. The *comC* is a distinct gene prepilin peptidase. The comF gene cluster comprises *comFA*, *comFB* and *comFC*, and has been shown to play a role in the process of DNA transport. It is evident that the *comG* operon comprises *comGA*, *comGB*, *comGC* and *comGD*, and that it is also involved in DNA transport. The *comE* operon comprises the genes *comEA*, *comEB* and *comEC*. The primary function of this operon is the replication and transportation of deoxyribonucleic acid (DNA). The manifestation of com gene clusters is instrumental in the development of genetic competence, thus facilitating the cell's capacity to internalise external DNA. ComE has been observed to enhance its own expression, as well as that of the *comC*.

Furthermore, it has been demonstrated that ComE has the capacity to enhance *comX* expression. The ComX precursor is cleaved to a 10-amino-acid linear peptide with a sequence of ADPITRQWGD, and is exported into the medium with the assistance of the membrane-associated regulatory protein ComQ, known as an isoprenyl transferase that modifies the ComX tryptophan. The ComX autoinducer has been observed to interact with ComP of a cell in which it is produced, or with a different neighbouring cell. This

interaction has been shown to cause a greater number of cells to switch their metabolic mode from active cell division to a stationary state. The *comX* and *srfA* operons are subject to the regulation of a positive feedback loop. An increase in the expression of *comX* results in elevated levels of surfactin, and this, in turn, leads to an escalation in *comX* expression.

Moreover, the process of biosynthesising surfactin is additionally subject to regulation by the second Rap-Phr quorum-sensing pathway. The composition of the subject is as follows: aspartate phosphatases RapC, RapF, RapK, RapG, RapH, RapA, RapE and RapB. During the exponential growth phase, RapC, RapF, RapK, RapG and RapH dephosphorylate ComA. This results in the inhibition of ComA activity and the prevention of the activation of the srfA operon. Consequently, this inhibits surfactin synthesis. It has been demonstrated that ComA-P exerts an effect on the activity of RapH, RapA and RapE by means of a negative feedback loop. The inhibition of the activity of the Rap phosphatases PhrC, PhrF, PhrK, PhrG, PhrH, PhrA and PhrE is done as a result of action translocase SecE, causing the secretion of the Rap phosphatases to the medium as peptides. Phr peptides have been identified as playing a pivotal role in the communication process between cells during the stationary phase. Through molecular binding, these peptides have been observed to interact with the oligopeptide ABC transporter OppA, thereby exerting a regulatory effect on the activity of Rap aspartate phosphatases. This regulatory mechanism has been shown to culminate in an augmentation of ComA activity and srfA operon expression, consequently leading to an elevated production of surfactin.

The degradation enzyme pathway exerts a direct influence on the expression of the *srfA* operon. In response to salt stress, the sensor histidine kinase DegS activates the phosphorylation of the DegU transcription regulator. The process in question has been shown to induce the expression of genes which encode for the production of degradative enzymes. Furthermore, it has been demonstrated to inhibit the expression of the *srfA* operon, thereby reducing the process of surfactin biosynthesis. However, it has been confirmed that RapG and RapH inhibit DegU activity during the exponential growth phase.

It is evident that a relationship has been established between the biosynthetic network of surfactin and the signalling pathway that is associated with sporulation. The process of sporulation is initiated by the action of phosphorylation sensor kinases, designated as KinA, KinB, KinC, KinD and KinE. KinA and KinB, in conjunction with the signal transduction factor KapB, exhibit a reaction to variable environmental conditions, thereby inducing a high concentration of phosphorylated response regulator Spo0F. This, in turn, instigates a phosphorylation cascade involving Spo0F, Spo0B, and Spo0A, leading to further sporulation. The KinC, in cooperation with the KinD and KinE, has been observed to phosphorylate Spo0F in low concentrations. The KinD has been demonstrated to directly phosphorylate Spo0A. The KinC is activated in response to reduced potassium levels in the cytoplasm when Spo0F-P is at a low concentration. This results in the inhibition of sporulation, but the activation of secondary metabolite biosynthesis pathways that are dependent on Spo0A. The occurrence of reduced potassium levels and membrane depolarisation can be attributed to the membrane damage induced by surfactin. During the exponential growth phase, RapH, RapA, RapE and RapB inhibit sporulation by dephosphorylating Spo0F. However, there is a controversy regarding the role of Phr peptides in the early stationary phase. These peptides have been observed to inhibit Rap aspartate phosphatases, thereby activating Spo0F and initiating the phosphorelay process associated with sporulation. Conversely, ComA-P has been observed to enhance the activity of RapH, RapA, and RapE, while concomitantly disrupting the process of sporulation. The outcome of cell fate is contingent upon the prevailing process; sporulation is a more probable outcome. In the cells responsible for synthesising surfactin, the process of sporulation is found to be inhibited.

8. Metabolic Pathways Regulated by Surfactin as a Signalling Molecule

As illustrated schematically in Figure 2, there are four metabolic pathways that are activated by surfactin. Surfactin, a compound that is characterised by its amphiphilic nature, has been demonstrated to bind to cell membranes, thus forming ion-conducting pores. This process has been shown to result in the occurrence of potassium leakage and membrane depolarisation. The KinC senses reduced potassium levels in the cytoplasm and activates the Spo0F-Spo0B-Spo0A sporulation phosphorelay. Upon activation of KinA, a process of phosphorylation of Spo0A at high concentrations ensues, thereby initiating the progression to subsequent phases of sporulation. This progression ultimately culminates in the formation of spores, which are defined as the metabolically inactive first cell subpopulation. The KinA is predominantly influenced by environmental conditions; conversely, the KinC is affected by surfactin. The latter exhibits reduced enzymatic activity relative to KinA, leading to diminished levels of phosphorylated Spo0A. A low concentration of Spo0A has been demonstrated to result in the inhibition of sporulation and the activation of variable secondary metabolite biosynthesis. This, in turn, has been shown to result in the formation of three different cell subpopulations, namely biofilm components, synthesising cells, exotoxin-producing cells or cannibals, and non-motile cells.

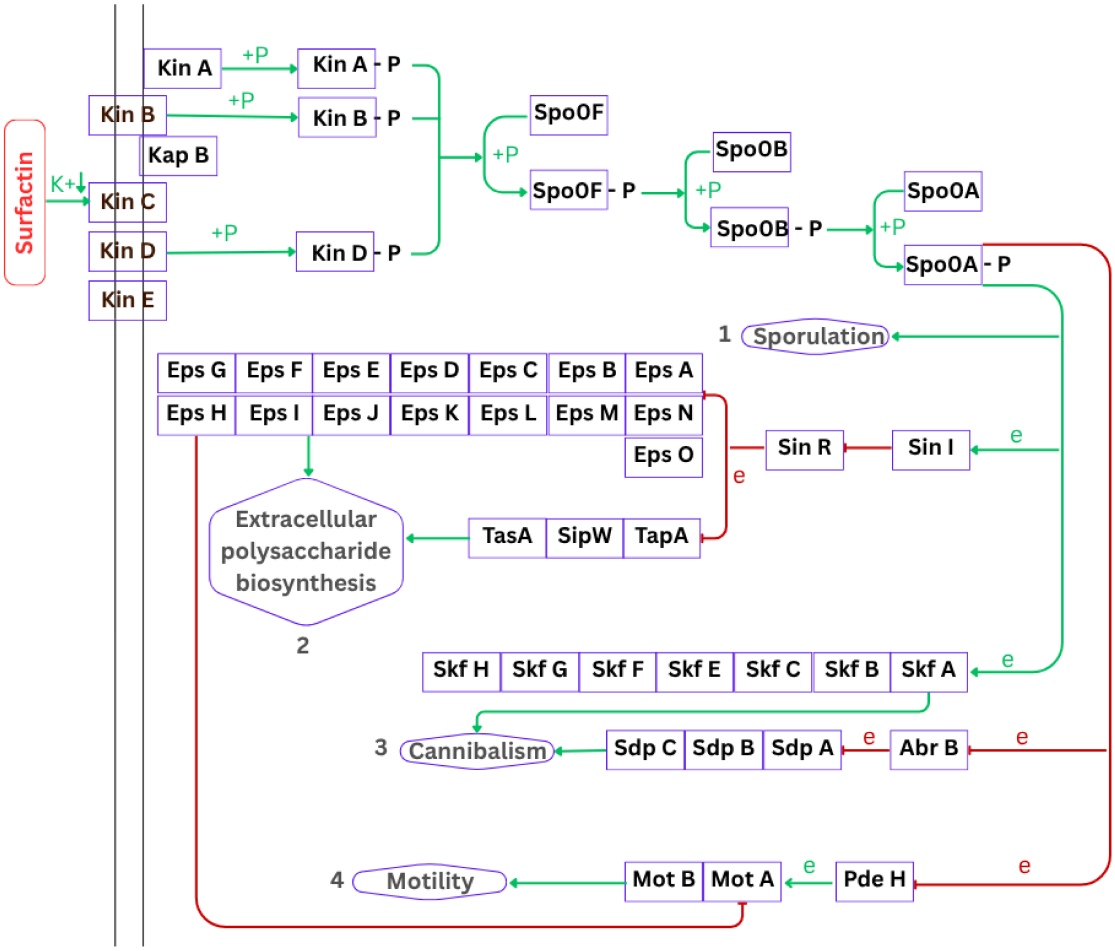

Figure 2. Schematic model for metabolic pathways regulated by surfactin as a signalling molecule, resulting in differentiation into 4 cell types. Double line represents cell membrane; green arrow shows activation; red dead-end line shows inhibition; 'e' represents gene expression regulation; 'P' represents phosphorylation.

The second subpopulation, known as biofilm matrix producers, is formed when Spo0A-P enhances the expression of *sinI*, the antagonist of SinR. The SinI has been demon-

strated to inhibit the activity of the master regulator of biofilm formation, SinR. During the exponential growth phase, the SinR gene has been observed to inhibit the expression of two operons. These operons are responsible for the biosynthesis and secretion of extracellular polysaccharides, namely eps and tasA-sipW-yqxM. In the stationary phase, the resultant inhibition of SinR by SinI is a consequence of the reaction to surfactin. Enhanced expression is observed in both operons involved in extracellular polysaccharide biosynthesis. The *eps* operon comprises 15 genes, from EpsA to EpsO, and contains all the enzymes required for the biosynthesis of the biofilm matrix: tyrosine kinases, epimerases, glycosyltransferases and acetyltransferases. The extracellular matrix component exporter, designated EpsK, is responsible for the secretion of extracellular polysaccharides into the medium. It has been demonstrated that EpsH has the capacity to inhibit the activity of the stator flagellar complex MotA, by interaction with FliG, thereby impeding motility. The tasA-sipW-yqxM operon contains three genes: TapA, which synthesises lipoprotein; SipW, which is a signal peptidase; and TasA, which is a biofilm matrix component. The tapA-sipW-tasA operon is responsible for the production of the lipopeptide fraction of biofilm.

The third subpopulation, which is known as the exotoxin-producing cells or cannibals, emerges when Spo0A-P activates expression of the *skf* operon, which consists of *skfA*, *skfB*, *skfC*, *skfD*, *skfE*, *skfF*, *skfG*, and *skfH*. Genes such as *skfB*, *skfC*, *skfD*, *skfG and skfH* act in concert as a unified enzymatic complex for biosynthesis and export of the killing factor SkfA through ABC transporters SkfE and SkfF. Within the same cell, Spo0A-P exerts a regulatory effect on the expression of the transcriptional regulator *abrB*. It is generally accepted that AbrB exerts its inhibitory influence on *sdp* operon expression. However, when *abrB* is deactivated, transcription of the *sdp* operon, which consists of genes *sdpA*, *sdpB* and *sdpC*, is initiated. The process of SdpC synthesis is activated by the precursor, which is subsequently exported into the medium by exporters SdpA and SdpB. This results in the activation of SdpC. It was found that the sporulation killing factor SkfA and the killing factor SdpC were responsible for the assassination and subsequent lysis of neighbouring cells, and that these were then consumed as a nutrient source.

The fourth subpopulation comprises non-motile cells. The inhibition of motility is observed when Spo0A-P suppresses the expression of the cyclic di-GMP phosphodiesterase PdeH. The PdeH enzyme has been shown to regulate motility by means of the degradation of cyclic diguanylate monophosphate (c-di-GMP). In the event of PdeH being blocked, an increase in c-di-GMP is observed, resulting in the inhibition of the stator flagellar complex MotA and, consequently, the inhibition of motility.

9. Discussion and Future Perspectives

As demonstrated in preceding research, the efficacy of genetic modifications within metabolic regulatory networks is contingent upon the specific modification implemented. To enhance surfactin production yield, the following modifications have been identified as effective: the regulation of amino acid and fatty acid precursor biosynthesis; modifications to quorum sensing pathways; the expression of efflux pumps; the repression of sporulation; the deletion of surfactin-regulated genes; and the knockout of competitive pathways.

It is evident that the metabolic engineering strategy of modifying precursor biosynthesis is not the most efficient. Despite the increase in the availability of branched-chain amino acids that this approach engenders, there is a lack of significant improvement in surfactin biosynthesis. The most effective modifications comprise the overexpression of the *sfp* gene, which is responsible for pantothenate production. This approach has been demonstrated to yield a slight 6% increase in surfactin production, despite a significant 17.8-fold increase in *srfA* operon expression levels [17]. The knockout of *lpdV* has been shown to result in a 1.6-fold increase in surfactin production, and the suppression of *murC* has been shown

to increase surfactin production [14,18,19]. A significant number of modifications have been proved to be either ineffective or detrimental. These include the knockout of *yvkC*, the overexpression of *ansZ*, the repression of the *pyrB* or *pyrC* genes, and the knockout of fatty acyl-CoA ligases *lcfA/yhfL* and *lcfA/lcfB* [14,19–21].

It has been observed that modifications of genes in quorum sensing pathways and global regulators are more efficient because the *srfA* promoter itself is part of the competence regulatory pathway. The most appropriate approach was the replacement of the *srfA* promoter with stronger promoters, such as *PRsuc*, *PRtpxi*, or *P43*. This resulted in a 10-fold increase in surfactin production and a 678-fold increase in transcription. There was a 1.4-fold increase in surfactin concentration in response to an increase in ComA activity [24]. The deletion of codY has been shown to result in a 51% increase in surfactin and decrease for other strain [20,29]. The replacement of the *srfA* promoter with constitutive promoters that did not react to quorum, such as *PHaII*, *P43*, or *PSB*, has been observed to result in a 98–99% reduction in surfactin production [17,24,25]. The alterations to the *abrB* gene deletion or expression did not exhibit significant outcomes. The process of communication between genes is characterised by a closed loop with feedback regulation, a process that is enhanced by *Phr-Rap* signalling. This suggests that the levels of surfactin should increase exponentially; however, this is rarely observed in practice. It can be hypothesised that surfactin biosynthesis is subject to a negative regulatory mechanism exerted by a proportion of cells responsible for its synthesis.

The most efficient scenario is the expression of efflux pumps. In this scenario, the outcome of YfiS led to a 37.4% increase in surfactin, *ycxA* showed an 89% increase, and YerP resulted in a 31.9% increase accompanied by an elevated biomass. *yerP* is contributed to increased surfactin transport and tolerance [17,19,20,36,42].

In the context of the stationary phase, it can be posited that the vast majority of the populations in evidence are spores. Therefore, the logical proposition is that the knockout of sporulation genes would be a beneficial strategy. However, it was observed that modifications in *spo0B* or *spo0H* resulted in a reduction in *srfA* expression [41]. Furthermore, the deletion of spo0A led to severe, unstable growth and the termination of surfactin biosynthesis [35,40].

In order to achieve maximum efficiency in the elimination of competing lipopeptide pathways, it is necessary to consider the results of the triple mutant, which is deficient in the *fenA*, *bac* and *pks* operons. The analysis of these results indicates that there was a 44.8% increase in surfactin. In addition, the analysis of the Δ*fenA* and Δ*pks* double mutant revealed a 30.2% increase in surfactin. The knockout of individual operons has been proved to result in a maximum increase in production of 33.3%. The combination of the *itu* and *fen* operons was found to result in a 10% improvement in surfactin production [20,24,43]. Nevertheless, fengycin and iturin A have been demonstrated to possess emulsifying properties that are more potent than those of surfactin itself. In addition, fengycin has been shown to exhibit considerable antifungal activity. It can thus be concluded that the deletion of the competitive pathway is only useful in the context of pure surfactin extraction. However, when the objective is biosurfactant production, the use of a mixed product is likely to be more effective.

The regulatory metabolic network that is activated in response to surfactin as a signalling molecule has not been the subject of sufficient study. In the case of effects on surfactin biosynthesis, there are a large number of publications about surfactin-induced cell differentiation by variable secondary metabolite production. However, it is probable that the deletion of surfactin-affected pathways can increase the number of surfactin-producing cell populations. This is because the knockout of biofilm-related pathways such as operon

eps and *tasA-sipW-yqxM* can increase the amount of surfactin produced. In particular, the single *eps* knockout mutant strain produced 4.1% more surfactin [20].

It is hypothesised that, in accordance with the findings of the regulatory metabolic network, the most efficacious course of action would be to create a triple mutant strain that incorporates all branches of the metabolic regulatory network. The primary modification is the *comA* overexpression, which directly enhances the *srfA* operon by binding to its promoter and activating surfactin biosynthesis. It has been demonstrated that *comA* is capable of activating RapH, RapA and RapE, which in turn inhibit Spo0F, thereby deactivating subsequent sporulation. This could result in a shift in the balance towards fewer sporulating cells and more surfactin-producing cells. The *comA*-overexpressing strain should not affect growth, because it is inactive during the exponential growth phase and is activated in the stationary phase by phosphorylation with histidine kinase ComP in response to ComX.

The second modification pertains to the overexpression of efflux transporters *ycxA* and *yerP*. The process has been shown to increase surfactin export from the cell to the medium. Additionally, it has been found to prevent surfactin from returning to the intracellular space and the cell-membrane damage that can result in resistance to higher surfactin concentrations in the medium. Surfactin has been found to be capable of being accumulated in the medium in higher concentrations without exerting toxicity on the culture or activating pathways induced by surfactin.

The third modification is the knockout of *kinC*, which results in the complete inhibition of surfactin-induced signalling, including biofilm formation, cannibalistic toxin production and motility regulation. A study was conducted to ascertain the interaction of Δ*kinC* mutants with surfactin and the quality of biofilms. However, no data were found for the performance of liquid cultures. Consequently, it was hypothesised that Δ*kinC* could cause unpredictable issues. Nevertheless, *kinA* expression is maintained in the *kinC* knockout mutant, which consequently enables sporulation.

10. Conclusions

A comprehensive analysis of the metabolic regulatory network revealed that the synthesis of surfactin is influenced by various physiological processes, including the pathways involved in the synthesis of amino and fatty acid precursors, the quorum sensing pathways, the activity of efflux pumps, the processes of sporulation, cell differentiation, and the formation of biofilms.

It has been demonstrated that modifications in precursor synthesis increase the availability of amino acids; however, these modifications exhibited a minor effect on surfactin yield. Quorum-sensing genes have been shown to directly impact the expression of the *srfA* operon, thus representing one of the most efficacious strategies for enhancing surfactin biosynthesis. The most efficient modification is the efflux pump overexpression, which improves surfactin export efficiency and self-resistance. It was hypothesised that a reduction in the process of sporulation would result in the redirection of available resources towards the production of other essential elements. However, further research is required to determine the impact of key sporulation genes on bacterial survival, as their deletion has been shown to reduce growth. The elimination of surfactin-induced biofilm formation pathways has been shown to increase surfactin biosynthesis. The deletion of competing lipopeptide metabolic pathways has been shown to redirect of resources towards surfactin synthesis.

The proposal entailed the creation of a triple-modified strain, integrating the overexpressed *comA* from the quorum sensing pathway, *ycxA* and *yerP* efflux pumps overexpressed, and *kinC*, which is regulated by surfactin. The objective of this integration was to achieve an industrial strain of *Bacillus* sp., with the aim of obtaining an improved surfactin yield and fermentation stability.

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